Shp-2 als neuer Regulator der Telomerase Reversen Transkriptase im Endothel

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(Sascha Jakob)

Düsseldorf, 30. November 2009

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1. Einleitung - Endothelzellalterung

Eine der häufigsten Erkrankungen in der westlichen Gesellschaft sind koronare Gefäß- und Herzerkrankungen. Krankheiten wie Atherosklerose werden gemeinhin als Volkskrankheiten bezeichnet. Bei der Atherosklerose handelt es sich um Gewebeveränderungen durch die Bildung von atherosklerotischen Plaques innerhalb des Gefäßes unter anderem in Folge von endothelialen Fehlfunktionen. Diese können eine Gefäßverengung oder sogar einen Gefäßverschluss zur Folge haben und im schlimmsten Fall zu Herzinfarkten führen. Ein wichtiger Faktor für die Entstehung der Atherosklerose ist der Kontakt des Endothels mit oxidierten LDL (low-density lipoprotein) (Rosenfeld et al., 1990). Dieser schädigt die Endothelzellen und führt über eine Reihe von Reaktionen, über die Einwanderung von Leukozyten und Einlagerung von Lipiden und daraus resultierenden Entzündungsreaktionen, zur Bildung von atherosklerotischen Plaques. Das Endothel ist die innerste Schicht des Blutgefäßes und bildet mit seinem „Monolayer“ aus Endothelzellen eine Barriere zwischen dem Blut und dem umgebenden Gewebe. An das Endothel schließt sich eine dickere Schicht aus glatten Muskelzellen an, welche an eine Bindegewebsschicht grenzt, die das Gefäß umgibt. Ein intaktes Endothel spielt eine wichtige Rolle für die Integrität der Blutgefäße (Ross, 1995). Es bietet eine nichtadherente Oberfläche, welche die Anheftung von Leukozyten und Blutplättchen verhindert, und bildet eine permeable Barriere, die die Aufnahme von Nährstoffen kontrolliert. Außerdem liefert es wichtige vasoaktive Substanzen und ist somit an der Aufrechterhaltung der vaskulären Stabilität beteiligt. Die Funktionalität des Endothels ist somit von essentieller Bedeutung für die Gefäße (Shimokawa, 1999).
Ein wichtiger Faktor, der mit Erkrankungen der Gefäße und des Herzens korreliert, ist das Alter. Ältere Menschen leiden wesentlich häufiger unter Gefäßproblemen als junge. Daher untersucht man insbesondere das Endothel als wichtigen Bestandteil der Gefäße in Bezug auf alterspezifische Erscheinungen. Während des Alterns kommt es zu Veränderungen der Endothelzellen. Zu diesen Veränderungen zählt auf molekularer Ebene vor allem die Verfügbarkeit von Stickstoffmonoxid (NO). NO ist ein kurzlebiges, radikales Gas, wirkt (auf das Gefäß) vasodilativ und ist an vielen biologischen Prozessen beteiligt. Synthetisiert wird es von den NO-Synthasen (NOS) aus der Aminosäure L-Arginin. Es existieren drei verschiedene Isoformen der NOS: die endotheliale (eNOS), die neuronale (nNOS) und die induzierbare (iNOS). Neue Studien zeigen, dass alle drei Isoformen auch im Endothel exprimiert werden (Tsutsui et al., 2009). Der wichtigste Vertreter der NO-Synthasen im Endothel ist allerdings eNOS. NO spielt eine wichtige Rolle in der Aufrechterhaltung der Funktionalität des Endothels. Es verhindert die Adhäsion von Leukozyten, inhibiert die Proliferation von glatten Muskelzellen und die Thrombozytenaggregation und –adhäsion (Vanhoutte, 1997). Des Weiteren inhibiert es die Apoptose von Endothelzellen, indem es die

Signalwege der Apoptose hemmt. Dies betrifft vor allem die Caspasen, eine Familie von Cystein Proteasen, die wesentlich zur Steuerung von Apoptoseprozessen beitragen (Kurokawa und Kornbluth, 2009). Die Aktivität der Caspasen wird durch eine S-Nitrosierung am essentiellen Cystein im katalytischen Zentrum des Enzyms inhibiert (Dimmeler et al., 1997; Li et al., 1997). Daher gehört NO zu den wichtigsten Schutzfaktoren des Endothels und somit gegen kardiovaskuäre Erkrankungen. In gealterten Endothelzellen wurde eine reduzierte eNOS-Aktivität und damit einhergehend weniger verfügbares NO beobachtet. Die Folge sind eine erhöhte Sensitivität der Endothelzellen gegenüber apoptotischen Prozessen (Hoffmann et al., 2001). Damit lassen sich die besonders im Alter auftretenden Gefäßveränderungen erklären. In gealterten Endothelzellen wurde außerdem ein Anstieg der reaktiven Sauerstoffspezies (ROS) beobachtet (Haendeler et al., 2004; Hamilton et al., 2001). Vertreter der ROS sind unter anderem das kurzlebige Sauerstoffradikal Anion ($O_2^{-\cdot}$) und das langlebigere Wasserstoffperoxid (H_2O_2). Die wichtigsten Quellen für endogene ROS in Endothelzellen sind die NADPH Oxidasen, die Mitochondrien und die entkoppelte eNOS, die wegen einer geringeren Verfügbarkeit von Tetrahydrobiopterin, einem wichtige Cofaktor von eNOS, im Alter statt NO verstärkt $O_2^{-\cdot}$ produziert (Higashi et al., 2006). Um eine Akkumulation von ROS zu vermeiden und das Redoxgleichgewicht aufrecht zu erhalten, existieren verschiedene antioxidative Systeme. Hierzu zählen Superoxiddismutasen, Peroxireduktasen, Katalasen, Gluthathion Peroxidasen, Glutaredoxin und das Thioredoxinsystem. Letzteres besteht aus zwei Oxidoreduktasen, dem Enzym Thioredoxin 1 (Trx-1) und der Thioredoxin Reduktase (Buechner, Schroeder, **Jakob** et al., 2008; Lukosz*, **Jakob*** et al., 2009). Im Alter kommt es zu einem Anstieg der ROS. Dies ist zum einen auf eine vermehrte Bildung der ROS zurückzuführen. Ein weiterer Grund für einen Anstieg der ROS ist eine reduzierte Expression der antioxidativen Systeme. So ist z. B. die Expression von Trx-1, einem der wichtigsten antioxidativen Enzyme im Endothel, in gealterten Endothelzellen stark reduziert (Altschmied und Haendeler, 2009).

Eine weiteres wichtiges Protein, das eine wesentliche Rolle im Alterungsprozess spielt, ist die Telomerase Reverse Transkriptase (TERT). TERT ist die katalytische Untereinheit der Telomerase. Die Telomerase wirkt der Verkürzung der Telomere, den Enden der Chromosomen entgegen. Diese tritt mit jeder Zellteilung auf und hat einen wesentlichen Einfluss auf die Stabilität der Chromosomen. In den meisten somatischen Zellen ist die Telomerase inaktiv, mit Ausnahme von Krebszellen, die eine sehr hohe Telomeraseaktivität aufweisen. Allerdings konnte auch in Endothelzellen Telomeraseaktivität sowohl in vitro als auch in vivo nachgewiesen werden (Haendeler et al., 2004; Hsiao et al., 1997; **Jakob** und Haendeler, 2007; Minamino et al., 2002). Kommt es zu einem Verlust der Telomeraseaktivität in Endothelzellen, werden diese seneszent, was zu Fehlfunktionen im Endothel führt, die wiederum die Bildung von Atherosklerose bewirken können. So wurden in

atherosklerotischen Regionen von humanen Arterien Endothelzellen gefunden, die einen seneszenzassoziierten Phänotyp aufwiesen (Minamino et al., 2002). Um den Einfluss der Telomeraseaktivität auf die Endothelzellalterung zu untersuchen, beschäftigte ich mich in meiner Arbeit mit den Gründen für den Verlust an Telomeraseaktivität und der daraus resultierenden Regulation von TERT.

2. Regulation von Telomeraseaktivität und TERT

Telomere sind die Enden der Chromosomen. Spezielle DNA Wiederholungen und Proteine bilden einen Komplex, der die Enden „abdeckt“ und die Chromosomen vor Erosion und Fusion mit anderen Chromosomenenden schützt. Die erste Telomersequenz wurde 1978 in *Tetrahymena* von Elizabeth Blackburn identifiziert (Blackburn und Gall, 1978), die für ihre Arbeit an den Telomeren dieses Jahr mit dem Nobelpreis ausgezeichnet wurde. Telomere in humanen Zellen bestehen aus der Basensequenz TTAGGG und sind zwischen 5 – 15 kb lang. Die Telomer-DNA endet als Einzelstrang von 75 bis 200 kb Länge und bildet zwei interne Ringe, den D-Loop und den T-Loop. Für die Bildung dieser Ringe sind neben der Basensequenz auch verschiedene Proteine notwendig, die zusammen mit der DNA einen, auch Shelterin genannten Schutzkomplex bilden (de Lange, 2005). Die Proteine TRF1 und TRF2 (telomeric repeat bindig factors) binden am Doppelstrangbereich der Telomere, das Protein POT1 (protein protection of telomeres 1) bindet direkt an den einzelsträngigen Bereich der Telomer-DNA und ist vermutlich für die Bildung des D-Loops mitverantwortlich. Weitere Proteine wie Rap1 (repressor activator protein 1), TPP1 (tripeptidyl peptidase 1) und TIN2 (TRF1-interacting nuclear factor 2) werden von TRF1 und TRF2 rekrutiert (Oeseburg et al., 2009). Während des Alterns verkürzen sich die Telomere aufgrund des End-replikations Problems mit jedem Zellzyklus. Dieser Begriff beschreibt den Effekt, dass das terminale Ende von linearen Chromosomen durch die DNA-Polymerase nicht repliziert werden kann. Dadurch verkürzen sich die Telomere mit jedem Zellzyklus. Ab einer kritischen Länge der Telomere kommt es zu einem Zellzyklusarrest (Hayflick und Moorhead, 1961; Vaziri et al., 1993), die Zelle teilt sich nicht mehr weiter. Dieser Zustand wird replikative Seneszenz genannt.

Der Verkürzung der Telomere wirkt das Enzym Telomerase entgegen. Dieses wurde 1985 von Carol Greider in humanen Zellen entdeckt (Greider und Blackburn, 1985), die für ihre Arbeit ebenfalls dieses Jahr mit dem Nobelpreis ausgezeichnet wurde. Die Telomerase ist ein Ribonukleoproteinkomplex, welcher aus zwei Hauptkomponenten besteht: der katalytischen Untereinheit Telomerase Reverse Transkriptase (TERT) und aus einer RNA Komponente (TERC), welche als Matrize für die Telomersynthese dient (Greider, 1996; Greider und Blackburn, 1989). Der Zusammenbau der aktiven Telomerase wird von den Chaperonen p23 und HSP90 (heat shock protein 90) gefördert. Diese binden an TERT und sind essentiell für den Aufbau des Holoenzyms und für dessen Aktivität (Holt et al., 1999). Im Gegensatz zu TERC wird TERT wird nicht in allen Geweben exprimiert, was erklärt, dass Telomeraseaktivität nicht in allen Geweben nachweisbar ist (Liu, 1999). Besonders in hochregenerativen Geweben ist die Telomeraseaktivität sehr hoch, z.B. in Stammzellen, Progenitorzellen, Lymphozyten, Hautkeratinozyten und Krebszellen. Interessanterweise wird

auch in Endothelzellen TERT exprimiert. In unserer Gruppe konnten wir in den verschiedenen Zellkompartimenten von Endothelzellen, Zytosol, Nukleus und Mitochondrium, Telomeraseaktivität nachweisen (**Jakob** und Haendeler, 2007). In vivo konnte im Laufe der Endothelzellalterung, induziert durch wiederholtes Passagieren der Zellen, der Verlust von Telomeraseaktivität beobachtet werden. Dies geht einher mit der Verkürzung der Telomere, was letztendlich zur replikativen Seneszenz der Endothelzellen führt (Vasa et al., 2000).

Nukleäres TERT besitzt unabhängig von seiner Rolle der Telomerverlängerung weitere Funktionen. In humanen Fibroblasten wurde eine antiapoptotische Wirkung nachgewiesen (Gorbunova et al., 2002). TERT-überexprimierende Fibroblasten zeigten eine erhöhte Apoptoseresistenz gegenüber H_2O_2, UV- und γ-Strahlung. Diese antiapoptotische Wirkung konnte auch in Endothelzellen nachgewiesen werden (Haendeler et al., 2003a).

In verschiedenen Studien wurde außerdem gezeigt, dass katalytisch aktives TERT die Regulation verschiedener Gene modulieren kann, die eine Rolle im Zellwachstum spielen. Ein Beispiel ist die DNA Methyltransferase 1 (Dnmt1). Mit TERT imortalisierte kapillare Endothelzellen von Rindern zeigen eine erhöhte Expression dieses Enzyms. Dies führt zu Methylierung der DNA und zur Inaktivierung weiterer Gene, die das Zellwachstum negativ regulieren (Veitonmaki et al., 2003).

Da die Telomerase eine zentrale Rolle in der Zellalterung, aber auch in der Krebsentstehung spielt, ist die Regulation dieses Enzyms von entscheidender Bedeutung. Wie bereits erwähnt wird TERC in fast allen, TERT hingegen nur in wenigen Geweben exprimiert. Dadurch wird TERT und seine Regulation zum „kritischen Element“ der Telomeraseaktivität. Es gibt sowohl transkriptionelle wie auch posttranslationale Mechanismen der Regulation von TERT. Auf transkriptioneller Ebene wird TERT durch diverse Transkriptionsfaktoren aktiviert. Hierzu zählen z. B. c-Myc (Wang et al., 1998) und Sp1 (Kyo et al., 2000), aber auch HIF-1 (hypoxia inducible factor 1) und AP-1 (activating enhancer-binding protein-1) (Kyo et al., 2008). Auch das Molekül NO kann die TERT-Expression und somit die Telomeraseaktivität beeinflussen. Vasa und Kollegen konnten eine Erhöhung der Telomeraseaktivität durch Zugabe von NO-Donoren feststellen (Vasa et al., 2000). Auch die Überexpression der eNOS zeigt diesen Effekt (Hayashi et al., 2006). Der Mechanismus dahinter ist noch nicht hinreichend bekannt. Es wird jedoch eine Verbindung zum Östrogen Rezeptor α angenommen, der zusammen mit der eNOS einen Komplex bildet und im Nukleus an den Promotor des TERT-Gens bindet und somit die Expression von TERT-Protein induziert (Grasselli et al., 2008).

Auf der Ebene der posttranslationalen Regulation stehen Phosphorylierung, Bindung an andere Proteine und die zelluläre Lokalisation, wobei diese Ereignisse oft miteinander verbunden sind. In Tumorzellen wird TERT mittels Phosphorylierung durch die Kinasen PKCα (proteine kinase α) (Li et al., 1998), ERK1/2 (extracellular regulated kinases 1 and 2)

(Seimiya et al., 1999) und Akt (Kang et al., 1999) aktiviert. In Endothelzellen konnte ebenfalls die Aktivierung durch Akt nachgewiesen werden (Breitschopf et al., 2001). Um die Aktivität von TERT aufrecht zu erhalten, ist die Bindung an HSP90 notwendig, da dieses die Dephosphorylierung von Akt und somit dessen Inaktivierung durch die Phosphatase PP2A verhindern kann. Dies führt zur Bildung eines nukleären Proteinkomplexes aus TERT, Akt und HSP90 (Haendeler et al., 2003b). Dieser Proteinkomplex ist essentiell zur Aufrechterhaltung der nukleären Telomeraseaktivität, da TERT in Endothelzellen durch Akt an Serin 823 phosphoryliert und damit aktiviert wird (Abb. 1).

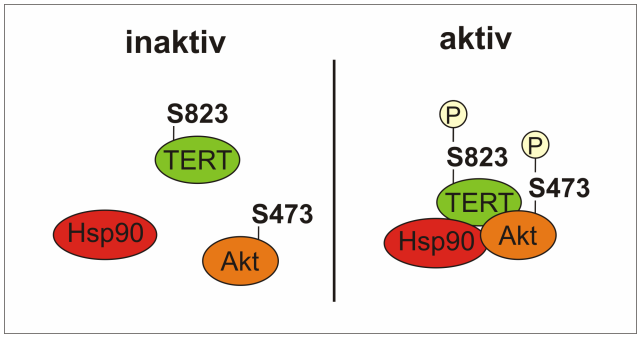

Abb. 1: Nukleärer Komplex aus Akt, Hsp90 und TERT. Akt phosphoryliert TERT am Serin 823, was zu dessen Aktivierung führt. Hsp90 schützt Akt vor der Inaktivierung.

Eine weiterer wichtiger Mechanismus der Telomeraseregulation ist die zelluläre Lokalisation von TERT. In unserer Gruppe wurde gezeigt, dass TERT unter Bedingungen des oxidativen Stresses aus dem Kern exportiert wird. Dieser Export wird durch Kinasen der Src-Kinase Familie vermittelt, die TERT an Tyrosin 707 phosphorylieren, wodurch TERT aus dem Kern exportiert wird. Der Export geschieht über die Kernporen und ist abhängig von der nukleären GTPase Ran und dem Export Rezeptor CRM-1 (chromosome region maintenance 1) (Haendeler et al., 2003a). CRM-1 gehört zur Gruppe der nukleären Transport Rezeptoren, die an Komplexen der Kernporen binden können. Des Weiteren besitzt CRM-1 eine Bindestelle für die GTPase Ran und für die jeweiligen, zu transportierenden Zielproteine. Für CRM-1 wurde gezeigt, dass es an TERT bindet (Haendeler et al., 2003a; Seimiya et al., 2000). Zusammen mit der GTPase Ran ist es für den Export von TERT verantwortlich (Abb. 2). Die Konsequenzen für die Zelle

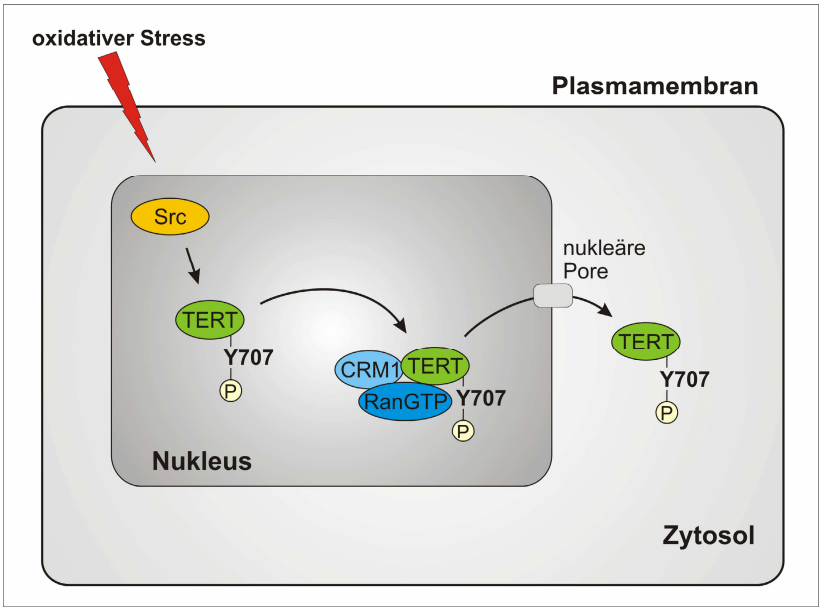

Abb. 2: Nukleärer TERT-Export. Oxidativer Stress induziert den nukleären TERT-Export. Oxidativer Stress erhöht die Aktivität der Src-Kinasen, was zur Phosphoyrlierung von TERT führt. In Folge dessen wird TERT abhängig von CRM1 und der GTPase Ran über die Kernpore exportiert.

sind eine erhöhte Sensitivität gegenüber Apoptose und eine beschleunigte Seneszenz.
In unsere Arbeitsgruppe konnten wir zeigen, dass es möglich ist, der Seneszenz von Endothelzellen durch Behandlung mit Antioxidantien wie N-Acetylcystein entgegenzuwirken (Haendeler et al., 2004). Außerdem führt eine Erhöhung der eNOS Aktivität durch Statine (Laufs et al., 1998) zu einer Verzögerung der Seneszenz von Endothelzellen (Haendeler et al., 2004). Statine sind HMG-CoA Reduktase Hemmer, die vornehmlich zur Senkung des Cholesterinsspiegels eingesetzt werden, von denen aber auch andere pleiotrope Effekte beschrieben wurden (Adam und Laufs, 2008). Es ist ebenfalls möglich, den nukleären TERT-Export und damit die Senszenz von Endothelzellen durch die Inhibition der Aktivität der Src-Kinasen mit dem Src-Kinase-Inhibitor PP2, zu verhindern (Haendeler et al., 2004).

2.1. Die Src-Kinase Familie induziert den nukleären TERT-Export

Die Familie der Src-Kinasen besteht aus mindestens neun Mitgliedern von Nicht-Rezeptortyrosinkinasen. Während einige Mitglieder, z.B. Src, Yes und Fyn in allen Zellen des Körpers zu finden sind, zeigen andere ein begrenztes, gewebespezifisches Expressionsmuster (Parsons und Parsons, 2004). Gerade die ubiquitär exprimierten Src-Kinasen zeigen die Eigenschaft, füreinander kompensieren zu können (Stein et al., 1994). Sie beeinflussen den Metabolismus der Zelle, die Zellteilung, das Zellüberleben und die Zellmigration (Parsons und Parsons, 2004). Wie alle Src-Kinasen spielen sie eine wichtige Rolle in der Signaltransduktion und vermitteln die Signalweiterleitung von Rezeptortyrosinkinasen zu anderen Kinasekaskaden, was letztendlich eine Zellantwort zur Folge hat. Die Kinase Src interagiert unter anderem mit dem PDGF (platelet derived growth factor)-Rezeptor, der durch den Wachstumsfaktor PDGF (platelet derived growth factor) stimuliert wird. Die Interaktion führt zur Phosphorylierung des Tyrosins 416 in Src, was seine Aktivierung zur Folge hat. Aktives Src phosphoryliert verschiedene andere Kinasen und reguliert so verschiedene Signalwege (Roskoski, 2004). Auch eine Erhöhung von ROS führt zu einer Aktivierung der Src-Kinase und einer damit verbundenen Signalweiterleitung. Allerdings wurde vor kurzem ein neuer Mechanismus entdeckt, in dem Src-Kinasen in Folge einer reversiblen Oxidation inaktiviert werden. Diese Oxidation findet am Cystein 277 in der katalytischen Domäne von Src statt, was eine Homodimerisierung von Src über eine Disulfidbrücke zur Folge hat. Diese spezifische Inaktivierung über das Cystein ist nur in drei Mitgliedern der Src-Kinase Familie zu finden, Src, Yes und Fgr, woraus man schließen kann, dass es sich herbei um einen spezifischen Regulationsmechanismus für bestimmte Kinasen handelt (Kemble und Sun, 2009; Sun und Kemble, 2009). Dies zeigt, das Src in komplexer Weise auf Zustände des oxidativen Stresses reagiert und wie wichtig somit die Aufrechterhaltung des Redoxgleichgewichts in der Zelle letztendlich ist.

Ein deutliches Beispiel der Folgen eines Ungleichgewichts im Redoxzustand der Zelle konnte unserer Arbeitsgruppe zeigen. Die erhöhte Aktivität der Src-Kinase Familie, ausgelöst durch oxidativen Stress, induziert den nukleären Export von TERT. Dies geschieht durch eine Tyrosinphosphorylierung von TERT am Aminosäurerest 707 durch Mitglieder der Src-Kinase Familie (Haendeler et al., 2003a). Da die Kinasefamilie recht groß ist, habe ich den Versuch unternommen, die Zahl der beteiligten Kinasen einzugrenzen. Zu diesem Zweck nutzte ich embryonale Mausfibroblasten, die einen dreifach-knockout der Kinasen Src, Fyn und Yes aufwiesen. Wie bereits erwähnt, sind diese drei Kinasen ubiquitär exprimiert und kompensieren sich gegenseitig (Stein et al., 1994). Der dreifach knockout zeigte diese Kompensation nicht mehr (Klinghoffer et al., 1999). Ich konnte in diesen Fibroblasten zeigen, dass es in diesen Zellen nicht mehr zu einem nukleären Export von TERT durch oxidativen Stress kommt (**Jakob** et al., 2008). Des Weiteren konnte ich die verantwortlichen Kinasen noch weiter eingrenzen, da ich nur die Kinasen Src und Yes im Nukleus nachweisen konnte, nicht jedoch Fyn. Da für die Phosphorylierung von TERT eine räumliche Nähe zu der Kinase unabdingbar ist, können nur Src und/oder Yes für den Export verantwortlich sein, nicht jedoch Fyn.

Bei dem Export von nukleärem TERT durch einen Anstieg der Src-Kinase Aktivität in Folge von ROS muss es sich um einen regulierten Prozess handeln. Ein kurzzeitiger Anstieg von ROS, der durch die intakten antioxidativen Systeme wieder herabreguliert wird, führt nicht zu einem endgültigen Verlust der nukleären Telomeraseaktivität. Der Prozess des nukleären Exportes muss also reversibel oder aufhaltbar sein. Das bedeutet, dass es entweder zu einem erneuten Import von TERT in den Nukleus kommt, oder dass der Export als solches verhindert wird. Ein möglicher Gegenspieler zu dem nukleären Export ist die Protein Tyrosin Phosphatase Shp-2.

2.2. Shp-2 als Gegenspieler des nukleären TERT-Exports

Shp-2 ist eine ubiquitär exprimierte zytosolische Phosphatase, von der bekannt ist, dass sie die Aktivität der Src-Kinase Familie regulieren kann (Doan et al., 2004). Die Bedeutung von Shp-2 für die Entwicklung der Zelle wird durch die Tatsache unterstrichen, dass Shp-2 knockout Mäuse embryonal letal sind (Saxton et al., 1997). Shp-2 enthält zwei N-terminalen Src Homologie 2 (SH2) Domänen und eine nahe zum C-Terminus liegende Protein Tyrosin Phosphatase Domäne. Dem C-Terminus des Shp-2 Proteins selbst wird bisher noch keine klare Rolle zugeschrieben. Es existieren verschiedene Tyrosin- und Serinphosphorylierungsstellen in diesem Bereich, die bislang kontrovers diskutierte Rollen aufweisen (Abb. 3). So wird für die Tyrosinphosphorylierungen einerseits eine Rolle für die Bindung an Adapterproteine angenommen (Bennett et al., 1994; Vogel und Ullrich, 1996), andererseits ein Einfluss auf die Shp-2-Aktivität, der jedoch sehr moderat zu sein scheint (Lu

et al., 2001). Die Rolle der N-terminalen SH-Domänen hingegen ist gut beschrieben. Sie sind wesentlich an der Regulation der Phosphatasefunktion beteiligt. Im inaktiven Zustand der Phosphatase haben die N-terminalen SH-Domänen direkten Kontakt mit der katalytischen Domäne. Teile der N-terminalen Domäne binden an geladene Reste um das katalytische Zentrum herum und ein Teil der SH-Domäne ragt ins katalytische Zentrum der Phosphatasedomäne. Auf diese Weise ist der Zugang von Substraten zum aktiven Zentrum blockiert. Durch Bindung von Phosphopeptiden, z. B. als Teil phosphorylierter Proteine, kommt es zu einer Konformationsänderung in der SH-Domäne, welche die Interaktionen zwischen SH-Domäne und Phosphatasedomäne unterbricht. Das aktive Zentrum ist daraufhin zugänglich für die Substrate von Shp-2.

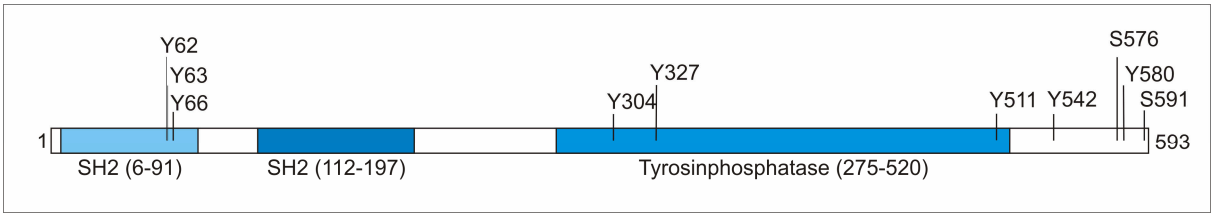

Abb. 3: Shp-2 Struktur. Im N-terminalen Bereich besitzt Shp-2 zwei SH2 Bindedomeänen, die Phosphatasedomäne befindet sich in der Nähe des C-terminalen Bereichs. Der C-Terminus selber besitzt verschieden Tyrosin- und Serin-Phosphorylierungsstellen.

Die Funktionen von zytosolischem Shp-2 sind bereits sehr gut untersucht und zeigen die wichtige Rolle von Shp-2 in vielen Signalwegen. Es ist bekannt, dass Shp-2 in verschiedene Zellsignalwege involviert ist und eine Rolle beim Zellwachstum, in der Zellentwicklung, bei Entzündungsreaktionen und bei der zellulären Chemotaxis spielt (Chong und Maiese, 2007). Der molekulare Mechanismus bei all diesen Funktionen läuft über die Dephosphorylierung von Zielproteinen. Um jedoch als Gegenspieler für den nukleären Export von TERT in Frage zu kommen, ist die physikalische Nähe zu TERT notwendig. Dazu muss sich Shp-2 im Kern befinden. 2002 konnte Chughtai mit seinen Kollegen zeigen, dass Shp-2 im Nukleus lokalisiert ist und dass es dort mit STAT5 (signal transducer and activator of transcription) assoziiert. Diese Beobachtungen wurden in Brustkrebszellen gemacht, in denen Shp-2 nach der Stimulation mit Prolactin einen Komplex mit STAT5 eingeht, der in den Nukleus translozierte. Die Bildung dieses Komplexes ist abhängig von einer Tyrosinphosphorylierung von STAT5. Der STAT5/Shp-2 Komplex bindet an die DNA und beeinflusst die Transkription von Genen der Milchproteine. Dieser Mechanismus ist unabhängig von der Funktion von Shp-2 als Phosphatase. Hier ist lediglich die Bindung und Erkennung von phosphoryliertem STAT5 wichtig. Allerdings wurde in einer anderen Studie eine phosphataseabhängige Funktion von Shp-2 im Nukleus gezeigt (Wu et al., 2002). In dieser wurde nachgewiesen, dass die Dephosphorylierung von STAT1 im Nukleus an Tyrosin- und Serinresten durch Shp-2 eine Inhibition der transkriptionellen Aktivität von STAT1 zur Folge hatte (Abb. 4).

Diese Arbeiten zeigten, dass Shp-2 zwei wichtigen Voraussetzungen erfüllen kann, um ein Gegenspieler des nukleären TERT-Export zu sein: Eine nukleäre Lokalisation und eine dort auftretende phosphataseabhängige Funktion.

Da die bisherigen Lokalisationsstudien in Tumorzellen durchgeführt wurden, untersuchte ich als erstes die zelluläre Lokalisation von Shp-2 in Endothelzellen. Ich konnte Shp-2 sowohl in der nukleären als auch in der zytosolischen Fraktion von HUVECs (human umbilical vein endothelial cells, humane venöse Nabelschnurendothelzellen) nachweisen. Über Immun-präzipitationsstudien konnte ich zudem zeigen, dass Shp-2 und TERT im Kern miteinander assoziiert sind. Somit war eine weitere notwendige Voraussetzung für eine Regulation von TERT, die physikalische Nähe zu diesem Protein, erfüllt. Da der TERT-Export abhängig von ROS ist, muss Shp-2 selbst durch oxidativen Stress reguliert werden. Ich konnte eine Reduktion der Proteinmenge von Shp-2 und seiner Aktivität in Folge einer Behandlung mit derselben H_2O_2-Konzentration nachweisen, die auch zu einem nukleären Export von TERT führt. Diese Reduktion der Shp-2-Aktivität stimmt mit Studien überein, die zeigen, dass Shp-2, wie andere Proteintyrosinphosphatasen, am katalytischen Cystein oxidiert werden kann. Diese reversible Oxidation kann über weitere Oxidationsstufen zu einer irreversiblen Oxidation und somit zu einem kompletten Verlust der Phosphataseaktivität führen. Shp-2 kann sich vor dieser irreversiblen Oxidation mittels zweier Cysteine schützen, die eine Disulfidbrücke bilden und somit das reduzierte, katalytisch aktive Cystein abschirmen (Chen et al., 2009). Um die Beteiligung von Shp-2 an dem nukleären Export von TERT nachzuweisen, führte ich Überexpressionsstudien durch. Ich verwendete hierzu ein Shp-2wt Konstrukt, welches für ein Shp-2-myc Fusionsprotein kodiert. Dieses Fusionsprotein zeigte die gleiche Zellkompartimentverteilung wie endogenes Shp-2. Die Überexpression von Shp-2wt konnte sowohl die Proteinmenge von TERT als auch dessen enzymatische Aktivität im Kern unter oxidativem Stress, künstlich hervorgerufen durch H_2O_2-Behandlung, aufrechterhalten (**Jakob** et al., 2008). Des Weiteren konnte ich nachweisen, dass hierfür die katalytische Aktivität von

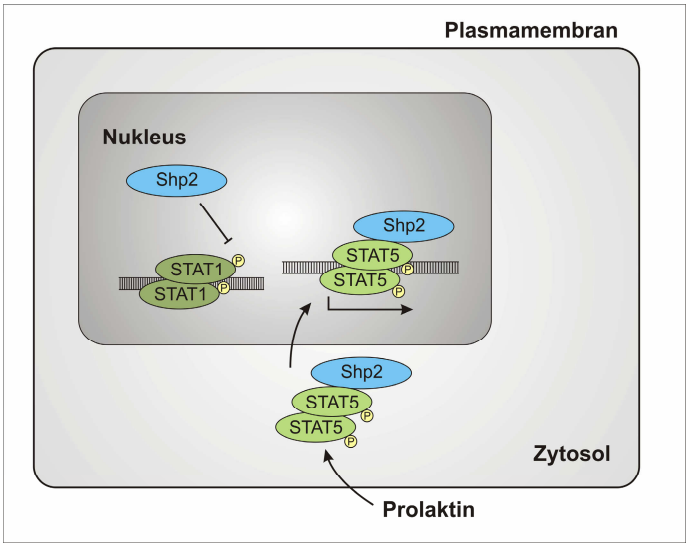

Abb. 4: Shp-2 und STAT. Shp-2 dephosphoryliert STAT1 im Nukleus und inhibiert dadurch dessen transkriptorische Aktivität. Die Stimulation von Brustkrebszellen mit Prolaktin führt zu einer Komplexbildung von STAT5 und Shp-2 und zu einem nukleären Import dieses Komplexes, der an die DNA bindet und die Genexpression von Milchproteinen reguliert.

Shp-2 notwendig ist. Die Überexpression einer dominant-negativen, katalytisch inaktiven Mutante von Shp-2, in der Cystein 459 gegen Serin ausgetauscht wurde (Shp-2(C459S)), die dieselbe Proteinverteilung zwischen Zytosol und Nukleus aufwies wie endogenes Shp-2, führte bereits ohne H_2O_2 Behandlung zu einem Verlust von TERT-Protein im Kern. Zusätzlich wurde von mir die Telomeraseaktivität in nukleären Extrakten aus Zellen, die entweder Shp-2wt oder Shp-2(C459S) überexprimierten, untersucht. Als Ergebnis zeigte sich, dass die Überexpression des Wildtyps zu einer Erhöhung der Telomeraseaktivität führte, wohingegen die Überexpression der Mutante die Reduktion der Telomeraseaktivität zur Folge hatte. Dieser Effekt scheint spezifisch für die Phosphatase Shp-2 zu sein, da eine andere Phosphatase, in diesem Fall PP2A keinen Effekt auf nukleäres TERT-Protein und nukleäre Telomeraseaktivität hatte. Die Überexpression von Shp-2wt konnte zudem den Verlust von Telomeraseaktivität, hervorgerufen durch H_2O_2-Behandlung, komplett verhindern. Die Überexpression von inaktivem Shp-2 jedoch reduzierte die durch H_2O_2 verringerte nukleäre Telomeraseaktivität zusätzlich. Für die Aufrechterhaltung von nukleärem TERT-Protein und nukleärer Telomeraseaktivität ist daher die Anwesenheit von katalytisch aktivem Shp-2 notwendig. Dies deckt sich mit der Beobachtung, dass eine H_2O_2-Behandlung Shp-2-Protein und Aktivität reduziert. Aufgrund dieser reduzierten Aktivität kann Shp-2 unter oxidativen Stressbedingungen den nukleären TERT Export nicht verhindern.

Der nächste Schritt war die Untersuchung des Mechanismus, mit dem Shp-2 den Export von TERT verhindert. Da es sich bei Shp-2 um eine Phosphatase handelt, lag es nahe, von einer Dephosphorylierung auszugehen. Wie bereits erwähnt, wird der TERT Export durch die Phosphorylierung von TERT an Tyrosin 707 induziert. Die Mutation dieses Tyrosins zu einem Phenylalanin (TERT(Y707F)) verhindert die Phosphorylierung und somit den nukleären Export von TERT, der durch oxidativen Stress ausgelöst wird (Haendeler et al., 2003a). Ich untersuchte nun, in welchem Zusammenhang diese Phosphorylierungsstelle von TERT mit Shp-2 stand. Ich konnte zeigen, dass die Assoziation von TERT mit Shp-2 durch die Phosphorylierung in Folge von oxidativem Stress zunimmt. Die nicht phosphorylierbare Mutante TERT(Y707F) zeigte keine Assoziation mit Shp-2, auch nicht in Gegenwart von H_2O_2. Auch auf die nukleäre Telomeraseaktivität von Zellen, die TERT(Y707F) überexprimieren, hatte weder Shp-2wt noch Shp-2(C459S) einen Einfluss. Dies deutet darauf hin, dass das Tyrosin 707 ein Ziel der Shp-2 Phosphataseaktivität ist. Diese Vermutung wird unterstützt von der Tatsache, dass die Verringerung der Shp-2 Menge durch Expression einer Shp-2 spezifischen shRNA zu einer Erhöhung der Tyrosinphosphorylierung von TERTwt in Gegenwart von H_2O_2 führt. Im Gegensatz dazu hat die Herabregulation von Shp-2 keinen Einfluss auf die Tyrosinphosphorylierung von TERT(Y707F). Diese Ergebnisse lassen vermuten, dass tyrosinphosphoryliertes TERT selbst das Ziel von Shp-2 ist und dass die Dephosphorylierung von TERT an Tyrosin 707 durch Shp-2 den nukleären Export von

TERT verhindert. Es ist jedoch nicht auszuschließen, dass Shp-2 auch einen Einfluss auf die Src-Kinasen hat, die für die Phosphorylierung von TERT verantwortlich sind. Da gezeigt wurde, dass diese Kinasen von Shp-2 reguliert werden können, wäre es alternativ auch möglich, dass Shp-2 deren Aktivität herabreguliert, wodurch es gar nicht erst zu einer Phosphorylierung von TERT und somit zu seinem Export kommt (Abb. 5). Auch die Bildung eines ternären Komplexes aus TERT, Src und Shp-2 wäre denkbar. In diesem Komplex könnte Shp-2 die Aktivität von Src inhibieren und somit die Phosphorylierung von TERT verhindern.

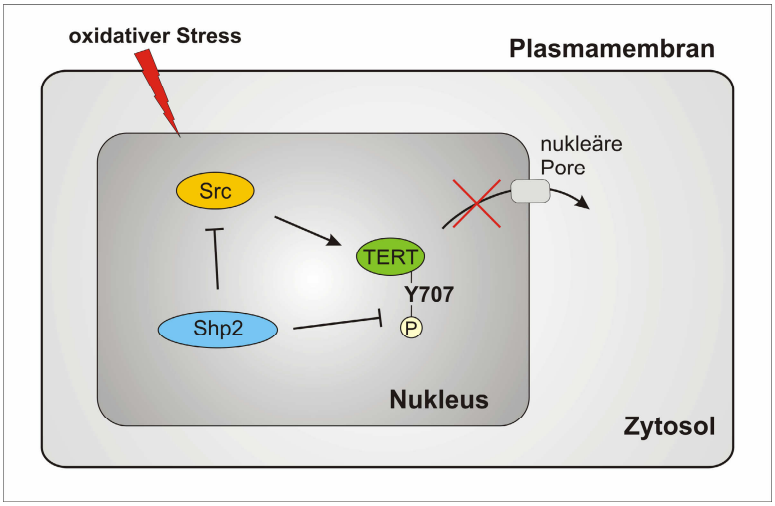

Abb. 5: Shp-2 inhibiert den nukleären TERT-Export. Dies geschieht entweder durch die Inhibition der Aktivität der Src-Kinasen mittels Shp-2 oder aufgrund der Dephosphorylierung von TERT durch Shp-2.

Durch die Aufrechterhaltung von TERT im Nukleus in Gegenwart von oxidativem Stress kann Shp-2 möglicherweise apoptotische Vorgänge inhibieren. Auf einen antiapoptotischen Effekt deuten auch erste Apoptosemessungen in Endothelzellen nach einer Überexpression mit Shp-2wt hin. Diese präliminären Daten zeigen, dass die Überexpression von Shp-2wt zu einer Reduktion der Apoptosesensitivität gegenüber H_2O_2 führt.

Weiterhin könnte nukleäres Shp-2 auch eine wichtige Rolle in Alterungsprozessen, zumindest in Endothelzellen, spielen. Es kann die Telomeraseaktivität durch die Inhibition des nukleären TERT-Exports aufrechterhalten. Da sowohl die Aktivität als auch die Proteinmenge von Shp-2 durch ROS herabreguliert wird, kann endogenes Shp-2 im Alter, aufgrund der erhöhten ROS-Level, den TERT-Export nicht verhindern. Eine Erhöhung der nukleären Shp-2 Aktivität könnte dem entgegenwirken und zur Aufrechterhaltung der Telomeraseaktivität beitragen. Diese Aktivitätserhöhung müsste jedoch spezifisch für den Nukleus sein, um zytosolisches Shp-2 nicht zu beeinflussen. Eine Möglichkeit der Erhöhung wäre die Steigerung des Imports von Shp-2 in den Nukleus. Um dies zu erreichen, ist es allerdings notwendig, den nukleären Importmechanismus aufzuklären. Dieser Fragestellung wird im Ausblick nachgegangen.

3. Mitochondrielle ROS-Produktion

Die bisherigen Beobachtungen waren verbunden mit einer Erhöhung von ROS in Endothelzellen. Eine der wichtigsten Quellen für endogene ROS sind die NADPH-Oxidasen (NOX). In Endothelzellen konnten bisher vier Mitglieder dieser Familie nachgewiesen werden: Nox1, jedoch unter physiologischen Bedingungen nur sehr schwach, Nox2, Nox4 und Nox5. Die stärkste Expression weist NOX4 auf, aber auch NOX2 ist in Endothelzellen exprimiert. NOX4 ist vor allem Im Nukleus zu finden, wohingegen NOX2 hauptsächlich an der Plasmamembran lokalisiert ist (Ushio-Fukai und Nakamura, 2008). In gealterten Endothelzellen konnte in unserer Arbeitsgruppe zudem ein Anstieg von NOX4 beobachtet werden. Die Rolle der NOX5 in Endothelzellen ist noch unklar. Eine weitere Hauptquelle für die ROS-Entstehung sind die Mitochondrien. Hier werden sie im Zuge der Respiration von den Komplexen I und III der Atmungskette gebildet. Komplex I überträgt Elektronen von NADH über das Coenzym Q10 auf den Komplex III. Von hier werden sie mittels Cytochrom C auf den Komplex IV transferiert, wo sie auf O_2 übertragen werden wodurch O_2 zu H_2O reduziert wird. Der Elektronentransfer ist verbunden mit einem Transport von Protonen über die Mitochondrienmembran von der Matrix in den Intermembranraum an den Komplexen I, III und IV, wodurch ein elektrochemischer Protonengradient aufgebaut wird. Der Rückfluss der Protonen über den Komplex V (ATP-Synthase) führt zur Bildung von ATP. Durch das fehlerhafte Übertragen der Elektronen von Komplex I bzw. III auf O_2 entstehen $O_2^{\cdot-}$-Radikale. Die Level der $O_2^{\cdot-}$- Radikale werden durch die lokale O_2 Konzentration beeinflusst und durch die Effizienz des mitochondriellen Elektronentransports, der sich im elektrochemischen Gradienten widerspiegelt. Die $O_2^{\cdot-}$-Radikale können im Mitochondrium durch die Mangansuperoxiddismutase zu H_2O_2 dismutiert werden. Das H_2O_2 wird von der Gluthathion Peroxidase oder vom Peroxiredoxin 3/Thioredoxin 2 System zu H_2O reduziert (Thomas et al., 2008). Die antioxidativen Systeme sind in den einzelnen Zellkompartimenten unterschiedlich. Im Zytosol stehen die Superoxiddismutasen, Katalasen, Glutathion Peroxidasen, Glutaredoxin und das Thioredoxinsystem im Vordergrund und im Nukleus sind besonders Trx-1 und die APEX nuclease 1(APEX 1) hervorzuheben (Lukosz*, **Jakob*** et al., 2009).

Im Alter kommt es zu einer Erhöhung der Sauerstoffradikale und damit verbunden zu Fehlfunktionen in den Mitochondrien (Zellen). Dies wird mit der Alterungstheorie der freien Radikale erklärt (Harman, 1956), nach der freie Radikale zu Schäden an der DNA, insbesondere an der mitochondriellen DNA (mtDNA), Proteinen und Membranlipiden führen. Besonders die mtDNA ist aufgrund ihrer Nähe zur Elektronentransportkette oxidativen Schäden ausgesetzt (Miquel, 1991). Diese Schäden manifestieren sich unter anderem in Form von Basenmodifikationen. Die Konsequenz sind fehlerhaft oder gar nicht mehr transkribierte mitochondrielle Gene. Der größte Teil der Komplexe der Atmungskette ist auf

der genomischen DNA kodiert, allerdings liegen Gene für 13 Untereinheiten der Atmungskettenkomplexe auf dem Mitochondriengenom (Mandavilli et al., 2002). Daher führen Schäden an der mtDNA und somit an Genen dieser Untereinheiten zu fehlerhaften Komponenten der Atmungskette. Dies kann zur Folge haben, dass mehr $O_2^{-\cdot}$-Radikale gebildet werden, was zu weiteren Schäden an der mtDNA führt. Somit entsteht eine Art Teufelskreis, der sich in vielen Alterungsprozessen niederschlägt (Mandavilli et al., 2002). Das Aufhalten dieses Teufelskreis könnte somit ein interessanter Therapieansatz für altersinduzierte Krankheiten sein. Zwei Möglichkeiten dafür werden nun im Folgenden diskutiert: 1. Aufrechterhaltung der Integrität der mtDNA; 2. Reduktion nicht-mitochondrieller und mitochondrieller ROS.

3.1. Aufrechterhaltung der mtDNA

Ein möglicher Ansatz könnte die Aufrechthaltung der mtDNA Integrität sein. Die durch ROS verursachten Schäden sind hier sehr viel größer, als bei der genomischen DNA, da die mitochondrielle DNA eine natürliche, größere Nähe zur Elektronentransportkette aufweist (Mandavilli et al., 2002). Es sind bereits Reparaturmechanismen für geschädigte mtDNA bekannt. Einer davon ist der mitochondrielle „base excision repair“. Dieser wird initiiert durch eine Glykolase, welche die glykosidische-Bindung zwischen der beschädigten Base und dem Zucker löst. Viele Glykolasen besitzen eine assoziierte Lyaseaktivität, die das Phosphodieesterrückgrat 3’ zur abasischen Seite spaltet. Die Apurinische/apyrimidinische (AP) Endonuklease kann dann die 5’-Phosphodiesterbindung hydrolysieren. Die dadurch entstehende ein Basenlücke wird von der DNA Polymerase γ aufgefüllt und von DNA Ligase III verschlossen. Sämtliche hierfür benötigten Enzyme konnten im Mitochondrium nachgewiesen werden (Mandavilli et al., 2002). Es konnte bereits gezeigt werden, dass Mäuse defizient für die mitochondrielle DNA Polymerase γ erhöhte mtDNA Schäden und einen Alterungsphänotyp aufweisen (Trifunovic et al., 2004). Daher wäre eine Aktivierung von mtDNA Reparaturenzymen ein möglicher therapeutischer Ansatz.

Interessanterweise wurde auch TERT in den Mitochondrien gefunden. Im Jahr 2004 konnten Santos und Kollegen TERT in den Mitochondrien nachweisen. Sie transfizierten hierfür ein Expressionskonstrukt für „enhanced green fluorescent protein“-gekoppeltes TERT in primäre Fibroblasten und konnten mittels Fluoreszenzmikroskopie eine mitochondrielle Lokalisation detektieren. Sie konnten zudem in Fibroblasten-Zelllinien, die nach retroviraler Transduktion TERT überexprimierten, eine mitochondrielle Telomeraseaktivität nachweisen. In diesen Zellen führte die Expression von TERT zu einer erhöhten Sensitivität gegenüber H_2O_2 induzierten DNA-Schäden und Apoptose (Santos et al., 2004; Santos et al., 2006). Im Gegensatz dazu haben Ahmed und Kollegen 2008 gezeigt, dass mitochondrielles TERT die Mitochondrien schützt. Auch sie konnten in Fibroblasten, die TERT überexprimieren, eine

mitochondrielle Lokalisation von TERT feststellen. In diesen Zellen kam es unter Bedingungen des oxidativen Stresses zu einem Export von TERT aus dem Nukleus, was wir auch für Endothelzellen nachweisen konnten (s. Kapitel 2) und im Gegenzug zu einer Akkumulation von TERT in den Mitochondrien. Die Autoren schließen daraus auf eine Translokation von TERT aus dem Nukleus ins Mitochondrium unter Bedingungen des oxidativen Stresses. Im Gegensatz zur Gruppe um Santos zeigte das Labor von Saretzki, dass mitochondrielles TERT die mitochondriellen ROS reduziert (Ahmed et al., 2008). In Übereinstimmung mit dieser Beobachtung konnten auch wir einen schützenden Effekt von mitochondriellem TERT auf die Mitochondrien in Endothelzellen zeigen. In unserer Studie konnten wir sowohl TERT-Protein als auch Telomeraseaktivität in den Mitochondrien nachweisen. Innerhalb der Matrix bindet TERT an die mitochondrielle DNA und zwar in einer Region, welche für Untereinheiten des Komplexes I der Atmungskette kodiert. Vermutlich ist die Bindung von TERT an die mtDNA mitverantwortlich für den oben erwähnten Schutz vor mtDNA-Schäden. Wir konnten zeigen, dass TERT in vitro den Abbau von mitochondrieller DNA, induziert durch UV-Strahlung, verhindert. In vivo konnten wir nachweisen, dass Fibroblasten von TERT knockout (TERT-/-) Mäusen eine erhöhte Sensitivität gegenüber UV-Strahlung aufwiesen bezüglich ihrer mitochondriellen Aktivität aufwiesen als entsprechende Wildtypzellen. Um auch die in vivo Relevanz dieser Ergebnisse nachzuweisen, untersuchten wir die Atmungskettenaktivität intakter Mitochondrien, welche aus Herz- und Leberzellen von TERT-/- Mäusen und den entsprechenden Wildtyp Tieren isoliert wurden. Tatsächlich konnten wir eine Reduktion der Atmungskettenaktivität in Mitochondrien aus Herzzellen der TERT-/- Tiere beobachten, jedoch nicht in den Mitochondrien der Leberzellen. Dies deutet auf einen gewebespezifischen Effekt von TERT hin.

Des Weiteren konnte ich im Gegensatz zu Santos et al. aber in Übereinstimmung mit Ahmed et al. eine Reduktion von mitochondriell gebildeten ROS durch TERT nachweisen, da die Herabregulation von TERT mittels shRNA zu einer Erhöhung des mitochondriellen ROS-Spiegels führte. Dies spiegelt sich auch umgekehrt in der reduzierten Sensitivität gegenüber H_2O_2-induzierter Apoptose in TERT überexprimierenden Zellen wider. Dieser Effekt wird durch Überexpression von TERT, das durch Fusion mit einer starken mitochondriellen Lokalisierungssequenz exklusiv in Mitochondrien transportiert wird, noch gesteigert. In diesen Zellen wird die H_2O_2-induzierte Apoptose komplett verhindert (Haendeler, Drose, Buechner, **Jakob** et al., 2009). Diese Daten lassen vermuten, dass mitochondrielles TERT eine Schlüsselrolle im Schutz vor Schäden an der mtDNA, mitochondriellen Fehlfunktionen und Apoptoseprozessen spielt (Buechner, Altschmied, **Jakob** et al., 2009). Für mögliche Therapieansätze, um die Schutzfunktion für die Mitochondrien durch TERT in Endothelzellen auch im Alter zu gewährleisten, wäre eine Erhöhung des mitochondriellen TERT-Spiegels zu überlegen. Hierfür wäre es notwendig, zuerst die Regulationsmechanismen von TERT in den

Mitochondrien zu untersuchen. Eine andere Möglichkeit wäre die Steigerung des Imports von TERT in die Mitochondrien. Auch hierzu müssten erst die Importmechanismen komplett aufgeklärt werden. Bei einem derartigen Ansatz wäre zu beachten, wie sich eine damit verbundene Depletion von nukleärem TERT auswirkt.

3.2. Reduktion von reaktiven Sauerstoffspezies

Ein zweiter Ansatz zum Aufhalten des Teufelskreises wäre die Reduktion der ROS selbst. Wie bereits beschrieben, kann mitochondrielles TERT die Produktion von mitochondriellen ROS reduzieren (Haendeler, Drose, Buechner, **Jakob** et al., 2009). Diese Beobachtung konnte ich auch für Shp-2 machen. Die Überexpression von Shp-2 reduzierte nicht nur die Produktion von ROS im Zytosol, sondern auch in den Mitochondrien (**Jakob** et al., 2009). Für die Messung der ROS im Zytosol wurde der Farbstoff H_2DCFDA (2',7'-Dichlorofluorescein Diacetat) verwendet. Dieser reagiert mit den ROS im Zytosol, unter Abspaltung des Diacetats wobei ein Fluoreszenzsignal abgegeben wird, welches unter anderem mittels FACS-Analyse gemessen werden kann. Für die Messung der mitochondriellen ROS wurde der Farbstoff Mitosox verwendet. Dieser Farbstoff wird in die Mitochondrien transportiert, wo er dann mit den dort vorhandenen ROS reagiert. Erste präliminäre Daten zeigen eine Erhöhung der ROS-Produktion sowohl im Zytosol als auch in den Mitochondrien nach einer Herabregulation von Shp-2 mittels shRNA. Dies bedeutet, dass Shp-2 in der Lage ist auch mitochondrielle ROS-Spiegel zu verringern. Dies basiert wahrscheinlich auf einer Lokalisation von Shp-2 in den Mitochondrien. In ersten Untersuchungen konnte ich zeigen, dass Mitochondrien Shp-2 enthalten. Dies steht im Einklang mit Beobachtungen von Arachiche et al, die ebenfalls eine mitochondrielle Lokalisation von Shp-2 nachgewiesen haben (Arachiche et al., 2008). Sie konnten außerdem die Kinase Src in Mitochondrien identifizieren und aufzeigen, dass einige Komplexe der Atmungskette Substrate von Src sind, was darauf hindeutet, dass die Aktivität der Atmungskette zumindest teilweise von Tyrosinphosphorylierungen der Komplexe abhängig ist. Daher ist es möglich, dass auch Shp-2, als Regulator von Src, in die Regulation der Aktivität der Mitochondrien involviert ist. Vor dem Hintergrund, dass auch Src in den Mitochondrien lokalisiert ist, lässt sich ebenfalls spekulieren, dass Shp-2 auch mitochondrielles TERT ebenso positiv regulieren kann wie im Nukleus und somit zusammen mit mitochondriellem TERT an der Aufrechterhaltung einer intakten Atmungskette beteiligt ist.

4. Ausblick

Zusammenfassend konnte ich zeigen, dass TERT in Folge von oxidativem Stress durch die Kinasen Src und/oder Yes aus dem Kern exportiert wird und dass Shp-2 diesen Export verhindern kann. Shp-2 benötigt dafür seine katalytische Aktivität und hat als Ziel das phosphorylierte Tyrosin 707 von TERT entweder direkt oder indirekt über eine Src und/oder Yes Inaktivierung. Auch im Mitochondrium konnte ich Shp-2 nachweisen und dass eine Herabregulation von Shp-2 sowohl nicht-mitochondrielle als auch mitochondrielle ROS reduziert. Daher scheinen sowohl nukleäres als auch mitochondrielles Shp-2 eine wesentliche Rolle für die TERT Regulation zu spielen (Abb. 6).

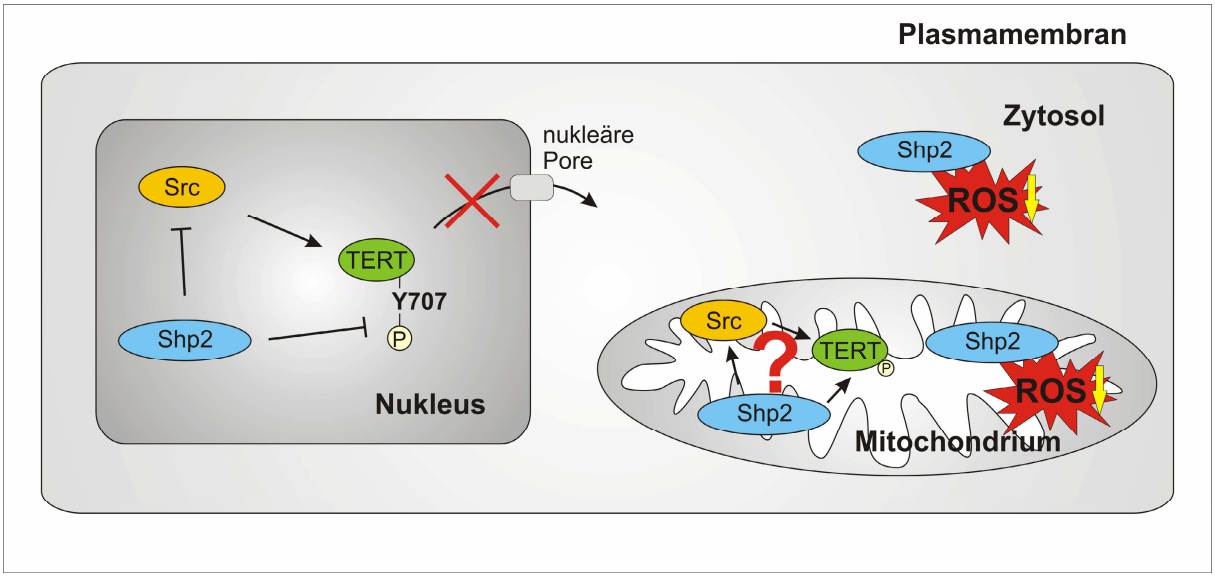

Abb. 6: Lokalisation von Shp-2 und seine neuen Funktionen. Im Nukleus verhindert Shp-2 den nukleären Export von TERT. Im Zytosol reduziert Shp-2 ROS, ebenso im Mitochondrium. Es ist zu vermuten, dass Shp-2 im Mitochondrium ebenfalls mit der Src-Kinase und/oder TERT interagiert.

Als Fragen bleiben offen, wie Shp-2 in die Mitochondrien und in den Kern gelangt. Shp-2 enthält im Gegensatz zu TERT keine mitochondrielle Lokalisierungssequenz. Jedoch ist bereits für andere Proteine, die keine derartige Lokalisierungssequenz haben, gezeigt worden, dass diese in die Mitochondrien importiert werden können. Dabei scheinen Phosphorylierungen eine wesentliche Rolle zu spielen (Gough et al., 2009; Wegrzyn et al., 2009). Interessanterweise gibt es im C-Terminus von Shp-2 mehrere Phosphorylierungsstellen, die bisher keinen bekannten Einfluss auf die Funktion von Shp-2 haben (Abb. 7). Hierzu zählen die Tyrosine 542 und 580 sowie die Serine 576 und 591. Die Tyrosine 542 und 580 scheinen eine Rolle für die Bindung an Grb-2 zu haben, jedoch keinen nachweisbaren Einfluss auf die Aktivität von Shp-2 (Araki et al., 2003; Bennett et al., 1994; Vogel und Ullrich, 1996). Von den Serinen 576 und 591 ist bisher nur bekannt, dass sie von verschiedenen Proteinkinase C (PKC) Isoformen phosphoryliert werden (Strack et al., 2002).

Einen Einfluss dieser Phosphorylierungen auf einen Signalweg ist bisher noch nicht nachgewiesen. Daher habe ich die Vermutung, dass eventuell eine oder mehrere der oben genannten Phosphorylierungstellen einen Einfluss auf die Lokalisation von Shp-2 haben.

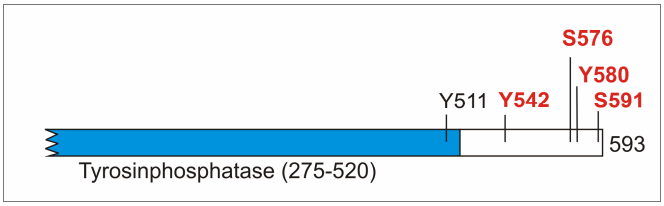

Abb. 7: Tyrosin- und Serinphosphorylierungsstellen des C-Terminus von Shp-2.

Um dies zu untersuchen, habe ich bisher beide Tyrosine mutiert und durch Phenylalanine ersetzt (Shp-2(Y542F/Y580F)), um eine Phosphorylierung dieser Stellen zu unterbinden. Alternativ habe ich die Tyrosine durch Glutaminsäure ersetzt (Shp-2(Y542E/Y580E)), wodurch das Protein saure Aminosäurereste an diesen Positionen trägt, die dadurch phosphomimetisch wirken. Diese Proteine habe ich in Endothelzellen überexprimiert. Nach einer Fraktionierung in Zytosol, Nukleus und Mitochondrium lässt sich als vorläufiges Ergebnis feststellen, dass die nicht tyrosinphosphorylierbare Doppelmutante (Shp-2(Y542F/Y580F)) nicht im Mitochondrium zu finden ist. Weitere Schritte werden sein, Einzelmutanten dieser Aminosäurereste in Shp-2 herzustellen und diese ebenfalls auf ihre Lokalisation zu untersuchen. Um zu überprüfen, ob Shp-2, ähnlich wie im Kern, TERT positiv regulieren kann müssen Überexpressionsstudien mit den entsprechenden Shp-2 Mutanten durchgeführt werden. Des Weiteren muss in diesen Studien auch der Einfluss von Shp-2 auf die Atmungskettenaktivität gemessen werden. Wie oben erwähnt, wird die Kinase Src im Mitochondrium mit der Aktivität der Atmungskettenkomplexe in Verbindung gebracht (Arachiche et al., 2008). Als ein Regulator von Src könnte Shp-2 somit einen Einfluss auf die Funktionalität der Atmungskette haben.

Auch der Kernimport für Shp-2 muss noch genauer untersucht werden. Auch hierfür benötigt Shp-2 einen Partner, da es keine nukleäre Lokalisierungssequenz besitzt. Ich vermute, dass diese Aufgabe von dem Protein Gab1 (growth factor receptor-bound protein 2 (Grb2)-associated binder-1) übernommen wird. Gab1 ist ein Adaptorprotein mit verschiedenen Bindemotiven, unter anderem auch für Shp-2 (Huang et al., 2002). Entscheidend ist, dass Gab1 eine nukleäre Importsequenz besitzt. Die Gruppe um Abe konnte eine nukleäre Lokalisation von ERK1/2 in Verbindung mit Gab1 nachweisen (Osawa et al., 2004). Für diesen nukleären Transport ist der Phosphorylierungszustand von ERK1/2 entscheidend. Ein ähnlicher Mechanismus lässt sich auch für Shp-2 vermuten. Abhängig von seinem Phosphorylierungszustand könnte es durch Gab1 in den Nukleus transportiert werden. Ein weiterer Schritt wird es daher sein, die Lokalisierung von Gab1 auch in Endothelzellen zu untersuchen und den Einfluss von Gab1 auf den nukleären Import von Shp-2. Hierzu habe ich bereits Gab1-Konstrukte generiert, in denen die nukleäre Importsequenz deletiert wurde

(Gab1ΔNLS). Eine Reduktion von nukleärem Shp-2 nach der Überexpression des Gab1ΔNLS würde auf Gab1 als Transportpartner hindeuten. Eine Herabregulation von Gab1 erscheint hingegen nicht sinnvoll, da dies wahrscheinlich den Transport einer großen Anzahl von Proteinen negativ beeinflussen würde und möglicherweise die Zellviabilität drastisch beinträchtigen könnte.

Die Beobachtungen dieser Arbeit zeigen für Shp-2 neue Funktionen im Kern und im Mitochondrium von Endothelzellen auf. Ein besseres Verständnis dieser Funktionen und der zugrunde liegenden Mechanismen könnten Shp-2 zu einem interessanten Kandidat für Therapien machen, die Alterungsprozesse inhibieren. In Bezug auf das Endothel bedeutet dies in erster Hinsicht eine Inhibition der Endotheldysfunktion, möglicherweise durch eine Steigerung kompartimentspezifischer Shp-2 Aktivität und der damit verbundenen Telomeraseaktivität. Die daraus resultierende Aufrechterhaltung der Integrität es Endothels könnte eine Reduktion von kardiovaskulären Erkrankungen zur Folge haben.

5. Zusammenfassung

Das Endothel, die innere Auskleidung der Gefäße, spielt eine wichtige Rolle in der Blutgefäßhomöostase und auch in pathologischen Prozessen, z.B. bei Gefäßverletzungen. Eine Verletzung der Endothelzellschicht führt zu einem Umbau der Gefäßwand, der gekennzeichnet ist durch Infiltration mit inflammatorischen Zellen, Proliferation von glatten Muskelzellen und auch Migration von Endothelzellen. Diese Prozesse führen zur Entwicklung atherosklerotischer Läsionen, welche die Hauptursache für koronare Gefäßerkrankungen darstellt. Die Endothelzellen spielen eine wichtige Rolle in der Krankheitsentstehung. Sie zeigen profunde Veränderungen im Verlauf des Alterns. Zahlreiche Studien belegen, dass es während des Alterungsprozesses zu einem Anstieg reaktiver Sauerstoffspezies (reactive oxygen species, ROS) und einer Verringerung der Stickstoffmonoxid-Bioverfügbarkeit kommt, was zu einer erhöhten Apoptose-Sensitivität der Endothelzellen führt. Ein weitere wichtige Rolle in der Endothelzellalterung spielt das Enzym Telomerase. Es wird für die Integrität der Chromosomenenden, der Telomere benötigt. Während jeder Zellteilung verkürzen sich die Telomere bis ein kritisches Limit erreicht wird, unterhalb dessen die Zellen seneszent werden. Telomerase mit ihrer katalytischen Untereinheit Telomerase Reverse Transkriptase (TERT) wirkt dieser Telomerverkürzung entgegen. Daher ist die Entschlüsselung der Regulationsmechanismen dieses Enzyms eine Voraussetzung zum Verständnis der Endothelzellalterung und somit Ziel dieser Arbeit. Kritische Parameter in der Regulation von TERT sind ihre Phosphorylierung und Lokalisation innerhalb der Zelle. Unter oxidativem Stress und während des Alterns von Endothelzellen wird TERT an seinem Tyrosinrest 707 durch Kinasen der Src-Familie phosphoryliert, was zu einem Export aus dem Zellkern führt. Ich konnte zeigen, dass die Tyrosin-Phosphatase Shp-2 diesem Kernexport entgegenwirkt. Dies beinhaltete den Nachweis, dass Shp-2, welches bisher als ein zytosolisches Enzym bekannt war, auch im Zellkern vorhanden ist, wo es mit TERT assoziiert. Dies führt zu einer Inhibition der Phosphorylierung von TERT, entweder durch Inhibition der Src Kinase-Aktivität oder durch direkte Dephosphorylierung von TERT. Interessanterweise konnte ich zeigen, dass Shp-2 neben seiner Funktion als Phosphatase die ROS-Bildung in Endothelzellen nicht nur im Zytosol, sondern auch in den Mitochondrien reduziert. Dies legt nahe, dass Shp-2 auch in diesem Zellorganell vorkommt, was ich in vorläufigen Experimenten nachweisen konnte. Da sowohl TERT als auch Src in Mitochondrien detektierbar sind, vermute ich, dass dort, ähnlich wie im Zellkern, ein regulatorisches Netzwerk mit Src, TERT und Shp-2 existiert, eine These, die ich in Zukunft untersuchen werde. Zusammenfassend lässt sich sagen, dass Shp-2 mit nukleärer TERT interagiert, wodurch diese vor dem Src-abhängigen Export geschützt wird, und dass es den zellulären ROS-Spiegel senkt, was einen Angriffspunkt zur Verzögerung der Endothelzell-

Seneszenz bieten könnte. Dies eröffnet die Möglichkeit therapeutische Strategien zur Erhaltung der Integrität des Endothels zu entwicklen, indem man spezifisch die Shp-2 Konzentration im Zellkern steigert. Dabei ist allerdings zu bedenken, dass derartige Therapiestrategien nicht mit der Shp-2 Funktion im Zytosol interferieren dürfen, um ein unkontrolliertes Zellwachstum als Nebenwirkung zu verhindern.

6. Summary

The endothelium, the inner lining of the vessels, plays an important role for the physiological maintenance of blood vessel homeostasis, but also for pathological processes such as vessel injury. Injury of the endothelial monolayer results in inflammatory remodelling of the vessel wall, which is characterized by invasion of inflammatory cells, proliferation of smooth muscle cells, but also in migration of endothelial cells. These processes lead to the development of atherosclerotic lesions, which are the major cause for coronary vascular disease. Endothelial cells, play an important role in the cascade of pathology, and these cells show profound changes with age. Recent studies demonstrated that aging is associated with an increase in reactive oxygen species (ROS) and reduced nitric oxide bioavailability leading to an enhanced apoptosis sensitivity of endothelial cells. Another important player in endothelial cell aging is telomerase. This enzyme is required to maintain the integrity of the telomeres, the ends of the chromosomes. During each cell division the telomeres are shortened until a critical limit is reached, when the cells become senescent. Telomerase with its catalytic subunit telomerase reverse transcriptase (TERT) counteracts this process. Therefore, decoding the regulation of this enzyme is a prerequisite to understand endothelial cell aging and is the aim of this study. Critical parameters in the regulation of TERT are its phosphorylation and intracellular localisation. Under oxidative stress and during aging TERT is phosphorylated on tyrosine 707 by Src family kinases resulting in its export from the nucleus in endothelial cells. I could show that this export is counteracted by the tyrosine phosphatase Shp-2. This involved the demonstration that Shp-2, which was previously known as a cytosolic enzyme, is also present in the nucleus, where it associates with TERT. This results in inhibition of net TERT phosphorylation either by inhibition of Src kinase activity or direct dephosphorylation of TERT. Interestingly, I demonstrated that Shp-2, besides its function as a phosphatase, reduces ROS formation in endothelial cells, not only in the cytosol, but also in mitochondria. This leads to the assumption that Shp-2 might be present in these organelles, which I could demonstrate in preliminary work. Knowing that TERT and Src are present in the mitochondria, I speculate that similar to the nucleus a regulatory circuit involving TERT, Src and Shp-2 exists there, which will be the subject of my future studies. In summary, Shp-2 interacts with nuclear TERT thereby protecting it against Src-dependent export and reduces cellular ROS levels, which might provide mechanisms to delay endothelial cell senescence. This offers the prospect to maintain the integrity of the endothelium by therapeutic strategies aimed at elevating specifically the nuclear levels of Shp-2. Such strategies must not interfere with cytosolic Shp-2 functions to avoid uncontrolled cell growth as a side effect.

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8. Eigene Veröffentlichungen

1. **Molecular mechanisms involved in endothelial cell aging: role of telomerase reverse transcriptase**
   S. Jakob, J. Haendeler
   Z Gerontol Geriatr *40*, 334-338, 2007

2. **Changes of MMP-1 and collagen type Ialpha1 by UVA, UVB and IRA are differentially regulated by Trx-1**
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3. **Nuclear protein tyrosine phosphatase Shp-2 is one important negative regulator of nuclear export of telomerase reverse transcriptase**
   S. Jakob, P. Schroeder, M. Lukosz, N. Büchner, I. Spyridopoulos, J. Altschmied, J. Haendeler
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4. **"Shping 2" different cellular localizations - a potential new player in aging processes**
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5. **Nuclear redox signaling**
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6. **Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage**
   J. Haendeler, S. Drose, N. Buechner, **S. Jakob**, J. Altschmied, C. Goy, I. Spyridopoulos, A.M. Zeiher, U. Brandt, S. Dimmeler
   Arterioscler. Thromb. Vasc. Biol., 29, 929-935, 2009

7. **Well-known signaling proteins exert new functions in the nucleus and mitochondria**
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Molecular mechanisms involved in endothelial cell aging: role of telomerase reverse transcriptase

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Molecular mechanisms involved in endothelial cell aging: role of telomerase reverse transcriptase

Molekulare Mechanismen in der Endothelzellalterung – Rolle der Telomerase Reversen Transkriptase

▶ **Abstract** Aging is one major risk factor for the incidence of coronary artery disease and the development of atherosclerosis. The functional integrity of the endothelial cell monolayer is essential to prevent lesion formation. Endothelial cells show profound changes with age. However, the molecular mechanisms are not well understood. Important players in the process of endothelial cell aging are reactive oxygen species, nitric oxide bioavailability, mitochondrial integrity and the activity of telomerase reverse transcriptase. This review will demonstrate the evidence that these processes are involved in endothelial cell aging and linked to each other. The future goal of understanding endothelial cell aging would allow for an anti-aging therapy to reduce the influence of aging in the development of atherosclerosis and coronary artery disease.

▶ **Key words** aging – endothelial cells – nitric oxide-bioavailability – oxidative stress – telomerase reverse transcriptase

▶ **Zusammenfassung** Altern ist einer der bekanntesten unabhängigen Risikofaktoren für das Auftreten der koronaren Gefäßerkrankung und die Entstehung der Atherosklerose. Die funktionale Integrität der Endothelzellen, der innersten Schicht der Blutgefäße, ist ein wesentlicher Schutz vor Atherosklerose-Entstehung. Jedoch kommt es bedingt durch das Altern zu einer Veränderung der Endothelzellen. Die molekularen Mechanismen, die in der Zelle zu diesen Veränderungen beitragen, sind jedoch nur unzureichend aufgeklärt. Wichtige Faktoren für die Entstehung der Endothelzellalterung scheinen reaktive Sauerstoffspezies, die Verfügbarkeit von Stickstoffmonoxid, die Funktionalität der Mitochondrien und die Aktivität der Telomerase Reversen Transkriptase zu sein. Dieser Übersichtsartikel will die wissenschafltichen Nachweise, dass diese Faktoren eine Rolle bei der Zellalterung spielen, vorstellen und eine mögliche Abhängigkeit dieser Faktoren von einander darlegen. Denn ein besseres Verständnis der Endothelzellalterung ist notwendig für ein mögliches therapeutisches Eingreifen, um den negativen Einfluss des Alterns bei koronaren Gefäßerkrankungen zu verringern.

▶ **Schlüsselwörter** Altern – Endothelzellen – NO-Verfügbarkeit – Oxidativer Stress – TERT

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Introduction

The incidence of coronary artery disease increases with age, and adults >65 years of age are four times more likely to experience coronary artery disease than those <49 years of age. Aging is indeed one of the major independent risk factors for the development of atherosclerosis and, therefore, for coronary artery disease. The functional integrity of the endothelial monolayer is essential to prevent vascular leakage and the formation of atherosclerotic lesions. Injury of the endothelial monolayer results in inflammatory remodelling of the vessel wall [1, 2]. This process is characterized by invasion of inflammatory cells and proliferation of smooth muscle cells thereby leading to the development of atherosclerotic lesions [2]. In the case of atherosclerosis, one relevant pathology occurs in the coronary arteries. The endothelial cells, which line these arteries, play an important role in the cascade of pathology, and these cells show profound changes with age [3, 4]. However, the molecular mechanisms underlying the process of aging are not well understood. Recent studies suggested that aging is associated with an increase in reactive oxygen species (ROS), a reduction in nitric oxide (NO)-bioavailability, mitochondrial dysfunction and reduced nuclear telomerase reverse transcriptase activity [3, 5, 6]. Thereby, the disturbance of the redox-balance seems to be linked to alterations in mitochondria function and dysregulation of telomerase reverse transcriptase. Thus, there is evidence for a critical role of cellular senescence in the process of aging and various age-related diseases, including atherosclerosis. Anti-aging therapy is now emerging as a novel strategy for the treatment of human atherosclerosis. One important candidate for an anti-aging therapy is telomerase reverse transcriptase (TERT). A number of reports have been demonstrated that TERT is activated by medications or humoral factors that are known to exert a beneficial effect on cardiovascular disease, such as aspirin, statins, estrogens and thiazolidinediones [6–9]. Alterations on mitochondrial and telomerase functions due to oxidative stress may contribute to aging processes in endothelial cells.

The following review will summarize the evidence for a potential link between ROS, NO-bioavailability, mitochondria and TERT in endothelial cell aging (Fig. 1).

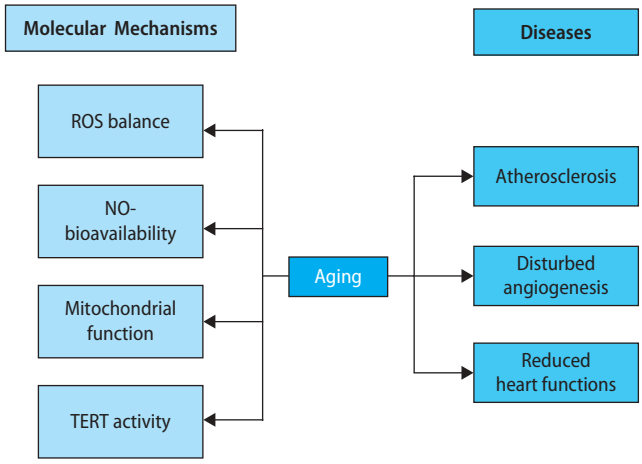

Fig. 1 Evidenced-based molecular mechanisms during the process of aging leading to cardiovascular disturbances and diseases

Molecular mechanisms in endothelial cell aging

NO is a short-lived free radical gas and a key molecule in regulating diverse biological processes. In the endothelium, the most relevant isoform for the production of NO in the pmol range is the endothelial nitric oxide synthase (eNOS) [10]. The important anti-atherosclerotic function of NO is evidenced by experimental and clinical studies. eNOS deficient mice exhibit reduced growth factor-induced angiogenesis and show accelerated atherosclerotic lesion formation [11, 12]. Several studies demonstrated that during the process of aging endothelial cells exhibit reduced NO-bioavailability. Mechanistically, aged endothelial cells show reduced eNOS activity and thereby, less bioactive NO [3, 13, 14]. Moreover, during the process of aging the formation of ROS increased in vitro and in vivo. Hamilton et al. showed that ROS formation was significantly greater in old rats compared with young rats. Of note, removal of the endothelium and N(G) nitro-L-arginine methyl ester treatment resulted in a significant reduction in ROS formation of old rats, demonstrating that the ROS are significantly generated from the endothelium as well [15]. In accordance with these findings, we showed that during the processes of endothelial cell aging, ROS formation increased prior to the onset of replicative senescence [6]. Thus, one may speculate that the reduction in bioactive NO is due to the increase in ROS formation. This hypothesis is supported by findings of van der Loo et al., who demonstrated an increase in peroxynitrite formation during the process of aging [16]. Recent studies suggested another mechanism how the reduction in bioactive NO occurs. During the process of aging tetrahydrobiopterin, a cofactor for eNOS, is reduced [17], which in turn leads to an uncoupling of eNOS. Thus, eNOS itself produced O_2^- radicals instead of NO and thereby less bioactive NO and more ROS are formed in aged endothelial cells (for review see [18]).

Another important process, which plays a role during the development of aging is mitochondrial function. A large body of evidence supports the hypothesis that impaired mitochondrial function is a major contributor of aging processes (for further details see the following reviews [19, 20]). Several human and animal studies have demonstrated an age-related decline of mitochondrial respiratory function and ATP synthase activity [21–23]. Moreover, oxidative mitochondrial DNA (mtDNA) mutations accumulated with age in human and animal organs and inversely correlated to maximal life span (for review see [24]). Further support for mitochondrial dysfunction in aging is obtained using genetically engineered mice containing a proof reading-deficient mtDNA polymerase-1 (PolgA) [25]. The homozygous knock in mice showed an increase in mtDNA point mutations that accumulated with increasing age. Consistent with this increase in mtDNA mutations, the mice have reduced respiratory chain activities resulting in less mitochondrial ATP, reduced life span and showed symptoms of accelerated aging such as weight and hair loss, and heart enlargement [25]. However, no increase in ROS levels was observed in these mice, arguing against a direct role of mitochondrial oxidative stress in aging. Given the fact that most of the ROS production during the process of aging is generated by the mitochondria, one possible explanation why no increase in ROS is demonstrated in these mice is that respiratory chain activities are reduced by more than 90%. Thus, we still cannot exclude from these mice that ROS has no direct role in the development of aging. On a molecular level, we showed that the increase in ROS formation occurred prior to a reduction in intact mtDNA in aged endothelial cells and that ROS formation is required for the senescent phenotype of endothelial cells [6].

Finally, one important target molecule for the process of aging is the telomerase reverse transcriptase (TERT). Telomeres are the physical ends of the chromosomes. They are necessary for the stability and integrity of the chromosomes [26, 27]. Because telomeres are shortened during each cell division they can function as a mitotic clock. This telomere shortening is prevented by the enzyme telomerase. The telomerase is a large ribonucleoprotein complex and consists of the reverse transcriptase subunit (TERT), which contains the catalytic activity of the enzyme, and the associated RNA component, which serves as the template for synthesis of the telomeric sequence [28–30]. The loss of TERT activity and thus the loss of telomere function induced endothelial dysfunction which can be observed in aged arteries. Vascular endothelial cells with senescence-associated phenotypes can be found in the atherosclerotic regions of human coronary arteries [14]. Therefore, the loss of TERT activity is associated with endothelial cell aging. This is further supported by a number of reports demonstrating that telomerase reverse transcriptase is activated by medications that exert beneficial effects on cardiovascular disease, such as aspirin, statins, estrogens and thiazolidinediones [6–9]. During the process of endothelial cell aging, nuclear TERT activity is reduced and preceded the onset of replicative senescence. Most importantly, this process was dependent on the increase in ROS formation during the process of endothelial cell aging. Mechanistically, the induction of oxidative stress led to Src-kinase family dependent nuclear export of TERT in endothelial cells, which can be blocked by antioxidants and statins [6]. Furthermore, in aged endothelial cells expression and activity of the Akt kinase is reduced [3]. The Akt kinase has been demonstrated to phosphorylate and thereby activate TERT [31, 32]. Therefore, a reduction in Akt kinase activity further reduced the activity of TERT in aged endothelial cells.

Moreover, TERT activity is enhanced by NO and endothelial senescence is delayed [33]. Thus, the enhanced ROS formation and the reduced NO-bioavailability subsequently induced the loss of nuclear TERT activity in aged endothelial cells, suggesting that the ROS formation, reduced bioactive NO and reduced nuclear TERT activity are inseparably tied together as mechanisms during the process of endothelial cell aging. Moreover, ROS formation has been demonstrated to result in damage of mtDNA and mitochondrial dysfunction has been associated with aging process. Therefore, one important question remains:

Is there a direct link between mitochondrial function and TERT? Recent studies showed that TERT activity can be measured in the mitochondria of several cell types [34]. It is interesting to note that overexpressing TERT into fibroblasts which do not express endogenous TERT increased their susceptibility to oxidative stress-induced mtDNA damage leading to cell death. In contrast, overexpressing nuclear targeted TERT, which can not enter the mitochondria is associated with diminished mtDNA damage, increased cell survival and protection against cellular senescence. Thus, introduction of TERT into cells, which do not express TERT endogenously, could result in a senescent phenotype. However, given the fact that cells which express TERT endogenously show TERT activity in the mitochondria and in the nucleus, mitochondrial TERT seems to have a mitochondrial function, which has to be determined in future studies [34, 35].

Thus, it is tempting to speculate that TERT is directly linked to mitochondria function. Our first data demonstrate that TERT activity can be measured in

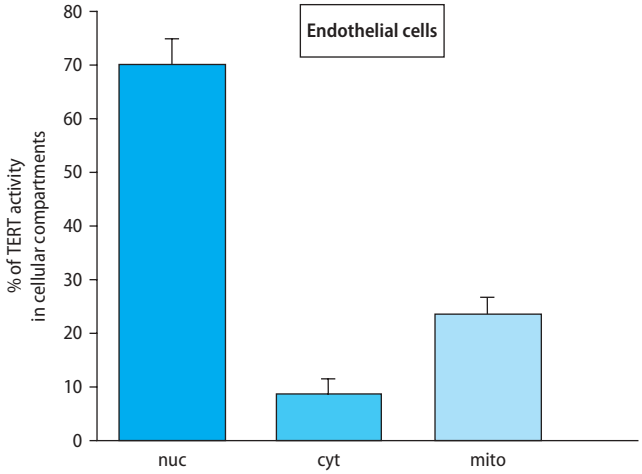

Fig. 2 Distribution of TERT activity in the nucleus (nuc), cytosol (cyt) and mitochondria of endothelial cells

the nucleus, cytosol and mitochondria of endothelial cells (Fig. 2) [36]. However, the underlying mechanisms, by means of which TERT can interfere with mtDNA and which is the functional role of TERT in the mitochondria and during the process of aging, is not clear yet. Further studies are needed to address these issues.

Conclusions

Aging is one major risk factor for several cardiovascular diseases. One interesting candidate for an anti-aging therapy could be TERT. Since, a number of reports have been demonstrated that TERT is activated by medications or humoral factors that are known to exert beneficial effects on cardiovascular diseases. However, given the new findings that mitochondrial TERT exists and that a functional role of mitochondrial TERT is unknown until now, one should first consider understanding this new field of "mitochondrial TERT biology" prior to the introduction of TERT as an anti-aging therapy.

Finally, it has to be noted that all of the molecular mechanisms of endothelial cell aging seems to be inseparably tied together and one of them predicts the others.

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Changes of MMP-1 and collagen type Ialpha1 by UVA, UVB and IRA are differentially regulated by Trx-1

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Changes of MMP-1 and collagen type Iα1 by UVA, UVB and IRA are differentially regulated by Trx-1

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ABSTRACT

Exposure of human skin to solar radiation, which includes ultraviolet (UV) radiation (UVA and UVB) visible light and infrared radiation, induces skin aging. The effects of light have been attributed to irradiation-induced reactive oxygen species (ROS) formation, but the specific signaling pathways are not well understood. Detrimental effects of solar radiation are dermal diseases and photoaging. Exposure of cultured human dermal fibroblasts to UVA, UVB or IRA increased ROS formation in vitro. One important redox regulator is the oxidoreductase thioredoxin-1 (Trx). Trx is ubiquitously expressed and has anti-oxidative and anti-apoptotic properties. Besides its function to reduce H_2O_2, Trx binds to and regulates transcription factors. The aim of this study was to investigate whether Trx influences the regulation of MMP-1 and collagen Iα1 by UVA, UVB and IRA. We irradiated human dermal fibroblasts with UVA, UVB and IRA. UVA, UVB and IRA upregulated MMP-1 expression. Trx inhibited UVA-induced MMP-1 upregulation in a NFκB dependent manner. UVA, UVB and IRA reduced collagen Iα1 expression. Incubation with Trx inhibited the effects of UVB and IRA on collagen Iα1 expression. In conclusion, MMP-1 and collagen Iα1, which play important roles in aging processes, seems to be regulated by different transcriptional mechanisms and Trx can only influence distinct signaling pathways induced by UVA, UVB and probably IRA. Thus, Trx may serve as an important contributor to an "anti-aging therapeutic cocktail".

1. Introduction

The skin acts as a physiological barrier protecting the organism against pathogens and chemical or physical damage. Aside from this important function, the skin shows age spots or wrinkles and signs of photoaging at the sun-exposed sites. It has become apparent that chronic sun exposure not only accelerates the formation of age spots and wrinkles, but also has various effects on skin function. Photoaging as a result of chronic sun exposure is characterized by dermal connective tissue changes, which give the skin a yellowish hue and leathery consistency. Histopathological studies of photodamaged skin have revealed reduced amounts of collagen and increased expression and activity of matrix metalloproteinases (MMPs). Further studies on the degradation system of collagen have shown that aging and irradiation accelerate the degradation of extracellular matrix, demonstrating a decrease in dermal collagen and an increase in the matrix metalloproteinase-1 (MMP-1) expression level, which cleaves interstitial collagen, associated with aging (Fligiel et al., 2003; Jenkins, 2002). One major signaling pathway, which contributes to photoaging, is the formation of reactive oxygen species (ROS) (Jenkins, 2002). UV (UVA and UVB) irradiation and infrared A (IRA) induce ROS formation in dermal fibroblasts (Russo and Halliday, 2006; Schroeder et al., 2007a). Dermal fibroblasts can be protected against the adverse effects of UVA, UVB and IRA irradiation by a number of both enzymatic and non-enzymatic anti-oxidants (Yan et al., 2005; Russo and Halliday, 2006; Schroeder et al., 2007a). The endogenous anti-oxidant capacity of the skin is a major determinant in its response to oxidative stress-mediated damage. Thus, anti-oxidants constitute an important group probably capable of preventing the occurrence and reducing the severity of irradiation-induced skin diseases and photoaging. One of the molecules, which is highly regulated by changes of the redox status in cells, is the oxidoreductase thioredoxin-1 (Trx). Trx is a 12-kDa protein, which is ubiquitously expressed in mammalian cells (Holmgren, 1989) and exerts its enzymatic activity as an oxidoreductase via cysteines 32 and 35 in the active site (Holmgren, 1989; Martin, 1995). Besides its well described function as an oxidoreductase, Trx exerts several other functions. By binding to different proteins, it modulates their function: Inhibition of binding to the apoptosis signaling kinase 1 and to the transcription factors AP1, Ref1 and NFκB modulates the ability of Trx to regulate cellular functions (Liu et al., 2000; Tanaka

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et al., 2001; Malik et al., 2006; Schroeder et al., 2007b). Therefore, we investigated the effects of Trx on UVA, UVB and IRA irradiation induced changes in MMP-1 and collagen I expression.

2. Materials and methods

2.1. Cell culture

Human dermal fibroblasts were isolated from foreskin of three different donors. Cells were cultivated in Eagle's minimum essential medium with Earle's salts (MEM, PAA Laboratories Pasching, Austria) supplemented with 10% fetal bovine serum (Gibco, Karlsruhe, Germany), 1% anti-biotics/anti-mycotics (penicillin, streptomycin, and amphotericin B), 1% glutamine (Gibco) and were cultivated on 100 mm plastic culture dishes (Greiner, Solingen, Germany) at 37 °C in humidified air with 5% CO_2. Cells were used between passages 5 and 10, grown to 100% confluence before treatment. Media was changed to serum-free MEM 24 h before irradiation with UVA, UVB and IRA respectively.

2.2. Irradiation

For irradiations, medium was replaced by phosphate buffered saline (PBS), lids were removed, and cells were exposed to radiation from a Hydrosun 500 H IRA device (Hydrosun Medizintechnik GmbH, Müllheim, Germany), or a UVA SUN 5000 Biomed UVA1 metal halogenide irradiation device, or a bank of four Philipps TL20W/12RS UVB fluorescent bulbs (Phillips, Hamburg, Germany). The IRA device was water-filtered and equipped with a black filter and emits wavelengths between 760 and 1400 nm leading to an irradiance of 360 mW/cm^2 at a distance of 20 cm measured through Hydrosun HBM1 irradiance measuring device (Hydrosun). The TL20W/12RS lamp emits most of its energy in the UVB range (290–320 nm) with an emission peak at 310 nm. The UVA1 output was determined with a UVAMETER (Mutzhas, Munich, Germany) and found to be 33.4 mW/cm^2 UVA1 at a lamp to target distance of 30 cm. The UVB output was measured with a UV-Dosimeter Type II equipped with a UV6 sensor (Waldmann Medizintechnik, Villingen-Schwenningen, Germany) and found to be 0.56 mW/cm^2 at a tube to target distance of 30 cm. The experimental doses were 30 J/cm^2 UVA (represents a duration of 15 min), 33 J/m^2 UVB (represents a duration of 5 s) and 240 J/cm^2 IRA (represents a duration of 15 min). The culture dishes were placed on a cooled plate connected to thermostated bath (Thermo Haake GmbH, Karlsruhe, Germany) to maintain temperatures below 37 °C during irradiation. Control cells were held on a 37 °C thermostated plate under similar conditions without irradiation. Human Trx (0.1–10 ng/ml, Sigma) and pyrrolidine dithiocarbamate (PDTC) (NFκB inhibitor, 10 μM, Sigma) were incubated directly after irradiation. Following the treatment cells were cultivated for 24 h with serum-free MEM culture medium at 37 °C.

2.3. Real-time PCR measurements of relative mRNA levels

RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction. The gene-specific mRNA expression of the isolated total RNA was determined by semi-quantitative differential real-time PCR. The RNA concentration was determined via photometric measurement at 260 nm. Aliquots of total RNA (100 ng) were applied for cDNA-Synthesis using the Superscript™ III First-Strand synthesis system for RT-PCR (Invitrogen, Karlsruhe, Germany). The PCR reactions were carried out on an Abi Prism 7000 (Applied Biosystems, Foster City, CA, USA) using SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany). For relative expression comparison between control cells and treated cells the 2-ΔΔCT method was used, utilizing the18S RNA as housekeeping gene. Three independent experiments were performed for each of three different dermal fibroblast cells with duplicate determinations each and the mean value of these was calculated. Primer was designed with the help of the online-software *Primer3*. The following primer pairs were used (5′–3′): 18S RNA: GCC GCT AGA GGT GAA ATT CTT G, CAT TCT TGGC AAA TGC TTT CG, MMP-1: CAT GAA AGG TGG ACC AAC AAT TT, CCA AGA GAA TGG CCG AGT TC Col1a1: CGC TAC TAC CGG GCT GAT GAT, GTC TTG GGG TTC TTG CTG ATG TA.

2.4. Immunoblot

Proteins were isolated after treatment and subjected to SDS–PAGE sample buffer and resolved on an 8% or 10% SDS–PAGE. Immunoblotting was performed with antibodies directed against MMP-1 (1:1000, overnight 4 °C, The binding site), collagen type I α1 (1:200, overnight 4 °C, Santa Cruz), actin (1:50,000, overnight 4 °C, Sigma), tubulin (1:50,000, Dianova) and GAPDH (1:50,000, overnight 4 °C Abcam). Antibodies were detected by the enhanced chemiluminescence system (Amersham).

2.5. Statistical analysis

Results are given as means ± standard error of the mean. Results were compared using a Mann–Whitney rank-sum test. A significance level of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of Trx on UVA, UVB and IRA induced MMP-1 mRNA

UVA, UVB and IRA all penetrate into the skin. In order to investigate the effects of UVA, UVB and IRA on MMP-1 expression, we irradiated dermal fibroblasts with 30 J/cm^2 of UVA, 33 J/m^2 of UVB and 240 J/cm^2 of IRA. These doses are physiologically relevant and can penetrate into the skin after sun exposure. All three doses did not induce cell death (data not shown). Twenty-four hours after irradiation, MMP-1 expression was measured by real-time PCR. UVA, UVB and IRA increased MMP-1 expression (Fig. 1A–C). It has previously been demonstrated that upregulation of MMP-1 expression by UV and IRA irradiation is dependent on ROS formation (Schroeder et al., 2007a; Yan et al., 2005). One important intracellular anti-oxidant is the protein Trx. Inhibition of Trx protein leads to dramatic cell death (Haendeler et al., 2004a) and Trx deficient mice are embryonic lethal (Matsui et al., 1996), demonstrating the absolute requirement of Trx for the organism. However, the effects of Trx in dermal fibroblasts have never been investigated. Therefore, we incubated dermal fibroblasts after UVA, UVB and IRA irradiation with 10 ng/ml Trx for 24 h. Surprisingly, Trx inhibited the upregulation of MMP-1 induced by UVA, but not by UVB (Fig. 1A and B). It has to be noted that the dose of IRA investigated here showed only a slight, but significant increase in MMP-1 expression ($n = 6$). Due to the huge interindividual differences of the donors, we did not observe a statistically significant effect of Trx on MMP-1 regulation after IRA exposure probably due to the minor effects observed (Fig. 1C).

3.2. Effects of Trx on collagen type I α1 (ColIα1) mRNA after exposure to UVA, UVB and IRA

ColIα1 is the most abundant protein in skin connective tissue, which also contains other types of collagen (III, V and VII), elastin, proteoglycans, fibronectin, and other extracellular matrix proteins.

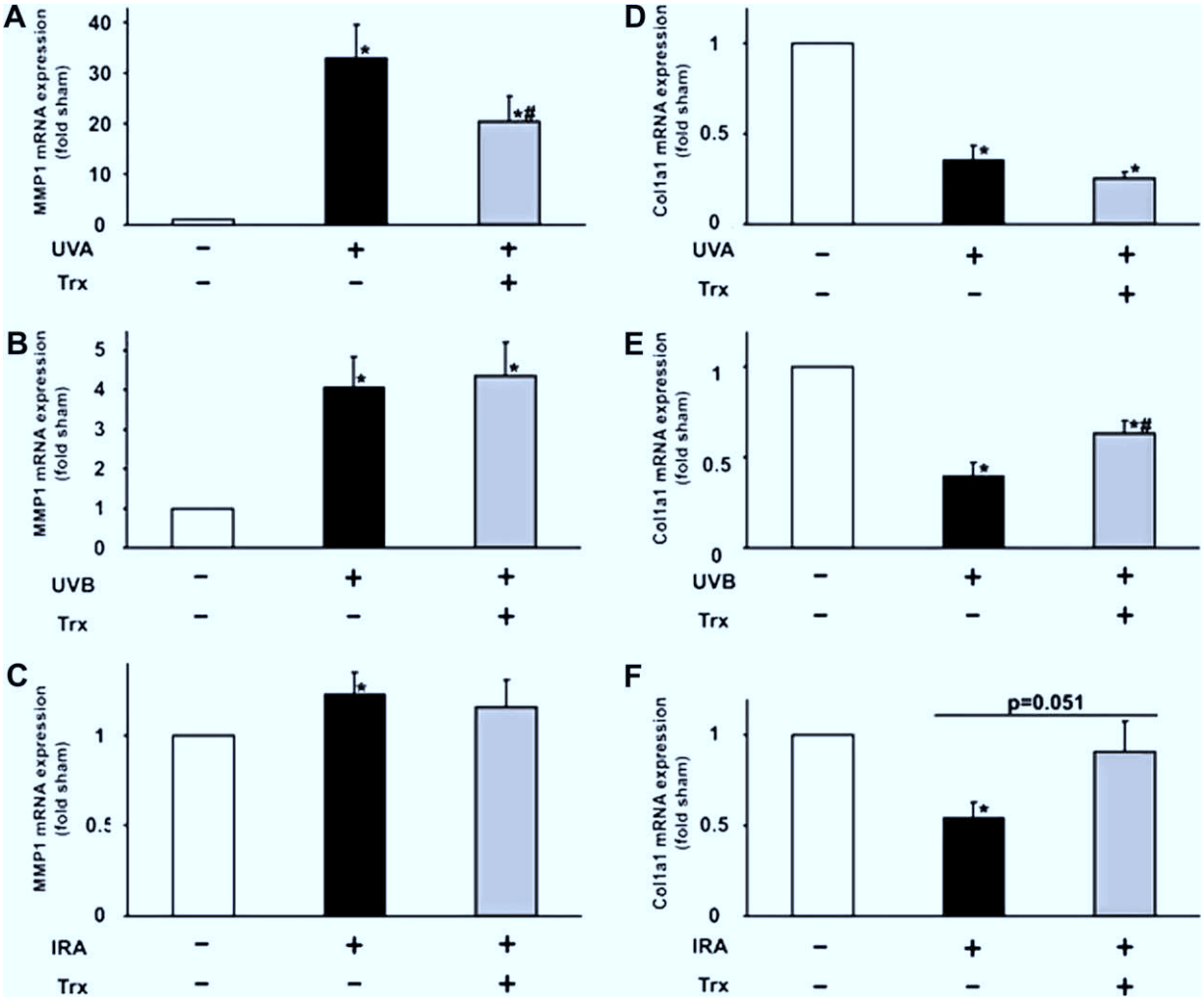

Fig. 1. MMP-1 and ColIα1 mRNA regulation by irradiation. (A) Fibroblasts were irradiated with 30 J/cm^2 UVA and incubated with 10 ng/ml Trx for 24 h. Data are means ± SEM of nine independent experiments. *Significantly different to respective sham ($p < 0.05$), #significantly different to respective irradiated sample without Trx ($p < 0.05$). (B) Fibroblasts were irradiated with 33 J/m^2 UVB and incubated with 10 ng/ml Trx for 24 h. Data are means ± SEM of nine independent experiments. *Significantly different to respective sham ($p < 0.05$). (C) Fibroblasts were irradiated with 240 J/cm^2 IRA and incubated with 10 ng/ml Trx for 24 h. Data are means ± SEM of six independent experiments. *Significantly different to respective sham ($p < 0.05$). (D) Fibroblasts were irradiated with 30 J/cm^2 UVA and incubated with 10 ng/ml Trx for 24 h. Data are means ± SEM of nine independent experiments. *Significantly different to respective sham ($p < 0.05$). (E) Fibroblasts were irradiated with 33 J/m^2 UVB and incubated with 10 ng/ml Trx for 24 h. Data are means ± SEM of nine independent experiments. *Significantly different to respective sham ($p < 0.05$), #significantly different to respective irradiated sample without Trx ($p < 0.05$). (F) Fibroblasts were irradiated with 240 J/cm^2 IRA and incubated with 10 ng/ml Trx for 24 h. Data are means ± SEM of six independent experiments. *Significantly different to respective sham ($p < 0.05$).

These extracellular matrix proteins are mainly synthesized by the dermal fibroblasts. Therefore, we investigated the expression of ColIα1 after UVA, UVB and IRA irradiation. UVA, UVB and IRA significantly reduced the expression of ColIα1 (Fig. 1D–F). Next, we analysed the effect of Trx on ColIα1. Unexpectedly, we found that Trx did not inhibit UVA induced reduction of ColIα1, but abrogated the effects of UVB on ColIα1 (Fig. 1D and E). Trx also inhibited the reduced ColIα1 expression in IRA irradiated samples ($n = 6$, $p = 0.051$, Fig. 1F).

3.3. Effects of Trx on MMP-1 and pro-ColIα1 protein levels after exposure to UVA, UVB and IRA

In order to confirm our data obtained by real-time PCR, we performed immunoblot analyses of MMP-1 and pro-ColIα1. As expected from the mRNA data, UVA, UVB and IRA also increased MMP-1 and decreased pro-ColIα1 protein levels (Fig. 2A–C). Incubation with Trx starting from 1 ng/ml to 10 ng/ml inhibited UVA induced MMP-1 upregulation (UVA vs. UVA + 10 ng/ml Trx, $p < 0.05$, Fig. 2A). However, reduction of pro-ColIα1 protein levels by UVA was not blocked by all Trx concentrations (Fig. 2A). In contrast, Trx blunted UVB-induced pro-ColIα1 downregulation starting from 0.1 ng/ml, whereas MMP-1 upregulation was not affected (Fig. 2B). Finally, Trx inhibited IRA induced upregulation of MMP-1 and downregulation of ColIα1 (Fig. 2C).

3.4. Blockade of NFκB abrogated the protective effect of Trx on UVA induced MMP-1 upregulation

In order to get insight in one of the mechanisms how Trx regulates MMP-1, we investigated the involvement of NFκB. It has previously been demonstrated that Trx binds to and thereby enhances DNA binding of the p50 subunit of NFκB and that UVA induces nuclear translocation of the p65 subunit of NFκB (Matthews et al., 1992; Wenk et al., 2004). Thus, Trx may alter the composition of the NFκB complex. Therefore, we incubated dermal fibroblasts with Trx in presence of the well established NFκB inhibitor PDTC. Indeed, incubation with PDTC abrogated the effects of Trx on UVA induced upregulation of MMP-1 (Fig. 3). In contrast, blockade of NFκB did not change pro-ColIα1

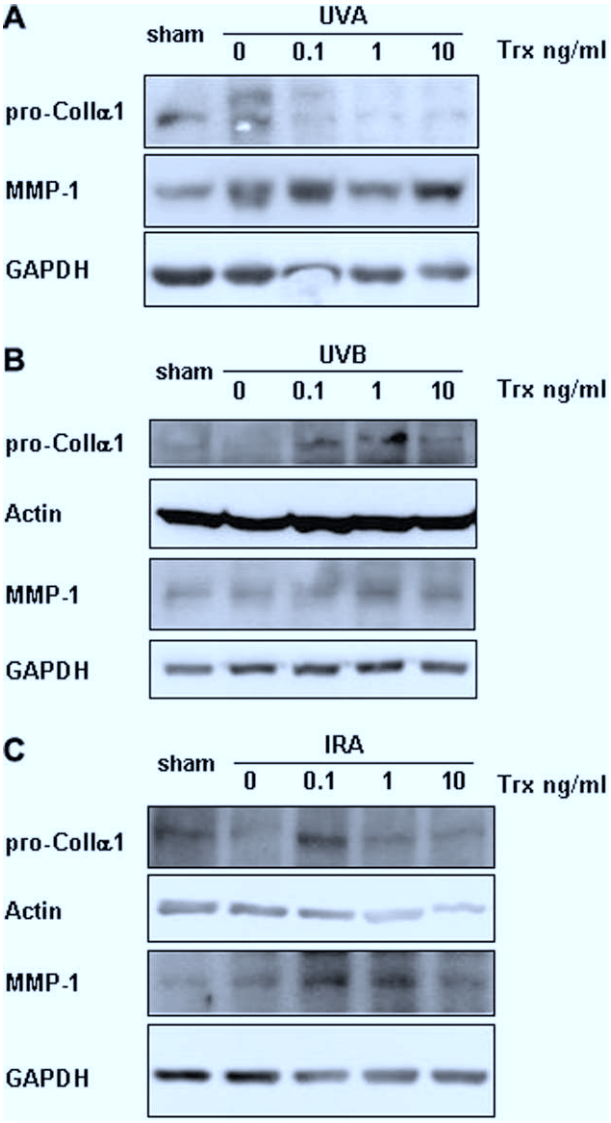

Fig. 2. MMP-1 and pro-Collα1 protein regulation by irradiation. (A) Fibroblasts were irradiated with 30 J/cm^2 UVA and incubated with 0.1, 1 and 10 ng/ml Trx for 24 h. Immunoblots were performed with antibodies recognizing pro-Collα1 and Collα1 (upper panel) and MMP-1 (middle panel). GAPDH was used for confirming equal loading (lower panel). (B) Fibroblasts were irradiated with 33 J/m^2 UVB and incubated with 0.1, 1 and 10 ng/ml Trx for 24 h. Immunoblots were performed with antibodies against pro-Collα1 and Collα1 (upper panel), actin (upper middle panel), MMP-1 (lower middle panel) and GAPDH (lower panel). Actin and GAPDH were used for confirming equal loading. (C) Fibroblasts were irradiated with 240 J/cm^2 IRA and incubated with 0.1, 1 and 10 ng/ml Trx for 24 h. Immunoblots were performed with antibodies recognizing pro-Collα1 and Collα1 (upper panel), actin (upper middle panel), MMP-1 (lower middle panel) and GAPDH (lower panel). Actin and GAPDH were used for confirming equal loading.

protein levels, indicating that regulation of collagen synthesis is not dependent on NFκB.

4. Discussion

The findings of our present study demonstrate that the expression of two major players in the process of photoaging, namely MMP-1 and Collα1, are regulated by UVA, UVB and IRA. Trx inhibited the UVA induced expression of MMP-1, but not of UVB. In contrast, Trx abrogated the reduction of Collα1 mRNA by UVB, but not by UVA. Moreover, Trx affected the IRA induced effects on Collα1 expression ($p = 0.051$).

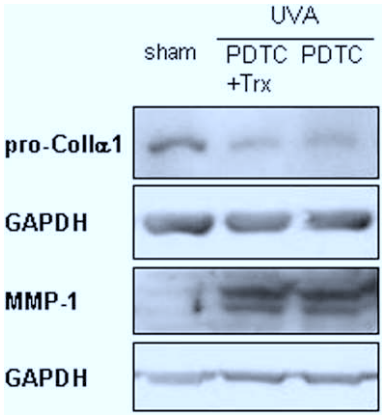

Fig. 3. Blockade of NFκB abrogated the protective effect of Trx on UVA induced MMP-1 upregulation. Fibroblasts were irradiated with UVA and incubated with 10 ng/ml Trx and 10 μM PDTC for 24 h. Immunoblots were performed with antibodies against pro-Collα1 and Collα1 (upper panel), GAPDH (upper middle panel), MMP-1 (lower middle panel) and GAPDH (lower panel). GAPDH was used for confirming equal loading.

Skin aging manifests as wrinkles, diminished structural integrity, and impaired wound healing, due to alterations in the extracellular matrix formed predominantly of Collα1 (for review see (Jenkins, 2002)). Decreased structural Collα1, increased elastin and higher levels of MMP-1, which degrades collagen, have been demonstrated in aged skin relative to young skin (Jenkins, 2002). Several applications have been shown to inhibit wrinkle formation by restoration of collagen formation, e.g. retinoic acid (Griffiths et al., 1993). The dermal fibroblasts investigated in our study are the predominant cells responsible for the alterations in extracellular matrix. Trx inhibited UVA induced MMP-1 exression in our study. This is in accordance with data from Yan et al., who demonstrated that tempol, one of the nitroxides, reduced MMP-1 induction by UVA (Yan et al., 2005). Moreover, we found that the protective effects of Trx on UVA induced upregulation of MMP-1 depend on NFκB. It has previously been shown that Trx enhanced DNA binding of the p50 subunit of NFκB and that blockade of the thioredoxin reductase inhibited Trx NFκB binding and reduced expression of a number of anti-apoptotic genes (Matthews et al., 1992; Lan et al., 2007). In contrast, Wenk et al. demonstrated that nuclear translocation of the p65 subunit occurred when dermal fibroblasts were exposed to UVA which consequently resulted in an increase of IL-6 production and MMP-1 upregulation (Wenk et al., 2004). Therefore, we hypothesized that enhanced binding of Trx to the p50 subunit of NFκB could change subunit composition of the NFκB complex, which may result in activation of a different subset of NFκB target genes. However, Trx did not influence reduction of Collα1 by UVA, suggesting that Collα1 expression is not regulated by NFκB. This is in accordance with our data presented here and with findings by Buttner et al., demonstrating that transcriptional regulation of Collα1 was independent of NFκB in fibroblasts (Buttner et al., 2004). Thus, one may speculate that UVA induced regulation of MMP-1 and NFκB occurred via different signaling pathways and that Trx only influenced the NFκB signaling.

Furthermore, we found that Trx prevented UVB induced reduction of Collα1 expression, but did not influence upregulation of MMP-1 expression by UVB. Thus, similar as for UVA separate pathways exist, which are regulated by UVB, but differentially influenced by Trx. Brenneisen et al. demonstrated that the UVB induced increase in MMP-1 is completely inhibited by LY294002, a specific inhibitor of PI3K, in dermal fibroblasts (Brenneisen et al., 2002). We have previously been shown that Trx increased PI3K/Akt pathway activity in endothelial cells (Haendeler et al.,

2004b). Therefore, Trx rather increased UVB induced upregulation of MMP-1 than decreased seems not surprising to us (Fig. 2B).

Finally, we demonstrated here that Trx inhibited IRA induced upregulation of MMP-1 and downregulation of Collα1. One may speculate that the anti-oxidative capacity of Trx may play a role in IRA signaling, since we previously demonstrated that IRA induced MMP-1 upregulation completely depends on mitochondrial ROS formation (Schroeder et al., 2007a) and that cells overexpressing Trx inhibit mitochondrial ROS formation (Zhou et al., 2007).

In conclusion, our study demonstrates that MMP-1 and Collα1 are at least in part regulated by different signaling pathways and that UVA and UVB induce several pathways, which regulate either the expression of MMP-1 or of Collα1. With respect to IRA, Trx can influence both MMP-1 and Collα1 expression. Moreover, Trx can only influence distinct signaling pathways induced by UVA and UVB. In the case of UVA induced MMP-1 expression, Trx seems to alter NFκB signaling. Thus, Trx in combination with other therapeutics, which affect other pathways, may serve as an anti-photoaging "cocktail".

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Nuclear protein tyrosine phosphatase Shp-2 is one important negative regulator of nuclear export of telomerase reverse transcriptase

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Nuclear Protein Tyrosine Phosphatase Shp-2 Is One Important Negative Regulator of Nuclear Export of Telomerase Reverse Transcriptase*[S]

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Aging is one major risk factor for numerous diseases. The enzyme telomerase reverse transcriptase (TERT) plays an important role for aging and apoptosis. Previously, we demonstrated that inhibition of oxidative stress-induced Src kinase family-dependent nuclear export of TERT results in delayed replicative senescence and reduced apoptosis sensitivity. Therefore, the aim of this study was to investigate mechanisms inhibiting nuclear export of TERT. First, we demonstrated that H_2O_2-induced nuclear export of TERT was abolished in Src, Fyn, and Yes-deficient embryonic fibroblasts. Next, we wanted to identify one potential negative regulator of this export process. One candidate is the protein tyrosine phosphatase Shp-2 (Shp-2), which can counteract activities of the Src kinase family. Indeed, Shp-2 was evenly distributed between the nucleus and cytosol. Nuclear Shp-2 associates with TERT in endothelial cells and dissociates from TERT prior to its nuclear export. Overexpression of Shp-2 wt inhibited H_2O_2-induced export of TERT. Overexpression of the catalytically inactive, dominant negative Shp-2 mutant (Shp-2(C459S)) reduced endogenous as well as overexpressed nuclear TERT protein and telomerase activity, whereas it had no influence on TERT(Y707F). Binding of TERT(Y707F) to Shp-2 is reduced compared with TERTwt. Ablation of Shp-2 expression led only to an increased tyrosine phosphorylation of TERTwt, but not of TERT(Y707F). Moreover, reduced Shp-2 expression decreased nuclear telomerase activity, whereas nuclear telomerase activity was increased in Shp-2-overexpressing endothelial cells. In conclusion, Shp-2 retains TERT in the nucleus by regulating tyrosine 707 phosphorylation.

Telomeres are the physical ends of the chromosomes. They maintain chromosome stability, genetic integrity and cell viability in a variety of different species (1, 2). Telomeres can also function as a mitotic clock, because telomeres are progressively shortened during each cell division. The enzyme telomerase, with its catalytic subunit telomerase reverse transcriptase (TERT),[5] counteracts the shortening of telomeres. Introduction of TERT into human cells extended both their life-span and their telomeres to lengths typical of young cells (3–5). In addition to this well known function of TERT, functions independent of telomere shortening have been described. TERT has been shown to inhibit apoptosis (6, 7). Recently, it has been demonstrated that TERT is also localized in the mitochondria, but its function there is discussed controversially (8–10). TERT is regulated by transcriptional and post-translational mechanisms. Phosphorylation, binding proteins, and cellular localization have been described for post-translational modifications of TERT (11–15). TERT can be phosphorylated and its activity is regulated by kinases like c-Abl, PKC, ERK1/2, and Akt (16–20). We demonstrated that TERT is tyrosine-phosphorylated by the Src kinase family under conditions of oxidative stress (21, 22). Functionally, this results in nuclear export of TERT disrupting the anti-apoptotic and telomere-extending potential of the enzyme, which subsequently leads to enhanced apoptosis sensitivity and accelerated senescence of cells (21, 22).

One important regulator of the Src kinase family activity is the protein tyrosine phosphatase Shp-2 (Shp-2). The importance of Shp-2 for survival has been documented by the Shp-2 knockout mice which are embryonic lethal (23). All the described mechanisms for the regulatory mechanisms of Shp-2 are associated with its ability to dephosphorylate target molecules such as Src kinase family members, which are bound to growth factor receptors at the membrane to inhibit the permanent activation of the receptors by kinase phosphorylation (for review see Ref. 24). Recently, it has been demonstrated that Shp-2 is also localized in the nucleus, where it binds to the transcription factor STAT5a and thereby regulates its function (25). Because the regulation, which leads to nuclear export of TERT has to occur in the nucleus, we hypothesized that Shp-2 might counteract the nuclear export of TERT.

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[5] The abbreviations used are: TERT, telomerase reverse transcriptase; Shp-2, protein tyrosine phosphatase Shp-2; EC, human umbilical venous endothelial cells; HEK, human embryonic kidney cells; MEF, mouse embryonic fibroblasts; wt, wild type; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Our data demonstrate that nuclear export of TERT is triggered by Src and/or Yes. One negative regulator of this export is Shp-2. Shp-2 is localized in the nucleus, is associated with TERT and dissociates from TERT prior to its export. The catalytic activity of Shp-2 is crucial for retaining TERT in the nucleus.

EXPERIMENTAL PROCEDURES

Cell Culture—Endothelial cells (EC) were cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/ml), bovine brain extract (12 μg/ml), gentamycin (50 μg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 10% fetal calf serum. After detachment with trypsin, cells were grown for at least 18 h (26, 27). Human embryonic kidney cells (HEK) were cultured in DMEM basal medium with 10% heat-inactivated fetal calf serum. Mouse embryonic fibroblasts deficient of Src, Fyn, and Yes and their wild-type counterparts were cultured in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum and gentamycin.

Plasmids—Human Shp-2 was cloned out of endothelial cell-derived cDNA incorporating EcoRI and BamHI restriction sites. The amplified PCR product was subcloned into pcDNA3.1 (−) vector containing a Myc tag (Invitrogen) or into pGFP vector creating a Shp-2 GFP fusion. The catalytically inactive mutant of Shp-2 (Shp-2(C459S)) was generated by site-directed mutagenesis (Stratagene) out of Shp-2 wt. The hTERT construct was kindly donated by Dr. Weinberg (28). TERT was subcloned into pcDNA3.1(−)Myc-His vector (TERTwt) containing the Myc tag at the C terminus. TERT(Y707F) was generated by site-directed mutagenesis. ShRNA vectors were obtained from Sigma (Mission shRNA series).

Transfection—HEK were transiently transfected with Lipofectamine/Plus according to the manufacturer's protocol (Invitrogen) as previously described (29) with a transfection efficiency of 90 ± 4%. EC were transiently transfected with Superfect (Qiagen) as described previously. Mouse embryonic fibroblasts were transiently transfected with Lipofectamine/Plus according to the manufacturer's protocol (Invitrogen). Transfection of siRNA was performed by using INTERFERRin™ according to the manufacturer's protocol (POLYPLUS transfection). Sequence of scrambled siRNA: 5′-AACUUGAGAAUCGCCUGAA-3′, sequence of Shp-2 siRNA: 5′-GAAGCACAGUACCGAUUUA-3′.

Telomerase Enzyme Activity Measurement—Telomerase enzyme activity was measured using a commercially available PCR-based assay according to the manufacturer's protocol (Roche Applied Science) as previously described (30). In brief, after PCR amplification, PCR products were either used for detection of telomerase enzyme activity by 1) ELISA or by 2) telomerase-mediated DNA laddering. 1) For ELISA, PCR products are immobilized via the biotin-labeled TS primers (provided with the assay) to a streptavidin-coated microtiter plate. The linearity of the assay was assured by the positive controls provided by the company, and as negative controls heat (95 °C, 2 min) and RNase-treated samples were used in the presence of the biotinylated primers (*inset*, Fig. 5*b*).

Separation of Nuclear and Cytosolic Extracts—Nuclear and cytosolic extracts were separated using a commercially available kit according to the manufacturer's protocol (Pierce). In brief, cells were scraped off the dish and centrifuged at 800 × *g* for 5 min at 4 °C. The resulting pellet was resolved in cytosolic extraction reagent I (CERI buffer) and incubated for 10 min at 4 °C. After adding cytosolic extraction reagent II (CERII buffer) and further incubation for 1 min at 4 °C, samples were centrifuged at 16,000 × *g* for 5 min at 4 °C. The resulting supernatant contained the cytosolic fraction. The resulting pellet was washed with phosphate-buffered saline and resuspended in nuclear extraction reagent (NER buffer) and incubated for 60 min at 4 °C. After centrifugation for 15 min at 16,000 × *g* at 4 °C, the resulting supernatant was obtained as nuclear fraction. Purity of the nuclear and cytosolic extracts was always assured by immunoblotting with topoisomerase 1 (nuclear) and HSP70 (cytosolic).

Immunoprecipitation and Immunoblotting—Lysates (250 μg) were immunoprecipitated with 2.5 μg of Shp-2 antibody or 2.5 μg of Myc antibody overnight at 4 °C. After incubation with A- and G-Sepharose (Amersham Biosciences) for 2 h at 4 °C, resulting beads were washed, subjected to SDS-PAGE sample buffer, and resolved on a 10% SDS-PAGE.

Immunoblotting was performed with antibodies directed against TERT (1:200, overnight, 4 °C, Calbiochem or 1:500, overnight, 4 °C, Rockland), actin (1:8000, overnight 4 °C, Sigma) and Shp-2, Myc, Hsp70, Ref-1, topoisomerase I (2 h, 1:250, all Santa Cruz). Antibodies were detected by the enhanced chemiluminescence system (Amersham Biosciences). Semi-quantitative analyses were performed on scanned immunoblots using Scion Image 1.6 (Scion Corp.).

Immunostaining—Cells were fixed in 4% paraformaldehyde and permeabilized using 0.3% Triton X-100 and 3% bovine serum albumin in phosphate-buffered saline. For immunostaining, cells were incubated with an antibody against TERT and stained with an anti-rabbit Texas-Red-conjugated Fab fragment. Nuclei were counterstained with Sytox-Blue or DAPI (1:1000, 5 min, Molecular Probes). Cells were visualized with confocal microscopy (Zeiss, LSM 510 META, magnification 1:40 oil).

Statistics—Statistical analysis was performed with Student's *t* test or Wilcoxon, Mann-Whitney test using XLSTAT 2008.

RESULTS

Nuclear Export of TERT Depends on Src and Yes—Recently, we demonstrated that short term exposure to oxidative stress as well as aging-induced reactive oxygen species formation led to a Src kinase family-dependent nuclear export of TERT. Loss of nuclear TERT resulted in increased apoptosis sensitivity and accelerated senescence (21, 22). To reduce the number of candidates of the Src kinase family, which are responsible for oxidative stress-induced nuclear export of TERT, we used embryonic mouse fibroblasts deficient for Src, Fyn, and Yes and overexpressed TERTwt in these cells. Treatment with 500 μM H_2O_2 for 6 h resulted in a reduction of nuclear TERT protein and an increase in cytosolic TERT protein in wildtype fibroblasts. In contrast, in fibroblasts deficient for Src, Fyn, and Yes, nuclear export of

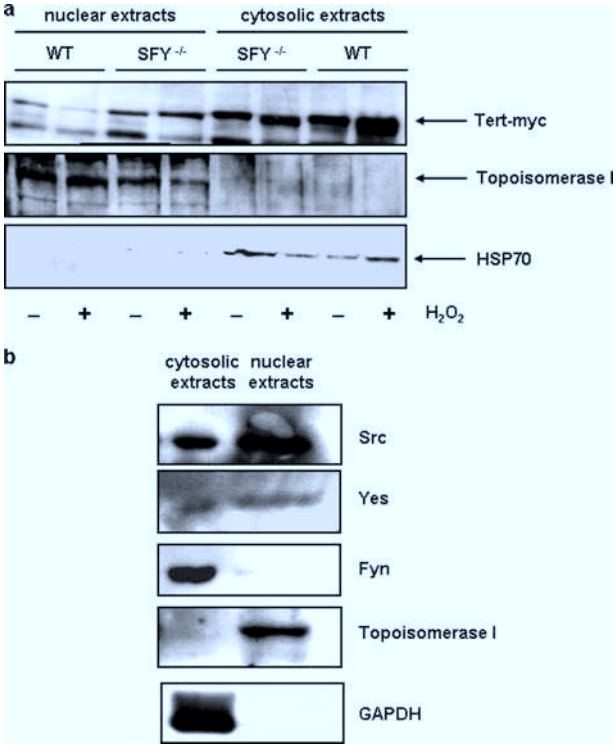

FIGURE 1. **Absence of Src, Fyn, and Yes completely abrogates nuclear export of TERT.** *a*, Src, Fyn, Yes-dependent nuclear export of TERT. Embryonic fibroblasts from mice deficient for Src, Fyn, and Yes ($SFY^{-/-}$) and from their wild-type counterparts (*WT*) were transfected with TERT-myc and incubated with 500 μM H_2O_2 for 6 h. Nuclear and cytosolic extracts were prepared as described under "Experimental Procedures" and used for immunoblotting; shown is a representative blot of three independent experiments. *Upper panel*, detection of TERT-myc with an anti-Myc antibody. The *middle* and *lower panel* show the purity of the nuclear and cytosolic extracts with the nuclear marker topoisomerase I and the cytosolic protein Hsp70. *b*, nuclear localization of Src and Yes. Nuclear and cytosolic extracts were prepared from EC and used for immunoblotting. The following proteins were detected with specific antibodies (from *top to bottom*): Src, Yes, Fyn, topoisomerase I (nuclear marker), and GAPDH (cytosolic marker).

TERT was completely abolished (Fig. 1*a*) demonstrating that these three kinases play a role in this process.

The functional relevance of these kinases in EC is substantiated by the fact that Src and Yes could be detected in nuclear extracts of them (Fig. 1*b*). Having demonstrated that Src and/or Yes are responsible for oxidative stress induced nuclear export of TERT, we next wanted to identify one of the counter players, which inhibits nuclear export of TERT. One known inhibitor of Src kinase family functions is Shp-2. However, to inhibit nuclear export of TERT, Shp-2, like Src and Yes must be localized in the nucleus. Therefore, we first investigated the cellular localization of Shp-2 in EC. We found that Shp-2 is evenly distributed between the nuclear and cytosolic extracts (Fig. 2*a*). Thus, we hypothesized that Shp-2 could be indeed the counter player for oxidative stress-induced nuclear export of TERT. Similar to endogenous Shp-2, overexpressed, myc- or GFP-tagged Shp-2 were also localized in the nucleus (Fig. 2*a* and supplemental Fig. S1).

If Shp-2 is involved in TERT nuclear export by oxidative stress, we hypothesized that Shp-2 itself must be regulated by H_2O_2 in EC. Indeed, incubation with 200 μM H_2O_2, which led to a nuclear export of TERT, resulted in a minor, but significant decrease of total Shp-2 protein and activity (Fig. 2, *b* and *c*).

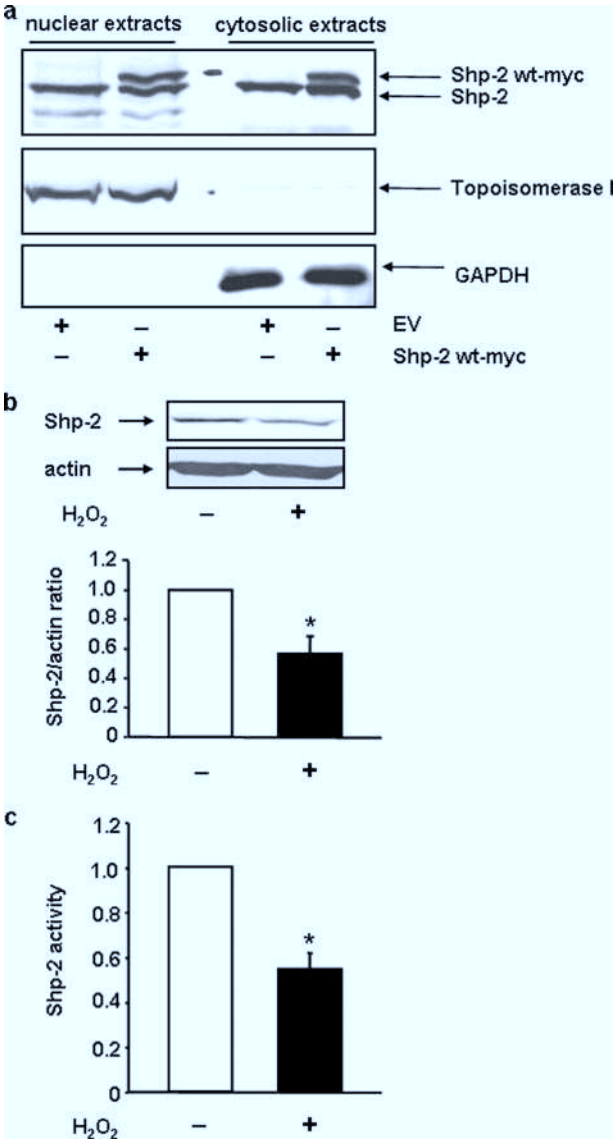

FIGURE 2. **Subcellular localization of Shp-2 and regulation by H_2O_2.** *a*, endogenous and overexpressed Shp-2 are evenly distributed between nucleus and cytoplasm in EC. Shp-2 wt-myc was overexpressed in EC. Nuclear and cytosolic extracts were prepared as described under "Experimental Procedures" and used for immunoblots. *Upper panel* shows an immunoblot with an anti-Shp-2 antibody, *middle* and *lower panels* demonstrate the purity of the extracts using antibodies against the nuclear marker topoisomerase I and the cytosolic marker GAPDH. One representative immunoblot is shown ($n = 6$). *EV*, empty vector; *b*, H_2O_2 reduces Shp-2 protein levels. *Upper panel*, EC were incubated with 200 μM H_2O_2 for 6 h and immunoblots were performed with an anti-Shp-2 antibody and anti-actin for normalization. *Lower panel*, semi-quantitative analysis of four independent experiments. Shown are the Shp-2 levels relative to actin, data are means ± S.E. (relative levels in untreated cells set to 1; *, $p < 0.05$). *c*, H_2O_2 reduces Shp-2 activity. EC were incubated with 200 μM H_2O_2 for 6 h, lysed, and Shp-2 activity was measured in the lysates. Data are means ± S.E. ($n = 6$) and are shown relative to Shp-2 activity in untreated cells (*, $p < 0.05$).

Shp-2 Associates with TERT and Retains It in the Nucleus—To get first insights whether Shp-2 could be a direct player in the regulation of TERT, we next determined whether TERT and Shp-2 associate with each other in the nucleus. Therefore, we co-immunoprecipitated endogenous Shp-2 and TERT-myc from nuclear extracts of TERT-myc-transfected cells. Indeed, the majority of TERT is associated with Shp-2 in the nucleus (Fig. 3). This association seems to be specific as we did not find

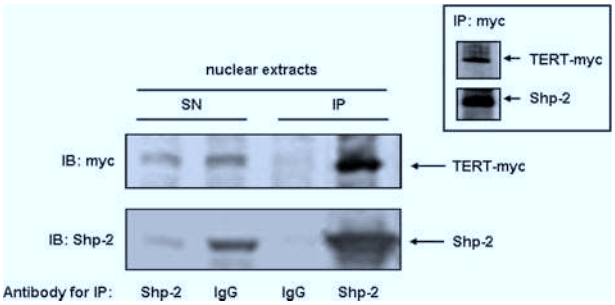

FIGURE 3. **Shp-2 associates with TERT.** HEK cells were transfected with TERT-myc and endogenous Shp-2 was immunoprecipitated from 250 μg of nuclear protein. Immunoblots with the precipitate (*IP*) and 25 μg of total protein from the supernatant (*SN*) were performed with an anti-Myc antibody (TERT-myc, *upper panel*) and anti-Shp-2 (*lower panel*). IgG served as negative control. The *inset* shows the reciprocal immunoprecipitation with an anti-Myc antibody.

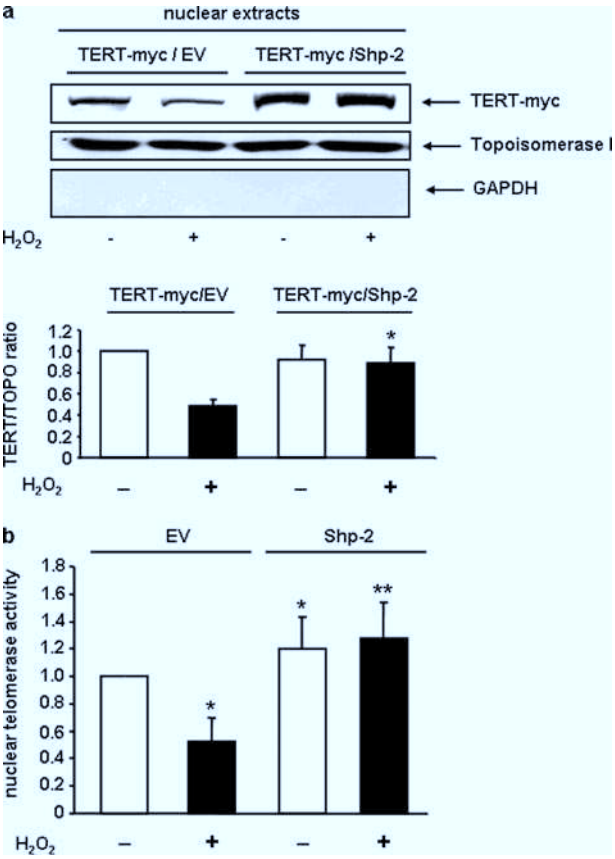

FIGURE 4. **Shp-2 wt rescues H_2O_2-induced loss of nuclear TERT protein and telomerase activity.** *a*, EC were transfected with either empty vector (*EV*) and TERT-myc or with Shp-2 and TERT-myc. After incubation with 200 μM H_2O_2, immunoblot against TERT-myc and topoisomerase I was performed in nuclear extracts. *a*, *upper panel* shows a representative immunoblot with an Myc antibody, the *upper middle panel* shows purity of nuclear fraction with an anti-topoisomerase I antibody and *upper lower panel* shows purity of nuclear fraction with an anti-GAPDH antibody ($n = 4$). *Lower panel* shows the semi-quantitative analysis of four independent experiments. TERT levels were normalized to topoisomerase I and are shown relative to the levels of cells transfected with EV/TERT-myc not treated with H_2O_2. Data are means ± S.E. (*, $p < 0.05$ *versus* TERT-myc/EV + H_2O_2). *b*, overexpression of Shp-2 rescued H_2O_2-induced reduction of nuclear telomerase activity. EC were transfected and treated as in *a*. Nuclear telomerase activity was measured as described under "Experimental Procedures" and is shown relative to the level in EV-transfected cells not treated with H_2O_2. Data are means ± S.E. ($n = 6$; *, $p < 0.05$ *versus* EV-H_2O_2. **, $p < 0.05$ *versus* EV + H_2O_2).

complexes between TERT-myc, Shp-2, and the transcription factor Ref-1 (supplemental Fig. S2). Having demonstrated that nuclear Shp-2 interacts with TERT, we next examined whether

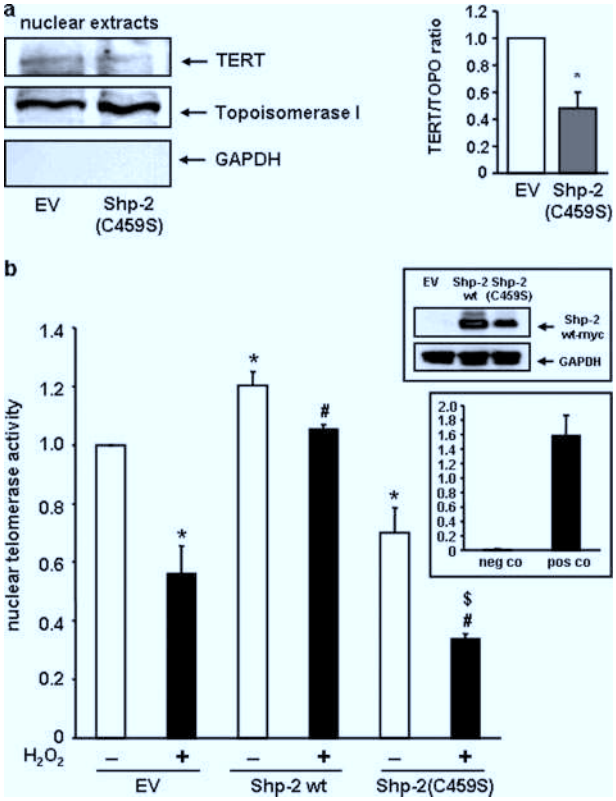

FIGURE 5. **Nuclear retention of TERT requires catalytically active Shp-2.** *a*, EC were transfected with empty vector (*EV*) or Shp-2(C459S). Immunoblot was performed with an anti-TERT antibody (*left panel*, *top*), an anti-topoisomerase I antibody (*left panel*, *middle*) and an anti-GAPDH antibody (*left panel*, *bottom*). TERT levels were determined by semi-quantitative analysis and normalized to topoisomerase I (*right panel*). Data are means ± S.E. of four independent experiments, the relative TERT levels in EV-transfected cells are set to 1 (*, $p < 0.05$). *b*, EC were transfected with empty vector (*EV*), Myc-tagged Shp-2 wt or Shp-2(C459S) and incubated with 200 μM H_2O_2 for 6 h. Nuclear telomerase activity was measured and is shown relative to the level in EV-transfected cells not treated with H_2O_2. Data are means ± S.E. ($n = 3$; *, $p < 0.05$ *versus* EV; #. $p < 0.05$ *versus* EV + H_2O_2; $, $p < 0.05$ *versus* Shp2(C459S)). *Lower inset* shows absolute values (A_{450}) of negative and positive controls; the measured absorbances of all samples were within the range of these controls. To assess the levels of both Shp-2 wt and Shp-2(C459S) immunoblot of whole cell lysates was performed with an anti-Myc antibody (*upper inset*, *top panel*), equal loading was confirmed using GAPDH (*upper inset*, *bottom panel*).

Shp-2 has an inhibitory effect on oxidative stress-induced nuclear export of TERT.

We overexpressed Shp-2 and TERT-myc in EC and incubated them for 6 h with 200 μM H_2O_2 to induce nuclear export of TERT. As shown in Fig. 4*a*, overexpression of Shp-2 completely abolished nuclear export of TERT. In line with this finding, overexpression of Shp-2 inhibited H_2O_2-induced reduction of endogenous, nuclear telomerase activity (Fig. 4*b*).

Nuclear Retention of TERT Requires Catalytically Active Shp-2—To determine whether the catalytic activity of Shp-2 is required for retaining TERT in the nucleus, we investigated the effects of the catalytically inactive, dominant negative Shp-2 mutant, Shp-2(C459S). First we verified that this mutant like the wild-type protein is evenly distributed between nucleus and cytoplasm (supplemental Fig. S3).

Overexpression of Shp-2(C459S) induced already under basal conditions reduction of nuclear TERT protein (Fig. 5*a*). In addition, we transfected EC with Shp-2 wt or Shp-2(C459S), incubated them with H_2O_2 and measured telomerase activity in

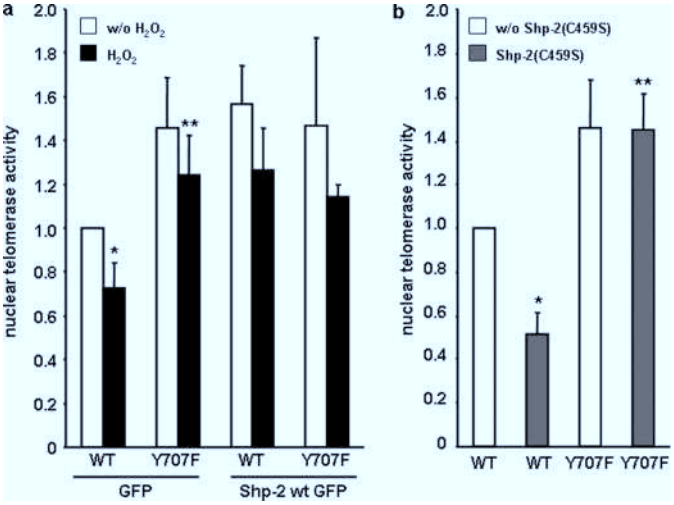

FIGURE 6. **Tyrosine 707 is a target for Shp-2.** *a*, EC were transfected with Myc-tagged TERT-wt (*WT*) or TERT(Y707F) (*Y707F*) together with GFP or Shp-2 wt GFP. After incubation with H_2O_2 nuclear extracts were prepared and nuclear telomerase activity was measured. Data are means ± S.E. ($n = 4$) relative to the values obtained with TERT-wt/GFP in untreated cells (*, $p < 0.05$ *versus* TERT wt/GFP w/o H_2O_2; **, $p < 0.05$ *versus* TERT wt/GFP with H_2O_2. *b*, EC were transfected with Myc-tagged TERT-wt (*WT*) or TERT Y707F (*Y707F*) together with GFP or Shp-2(C459S)-GFP. 24 h after transfection, nuclear extracts were prepared, and nuclear telomerase activity was measured. Data are means ± S.E. ($n = 4$) relative to the values obtained with TERT-wt/GFP (*, $p < 0.05$ *versus* TERT-wt/GFP; **, $p < 0.05$ *versus* TERT-wt/Shp2(C459S)-GFP).

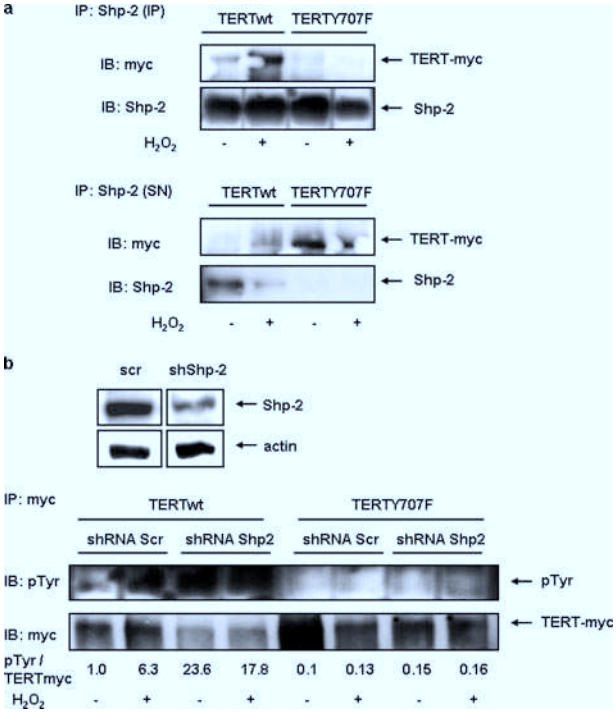

FIGURE 7. **Tyrosine phosphorylation of TERT depends on Shp-2.** *a*, HEK cells were transfected with Myc-tagged TERT-wt (*WT*) or TERT(Y707F) (*Y707F*), incubated with H_2O_2 for 1 h as indicated and immunoprecipitated with an anti-Shp-2-antibody from nuclear extracts. Immunoblots with the precipitates (*IP*) and the supernatants of the IP (*SN*) were performed with an anti-Myc antibody (*upper panel*) or an anti-Shp-2 (*lower panel*). *b*, HEK cells were first transfected with a shRNA vector targeting Shp-2 (shShp-2) or a scrambled control (*scr*) and 24 h later with TERT-wt (*WT*) or TERT(Y707F) (*Y707F*). Another 24 h later, cells were treated with H_2O_2 for 1 h, and nuclear lysates used for immunoprecipitation (*IP*) with an anti-Myc antibody. Immunoblots with the precipitates were performed with an anti-phosphotyrosine antibody (*upper panel*) or an anti-Myc antibody (*lower panel*).

nuclear extracts. Shp-2 wt increased, whereas Shp-2(C459S) reduced endogenous, nuclear telomerase activity under basal conditions. This effect seems to be specific for Shp-2, because overexpression of another phosphatase, namely the protein phosphatase 2a (PP2A) did not alter nuclear TERT protein and telomerase activity (supplemental Fig. S4). Shp-2 wt completely abrogated the effect of H_2O_2 on endogenous, nuclear telomerase activity, which is in accordance with our data presented in Fig. 4*a*. Interestingly Shp-2(C459S)-overexpressing cells showed a stronger effect on endogenous, nuclear telomerase activity compared with empty vector transfected cells under H_2O_2 treatment (Fig. 5*b*). These data clearly demonstrate that nuclear retention of TERT depends on the presence of Shp-2 protein and its activity. This is in accordance with the findings shown in Fig. 2 that H_2O_2 treatment reduces total Shp-2 protein and activity, providing one possible explanation for the H_2O_2-induced nuclear export of TERT.

We previously reported that TERT(Y707F) is retained in the nucleus under H_2O_2 treatment, suggesting that this tyrosine is crucial for nuclear export (21). The involvement of Shp-2 in this process suggests that phosphorylation/dephosphorylation regulates subcellular TERT localization. As expected, Shp-2 wt did not alter nuclear telomerase activity in TERT(Y707F)-overexpressing cells even after H_2O_2 treatment because this mutant cannot be phosphorylated at position 707 and thus does not require dephosphorylation for nuclear retention (Fig. 6*a* and Ref. 21). Moreover, under basal conditions, overexpression of dominant negative Shp-2(C459S) reduced nuclear telomerase activity in TERT-wt transfected cells, whereas it had no influence on TERT(Y707F) (Fig. 6*b*), suggesting that tyrosine 707 in TERT is the target for Shp-2 phosphatase activity. Next, we investigated the influence of tyrosine 707 on the TERT Shp-2 association in the nucleus in the presence and absence of H_2O_2. Association of TERT(Y707F) to Shp-2 is markedly reduced compared with TERT-wt. This is also reflected in the supernatants of the immunoprecipitations where more unbound TERT(Y707F) is found (Fig. 7*a*). Finally, ablation of Shp-2 increased tyrosine phosphorylation of nuclear TERT-wt, whereas TERT(Y707F) was unaffected (Fig. 7*b*). Similarly, reduced Shp-2 expression decreased nuclear telomerase activity and TERT protein already under basal conditions (supplemental Fig. S5 and Fig. 7*b*).

DISCUSSION

The present study demonstrates that nuclear Shp-2 is associated with nuclear TERT and dissociates from TERT prior to its export, that Shp-2 inhibits nuclear export of TERT and that tyrosine 707 in TERT is a target for Shp-2.

Shp-2 was identified as a cytosolic SH2 domain containing tyrosine phosphatase, which is ubiquitously expressed. The wide distribution of the enzyme indicates that it might regulate various physiological functions. Homozygous Shp-2 knockout mice are embryonic lethal underscoring the importance of the enzyme. It is clear that Shp-2 plays a critical role in regulating signal transduction; however, a profound biochemical basis for the different functions of this phosphatase remains to be elucidated. Our data now add another important issue to the list of

unaddressed questions: What is the function for nuclear Shp-2? Chughtai *et al.* (25) reported that Shp-2 exists in a complex with the transcription factor Stat5a. The authors speculate that active Shp-2 and a tyrosine-phosphorylated Stat5a translocate to the nucleus and potentially function in the nucleus to regulate transcription as both Shp-2 and Stat5a as a complex bind DNA. Our data now provide evidence that Shp-2 is localized in the nucleus already under basal conditions, where it is associated with TERT. Thus, it is tempting to speculate that some of the physiological functions of Shp-2 have to be addressed to its nuclear localization. The question remains how Shp-2 is imported into the nucleus, because it lacks a nuclear localization sequence. Therefore, a binding protein for Shp-2 could be required to induce its nuclear import. A possible candidate is Gab1, which has a nuclear import sequence (31) and has been shown to bind to Shp-2 (32). Preliminary data from our laboratory indicate that overexpression of a nuclear localization deficient Gab1 mutant prevents nuclear import of Shp-2.[6]

Under oxidative stress, TERT is tyrosine-phosphorylated and thereby exported from the nucleus in a Src kinase family/CRM1-dependent manner (21). In this study we demonstrate that Src and Yes are also localized in the nucleus of EC and thereby could be responsible for tyrosine phosphorylation of TERT. Moreover, the Src kinase family has been implicated in aging processes. This is mainly due to the fact that reactive oxygen species, which accumulate with age and induce lipid peroxidation, protein modification, DNA strand breaks, and cause oxidative damage, enhance the activity of the Src kinase family, which results in a progressive loss of cell function, a hallmark for aging processes (22, 33–35). We provide functional evidence that Src, Fyn, and Yes could play a role in aging processes, because cells deficient in these kinases can retain TERT in the nucleus under oxidative stress.

It has to be assumed that the export of nuclear TERT is regulated. Here, we demonstrate that Shp-2 is associated with TERT in the nucleus under basal conditions. More importantly, endogenous Shp-2 dissociates from nuclear TERT prior to its export and down-regulation of endogenous Shp-2 expression reduced nuclear telomerase activity. Thus, one may speculate that Shp-2 protects TERT from nuclear export by complex formation with TERT. Our data also suggest that tyrosine 707 in TERT is a target for the phosphatase activity of Shp-2. Therefore, Shp-2 might indeed be the unknown counterplayer for oxidative stress-induced nuclear export of TERT. However, we still do not know whether it acts solely via dephosphorylation. However, our data presented here strongly suggested that tyrosine 707 in TERT is a target of Shp-2 because the phosphorylation status of TERT(Y707F) is unaffected by Shp-2 and more importantly tyrosine phosphorylation of TERT-wt is negatively regulated by Shp-2. As a functional consequence nuclear Shp-2 could protect the cell either against accelerated senescence or increased apoptosis sensitivity or even both.

To our knowledge Shp-2, or more precise nuclear Shp-2, has not been implicated in aging processes until now. It has only been suggested that Shp-1 is responsible for aging-related attenuation of EGF receptor signaling in dermal fibroblasts (36), which suggests that Shp-1 negatively contributes to aging processes. This is in line with findings that Shp-1 plays a negative role in transducing signals for cellular responses (24). Previous biochemical evidence has shown that the enzymatic activity of Shp-2 is required for its function in signal transduction (37, 38). Replacing cysteine 459 with serine completely abolished its enzymatic activity. Binding of this mutant to other proteins via its SH2 domains remained unaltered. However, introduction of this mutant markedly inhibited the activation of MAP kinases in response to EGF and insulin (37, 39). This is in line with our data presented here that Shp-2(C459S) has also a nuclear localization (data not shown), but reduced nuclear telomerase activity and TERT protein already under basal conditions.

In summary, our study demonstrates a new important function for nuclear Shp-2 in retaining TERT in the nucleus. In addition, tyrosine 707 is a target for Shp-2. It counteracts the Src and Yes effects on TERT protein and telomerase activity in the nucleus under conditions of oxidative stress, most likely by regulating the net phosphorylation status of tyrosine 707 in TERT. This suggests that keeping Shp-2 in the nucleus delays aging processes and inhibits apoptosis.

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"Shping 2" different cellular localizations - a potential new player in aging processes

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J. Haendeler: Senior Autor, hatte die Idee zum Manuskript, korrigierte den Text und gab wertvolles Feedback für das Manuskript.

Research perspective

"Shping 2" different cellular localizations - a potential new player in aging processes

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Abstract: The functions of the ubiquitously expressed protein tyrosine phosphatase Shp-2 are dependent on its localization. Cytosolic Shp-2 is known to modulate different pathways involved in cell growth, cell development, tissue inflammation and cellular chemotaxis. But Shp-2 is also localized in the nucleus and the mitochondria. Nuclear Shp-2 forms a complex with the signal transducer and activator of transcription 5 (STAT5) which then binds to DNA and regulates transcription of milk genes. In contrast, nuclear Shp-2 dephosphorylates STAT1 and thereby inhibits gene transcription. In addition, it counteracts the oxidative stress dependent nuclear export of Telomerase Reverse Transcriptase (TERT) mediated by members of the Src kinase family, a process leading to replicative senescence. For the recently found mitochondrial Shp-2 an involvement in the regulation of the cellular redox balance is discussed. Shp-2 shows the ability to regulate reactive oxygen species formation in the mitochondria. There are hints that mitochondrial Shp-2 and Src are involved in the regulation of respiratory chain activity. Since a substantial fraction of TERT has been found in the mitochondria, it is hypothesized that mitochondrial Shp-2 acts as a positive regulator of TERT in the mitochondria, similar to its nuclear role. Taken together, Shp-2 seems to be a new player in aging processes.

Shp-2 is a ubiquitously expressed protein tyrosine phosphatase, which contains two N-terminal Src homology 2 (SH2) domains and a C-terminal protein tyrosine phosphatase domain. Several years of research established an important role for cytosolic Shp-2. It is known to modulate different pathways involved in cell growth, cell development, tissue inflammation and cellular chemotaxis due to its well described function to dephosphorylate receptor tyrosine kinases (reviewed in [1]). However, over the last years it has become clear that Shp-2 is also localized in the nucleus and in the mitochondria where it exerts different functions.

In 2002 Chughtai et al reported a nuclear localization of Shp-2 associated with the signal transducer and activator of transcription 5 (STAT5). The stimulation of mammary cells with prolactin induced the nuclear translocation of Shp-2 in a complex with STAT5. Formation of this complex and tyrosine phosphorylation of STAT5 in response to prolactin requires the SH2 domain closer to the C-terminus and the catalytic activity of Shp-2. The authors speculated that the nuclear Shp-2/STAT5 complex binds to DNA and regulates transcription of milk protein genes [2], demonstrating a transcriptional regulation by nuclear Shp-2. This provided for the first time evidence for a function of Shp-2 besides dephosphorylation. In contrast, it has been demonstrated that Shp-2 dephosphorylates STAT1 at tyrosine and serine residues in the nucleus and thereby inhibits its transcriptional

activity [3]. One may speculate that depending on the mode of its action Shp-2 differently regulates specific STAT proteins. Just recently, we discovered that nuclear Shp-2 seems to be involved in aging processes. Previous findings from our group demonstrated that the enzyme Telomerase Reverse Transcriptase (TERT), which is important for maintaining telomere length and known to delay aging processes, when overexpressed, is tyrosine phosphorylated by Src kinases in the nucleus under conditions of oxidative stress in several cell types, including endothelial cells [4, 5]. This tyrosine phosphorylation triggers nuclear export of TERT. Taking into account that cytosolic Shp-2 and the cytosolic Src kinase family can regulate and antagonize each other under certain conditions, we hypothesized that a nuclear Shp-2 also exists in endothelial cells and that this may counteract the Src kinase dependent nuclear export of TERT. Indeed, ablation of endogenous Shp-2 results in increased tyrosine phosphorylation of nuclear TERT and a reduction of telomerase activity in the nucleus. Moreover, overexpression of Shp-2 inhibited oxidative stress induced tyrosine phosphorylation and export of TERT from the nucleus. It has to be noted that this process requires the catalytic activity of Shp-2, since the catalytically inactive mutant Shp-2(C459S) can not prevent nuclear export of TERT. Interestingly, overexpression of Shp-2(C459S) reduced nuclear telomerase activity already under basal conditions. This effect was dependent on tyrosine 707 in TERT [6]. One possible explanation for the nuclear export of TERT induced by Shp-2(C459S) under basal conditions could be the significant increase in reactive oxygen species (ROS), which are known to activate the Src kinase family. Indeed, ROS formation is enhanced upon overexpression of Shp-2(C459S) in endothelial cells (Figure 1). These data point to a regulatory role of Shp-2 in the redox balance of cells.

ROS are important signalling molecules for cellular signal transduction. An imbalance of the redox status with a reduced antioxidative capacity and an increased ROS production has been described to play an important role in aging processes as well as in several diseases. Increased ROS can directly damage DNA, proteins and membrane lipids. This leads among others to damage of the electron transport chain, which results in an increased formation of ROS which in turn cause further damage to DNA, proteins and lipids. This vicious cycle seems to play an important role in aging processes and age-related diseases (for review see [7]).

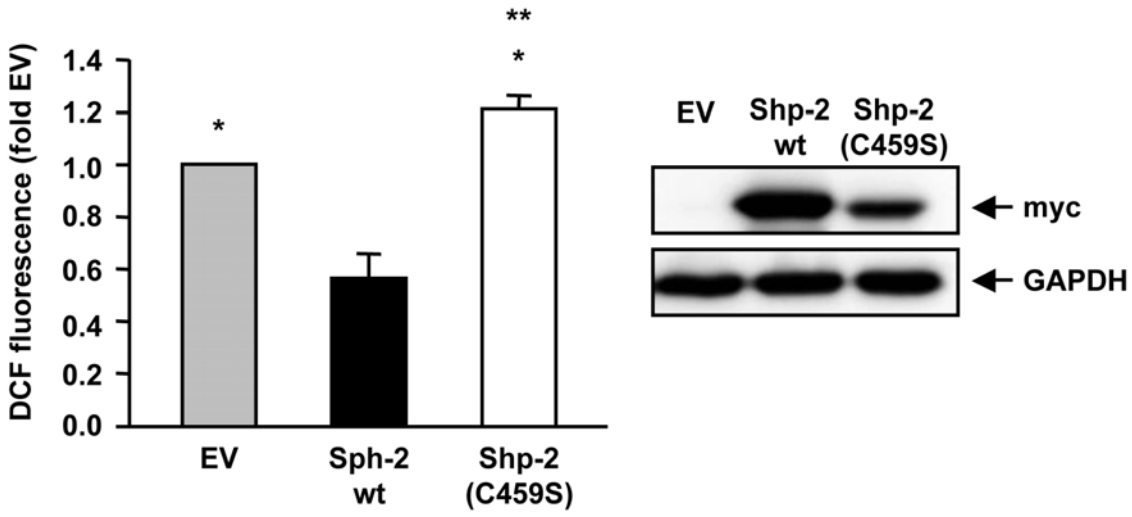

Figure 1. Shp-2 reduces endogenous ROS formation. Endothelial cells were transfected with empty vector (EV), Shp-2 wt or Shp-2(C459S) and endogenous ROS formation was measured using FACS analysis. *p<0.05 versus Shp-2 wt. **p<0.05 versus EV. Data are means +/- SEM (n=6).
The Western blot on the right demonstrates expression of Shp-2 wt and Shp-2(C459S), which were detected with anti-myc antibody.

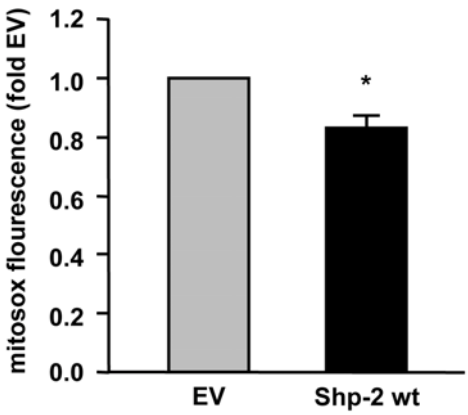

Figure 2. Shp-2 reduces endogenous mitochondrial ROS formation. Endothelial cells were transfected with empty vector (EV) and Shp-2 wt. Mitochondrial ROS formation was measured using mitosox and FACS analysis. *p<0.05 versus EV. Data are means +/- SEM (n=3).

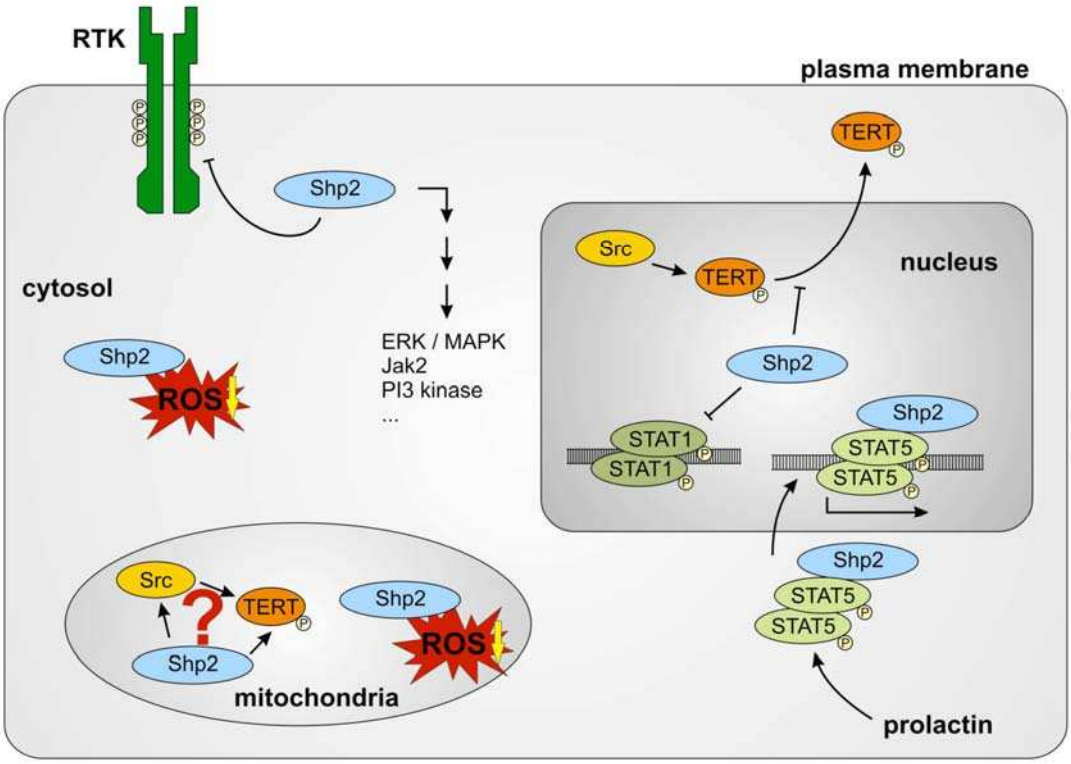

Figure 3. Different functions of Shp-2 in different cell compartments. Cytosolic Shp-2 modulates different pathways by dephosphorylation of receptor tyrosine kinases (RTK). It also decreases cytosolic ROS levels. Nuclear Shp-2 inhibits ROS induced nuclear export of TERT and DNA-binding of STAT1 dimers by dephosphorylation. Prolactin induces the association of Shp-2 and STAT5 and nuclear import of this complex. Shp-2/STAT5 complex binds to DNA and induces transcription of milk genes. Functions of mitochondrial Shp-2 remain unclear. A connection between mitochondrial Src, Shp-2 and TERT may exist. Reduction of mitochondrial ROS formation seems to depend on Shp-2.

Therefore, controlling ROS formation seems to be an interesting tool in delaying aging processes. It is tempting to speculate that nuclear Shp-2 plays an important role in nuclear based aging processes by reducing export of TERT from the nucleus and by reducing ROS formation (figure 1). However, we have also new hints, that Shp-2 may affect mitochondrial ROS production and thus, aging processes which depend on reduced mitochondrial function. New data from our laboratory demonstrate that overexpression of Shp-2 decreases not only ROS production in the cytosol (Figure 1) but also in the mitochondria (Figure 2). Moreover, preliminary results suggest that ablation of Shp-2 increases mitochondrial ROS levels. To specifically measure mitochondrial ROS levels, we used mitosox, a redox-sensitive dye, which first has to enter mitochondria before it can react with ROS. One can speculate, that the observed reduction of mitochondrially derived ROS is connected to a localization of Shp-2 in the mitochondria. Indeed, Salvi et al detected a tyrosine phosphatase activity in the mitochondria of rat brains and identified the responsible phosphatase as Shp-2 [8]. Recently, Arachiche et al showed also the mitochondrial localization of Shp-2 and of the tyrosine kinase Src, which is regulated by Shp-2 [9]. They demonstrated that the complexes of the respiratory chain are substrates of Src, which indicates that respiratory chain activity is partially dependent on tyrosine phosphorylation. Since Shp-2 is an important regulator of Src, Shp-2 is possibly involved in regulation of mitochondrial activity. In line with these findings, we recently demonstrated that TERT is localized in the mitochondria and importantly contributes to respiratory chain activity [10]. TERT deficient mice derived from heterozygous breeding pairs, which show no reduction in telomere length and thus no premature aging phenotype, demonstrated reduced respiratory chain activity in the heart, suggesting an important role for TERT in respiration in vivo [10]. Given the facts that Src kinase family members as well as Shp-2 show mitochondrial localization [9], it is tempting to speculate that similar to nuclear TERT also mitochondrial TERT is positively regulated by Shp-2 in these organelles. This could implicate that mitochondrial Shp-2 in concert with TERT accounts for an intact respiratory chain activity and for reduced mitochondrial ROS formation. Therefore, mitochondrial Shp-2 and TERT could break the above mentioned vicious cycle and thereby may delay aging processes, which depend on mitochondrial dysfunction.

In summary, Shp-2 has the potential to be a yet unknown new important key player in aging processes. Its regulatory function seems to be dependent on its localization within the cell (Figure 3). Nuclear localized Shp-2 counteracts replicative senescence induced by nuclear TERT export and mitochondrial Shp-2 could delay aging processes induced by elevated ROS levels. Therefore, Shp-2 could be an important target for the therapy of diseases connected to aging processes. However, therapeutic interventions aimed at the activation of Shp-2 should take into account the compartment specific functions of this protein.

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CONFLICT OF INTERESTS STATEMENT

The authors of this manuscript have no conflict of interests to declare.

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Nuclear redox signaling

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COMPREHENSIVE INVITED REVIEW

Nuclear Redox Signaling

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Abstract

Reactive oxygen species have been described to modulate proteins within the cell, a process called redox regulation. However, the importance of compartment-specific redox regulation has been neglected for a long time. In the early 1980s and 1990s, many *in vitro* studies introduced the possibility that nuclear redox signaling exists. However, the functional relevance for that has been greatly disregarded. Recently, it has become evident that nuclear redox signaling is indeed one important signaling mechanism regulating a variety of cellular functions. Transcription factors, and even kinases and phosphatases, have been described to be redox regulated in the nucleus. This review describes several of these proteins in closer detail and explains their functions resulting from nuclear localization and redox regulation. Moreover, the redox state of the nucleus and several important nuclear redox regulators [thioredoxin-1 (Trx-1), glutaredoxins (Grxs), peroxiredoxins (Prxs), and APEX nuclease (multifunctional DNA-repair enzyme) 1 (APEX1)] are introduced more precisely, and their necessity for regulation of transcription factors is emphasized. *Antioxid. Redox Signal.* 12, 0000–0000.

I. Introduction

Oxygen is one of the most relevant molecules for all aerobic organisms. For many years, it has been clear that aerobic organisms produce reactive oxygen species (ROS) from oxygen. The molecules, which belong to the ROS, are superoxide anion $O_2^{-\bullet}$, hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2). Even under physiologic conditions, all of these molecules are produced within cells.

Several oxidative systems exist generate $O_2^{-\bullet}$ from oxygen. Potential sources of this ROS production are, for example, the NADPH oxidases, the respiratory chain within the mitochondria, and the xanthine/xanthine oxidase system (Fig. 1A). Conversely, a pool of several antioxidative enzymes scavenge, reduce, or inactivate ROS (Fig. 1A). Specifically, $O_2^{-\bullet}$ is reduced by superoxide dismutases (SODs) to H_2O_2. H_2O_2 is then metabolized to H_2O by several enzymes, mainly by catalase, but also by glutathione peroxidase (GPx), which in turn results in oxidation of glutathione. The glutathione disulfide (GSSG) itself is then reduced to glutathione by the glutathione oxidoreductase (GR) to feed back into this cycle (Fig. 2). Another important antioxidative system, the thioredoxin/thioredoxin-reductase (Trx/TR) also has been described to metabolize H_2O_2 directly. However, this seems to be a rather rare event in cells, and the Trx/TR system mainly reduces oxidized proteins. In the course of this process, reduced Trx itself is oxidized. Reduced Trx is then regenerated by TR by using the cofactor NADPH to be further available for the reduction of oxidized proteins (Fig. 3).

Therefore, a controlled redox balance exists in cells. Perturbation of this balance either by increased production of ROS or by reduced antioxidative capacity will result in so-called oxidative stress (Fig. 1B). Increased ROS lead to modifications of biologic molecules, including proteins, DNA, and lipids. In line with this, under conditions of oxidative stress, $O_2^{-\bullet}$ can directly react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), which readily crosses cell membranes. Although possessing a short half-life, $ONOO^-$ interacts with target molecules even in neighboring cells. Peroxynitrite is involved in protein nitration by nitrating tyrosine residues irreversibly to form 3-nitrotyrosine (95). The interplay of $ONOO^-$ with ROS to damage cells is then termed nitrosative stress. $ONOO^-$ itself is extremely toxic to cells because it is readily converted to two other radical species, $OH^{\bullet}$ and $NO_2^{\bullet}$, by hemolytic decomposition (92). Therefore, these species are often referred to as ROS/RNS (reactive nitrogen species). Oxidative and nitrosative stress–induced modifications of biologic molecules have been implicated in a variety of diseases, such as cardiovascular diseases, neurologic disorders, and cancer. Over a long period, it was believed that the production of ROS is, in principle, bad for cells, and therefore, the term "redox signaling" was inappropriate. However, several lines of evidence have established that redox signaling exists and is required for organisms to survive. Moreover, ROS have a wide range of action because of their ability to interact with almost all biologic molecules. The mode of action of ROS is also dependent on the localization of their target molecules. Thus, the modifications induced by ROS can be separated depending on the cellular compartments they affect. Several studies investigated whether mitochondrial and nuclear redox signaling exist, in addition to the well-described cytosolic (previously termed cellular) redox-dependent events. Under physiologic conditions, ROS can induce changes in gene expression (203), whereas under conditions of oxidative and nitrosative stress, ROS and RNS can directly damage DNA in the nucleus, which can result in apoptosis or malignant transformation (140, 233).

This review focuses on nuclear redox signaling and oxidative stress–induced nuclear translocation of proteins and their resultant nuclear modes of actions. The antioxidative enzymes, Trx-1 and the APEX nuclease (multifunctional DNA repair enzyme) 1 (APEX1, also known as Ape/Ref-1), and their important role in nuclear redox signaling are highlighted. A number of proteins, which belong to the classes of transcription factors, chromatin-modifying enzymes, kinases, and phosphatases, are discussed in detail. All of the mentioned proteins have in common a nuclear localization function and are redox regulated or regulated by oxidative stress in higher eukaryotes. Although this review tries to be as comprehensive as possible, we are aware that we might not have discussed all of them.

II. Nuclear Redox Regulators

A. Redox state of the nucleus

The term redox state describes the balance between the oxidized and reduced forms of biologically relevant redox pairs, including $NADH/NAD^+$, $NADPH/NADP^+$, and GSH/

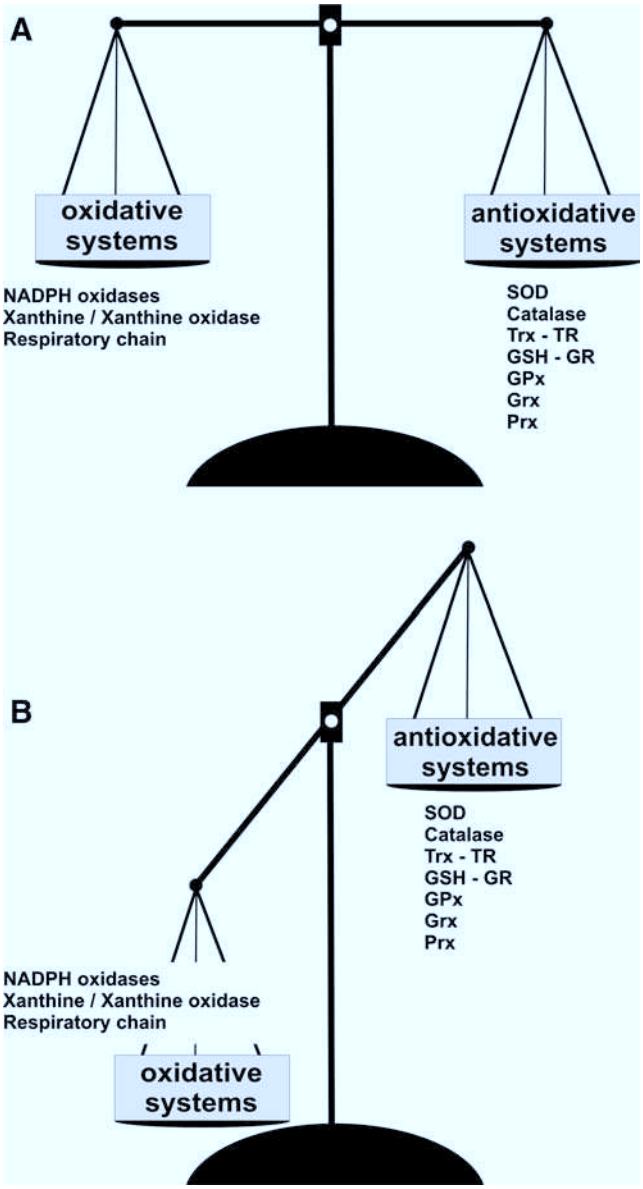

FIG. 1. Balance between oxidative and antioxidative systems. (**A**) The generation of ROS by oxidative systems (*e.g.*, NADPH oxidase, xanthine/xanthine oxidase, and the respiratory chain) is controlled by the activity of antioxidant systems, like superoxide dismutase (SOD), catalase, thioredoxin/thioredoxin reductase (Trx/TR), glutathione/glutathione reductase (GSH/GR), glutathione peroxidase (GPx), glutaredoxin (Grx), and peroxiredoxin (Prx). They scavenge ROS or reduce their levels and thus maintain the redox balance in healthy cells and tissues. (**B**) A state of oxidative stress occurs either by increased activity of the oxidative systems or by reduced antioxidative capacity of the cells.

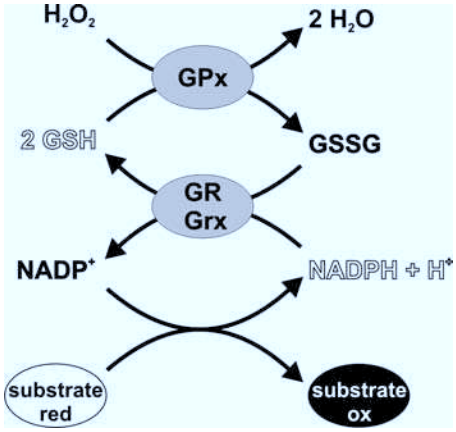

FIG. 2. The GSH/GR/GPx system: a general model of the GSH/GR/GPx system. Glutathione peroxidase (GPx) reduces H_2O_2 to H_2O and thereby oxidizes GSH to GSSG. GSSG itself is reduced by glutathione reductase (GR) to regenerate GSH. The reductant in this reaction is $NADPH + H^+$, created by metabolic substrate oxidation.

GSSG. It is reflected in the balance of several sets of metabolites (*e.g.*, lactate and pyruvate) whose interconversion is dependent on these ratios. An abnormal redox state can develop in a variety of deleterious situations, such as hypoxia, shock, and sepsis. Determination of the relative concentrations of the components of these redox pairs showed that the GSH/GSSG and $NADPH/NADP^+$ ratios are greater than 1 (234, 236, Hwang, 1995 #1311), whereas the $NADH/NAD^+$ ratio is less than 1 (221, 261). Generally, the nucleus provides a reductive environment.

NAD^+/NADH is required as a coenzyme for metabolic processes. The high NAD^+/NADH ratio allows this coenzyme to act as both an oxidizing and a reducing agent. In contrast, the main function of $NADP^+$ is as a reducing agent in anabolism. Because NADPH is needed to drive redox reactions as a strong reducing agent, the $NADPH/NADP^+$ ratio is kept high. Because no barrier exists to diffusion of these dinucleotide coenzymes across the nuclear membrane (160, 261), it is assumed that the NAD^+/NADH and $NADP^+$/NADPH ratios are the same in the cytoplasm and the nucleus. The majority of these coenzymes are protein bound, and the concentration of free NADH in the nucleus has been calculated to be 130 n*M* (261). It is assumed that the NADPH/NADH ratio is about 4 (210); therefore, the concentration of free NADPH in the nucleus must be $\sim$500 n*M*.

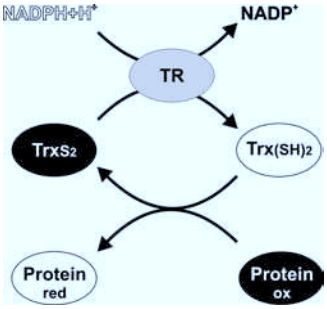

FIG. 3. The Trx/TR system: simplified model of the Trx/TR system and Trx function. The Trx/TR system consists of two oxidoreductase enzymes, thioredoxin (Trx) and thioredoxin reductase (TR). Reduced Trx ($Trx_{(SH)2}$) directly interacts with oxidized proteins by forming disulfide bridges. This involves formation of a mixed-disulfide intermediate in the thiol-disulfide exchange reaction (124). As a consequence of this two-step reaction, the protein is reduced, and Trx itself is oxidized (Trx_{S2}). The regeneration of reduced Trx from its oxidized form is catalyzed by TR by using $NADPH + H^+$.

AU4 ▶

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is a ubiquitous thiol tripeptide and is the most abundant thiol present inside the cell. GSH has multiple direct and indirect functions in many critical cellular processes like synthesis of proteins and DNA, amino acid transport, enzyme activity, and metabolism (149). GSH also serves as a reductant to destroy free radicals, hydrogen peroxide, and other peroxides and as a storage form of cysteine. The redox state of the GSH/GSSG couple is often used as an indicator of the overall redox environment of the cell (200). GSH is found in a fairly high concentration of ~10 m*M* within cells. As it can freely diffuse (160, 172), the cytoplasmic and nuclear concentrations are similar (215, 236). Interestingly, the nuclear levels change during the cell cycle, with the highest levels found in the S and G_2/M phases (137). In line with these findings, depletion of GSH leads to reduced proliferation and apoptosis (138, 151). Taken together, one would hypothesize that during the G_2/M phase, a more reductive nuclear environment is required for cell proliferation to proceed. This was corroborated by a combined *in vitro*/bioinformatic investigation, which showed that 69 proteins containing redox-sensitive motifs have functions in central cell-cycle processes like transcription, nucleotide metabolism, (de)phosphorylation, and (de)ubiquitinylation. The majority of these oxidant-sensitive proteins function during the G_2/M phase, indicating that oxidant-sensitive proteins may be temporally regulated by oscillation of the intracellular redox environment (39).

B. Antioxidative enzymes in the nucleus

As mentioned earlier, this review focuses on nuclear redox signaling. Therefore, it is important to introduce the antioxidative systems that have been described to be localized in the nucleus. Already in the late 1980s, Cu/Zn SOD and catalase were detected in the nuclear fractions of the developing rat cerebral cortex (43). In contrast, in adult Langerhans cells in catalase-overexpressing mice, catalase could not be detected in the nucleus (34). These findings point out that the presence of catalase in the nucleus seems to be dependent on cell type and perhaps also on age.

Several studies investigated the nuclear localization and activity of enzymes of the glutathione system, including GR, GPx, and glutathione *S*-transferases (GSTs). In rat liver cells, all proteins were found to be localized in the nucleus. Moreover, enzymatic activity could be measured in nuclear extracts (191). Recently, the exact localization of GSTα-a was determined to be at the nuclear membrane. Thus, the authors speculated that GSTα-a probably has a role as a defense barrier at the nuclear envelope (220). Several lines of evidence demonstrated that Trx-1 is localized in the nucleus, and its major nuclear function seems to be the binding to and reduction of transcription factors, thereby modulating their activities. Other thiol reductases, namely nucleoredoxin and glutaredoxin (Grx), which have similar, but also distinct functions from those of Trx-1, have been reported to be localized in the nucleus (80). Another important enzyme this context is APEX1. It is known that the DNA base excision-repair pathway is responsible for the repair of alkylation and oxidative DNA damage. A crucial step in the base excision-repair pathway involves the cleavage of an apurinic/apyrimidinic (AP) site in DNA by AP endonucleases (248). The major AP endonuclease in mammalian cells is APEX1, a multifunctional enzyme that acts not only as an AP endonuclease but also as a redox-modifying factor for a variety of transcription factors. Moreover, several studies demonstrated that APEX1 and Trx-1 act in concert in regulating transcription factors, which is discussed in more detail later in this review.

The variety of antioxidative enzymes reported to be localized in the nucleus and to be present in their active forms underscores the existence of a nuclear redox signaling network. In several nuclear proteins, the activity and functions depend on the redox balance and on nuclear redox signaling.

C. Thioredoxin-1

One major redox regulator in cells besides the glutathione system is the thioredoxin system. Thioredoxin was first discovered by Peter Reichard and co-workers (118) in 1964 as an electron donor for ribonucleotide reductase from *Escherichia coli*. In 1968 Holmgren and co-workers (85) determined the amino acid sequence of thioredoxin from *E. coli* after isolation of the pure protein and showed the classic active site -Cys-Gly-Pro-Cys-. The crystal structure of *E. coli* Trx in its oxidized form resulted in the definition of the thioredoxin fold: a central β-sheet surrounded by α-helices with the active site at the end of a β-strand and in the beginning of an α-helix (88). This structure defines a large superfamily of proteins (124). It is adopted by bacterial glutaredoxins (Grx) and appears in the other members of the family as a substructure or domain (9, 50, 139). Besides Trxs and Grxs, protein disulfide isomerases, GSTs, GPxs, peroxiredoxins, and chloride intracellular channels (CLICs) are members of the Trx superfamily (12, 139, 202). Mammalian cells contain two Trxs, Trx-1 and Trx-2 (exclusively localized in mitochondria) and three TRs: cytosolic TR1, mitochondrial TR2, and the testis-specific thioredoxin glutathione reductase (TGR). In 1985, Holmgren (87) introduced Trx-1 as a small, ubiquitous protein with two redox-active cysteine residues in an exposed active center, having the same amino acid sequence as *E. coli* Trx -Cys-Gly-Pro-Cys- (Cys 32 and Cys 35 within Trx-1), which is essential for its redox-regulatory function. The thioredoxin-1 system consists of Trx-1 and TR1. The regeneration of reduced Trx-1 from its oxidized form is catalyzed by TR1 by using NADPH (162). One important function of Trx-1 is the reduction of oxidized proteins, which depends on cysteine 32 and cysteine 35. The functions of Trx-1 are dependent on its cellular localization. Cytosolic Trx-1 interacts with its active-site cysteines with several proteins by forming disulfide bridges and thereby inhibits apoptosis induction. Trx-1 associates with ASK1 and the vitamin D_3–upregulated protein 1 (Txnip, also named VDUP-1) (194, 258). Thereby, reduced Trx-1 protects cells from apoptosis through an inhibitory binding to ASK1, whereas this binding is lost when Trx-1 is oxidized (194). Similarly, binding of Trx-1 to Txnip completely abrogates the antiproliferative function of Txnip (204). It has been demonstrated that, under certain conditions, Trx-1 accumulates in the nucleus and directly or indirectly interacts with different transcription factors (203). In the case of direct association, Trx-1 reduces oxidized transcription factors and thereby allows them to interact with DNA. The capacity of Trx-1 to interact with several transcription factors and thereby alter their functions is discussed in more detail later in this review. In case of an indirect action on transcription factors, Trx-1 binds to APEX1, and APEX1 itself reduces the oxidized

transcription factors. APEX1 is a complex protein with several functions, including endonuclease activity, redox factor, and redox chaperone properties. Because of its importance in nuclear redox signaling, APEX1 is introduced in more detail later in this review.

D. Glutaredoxins and peroxiredoxins

Glutaredoxins (Grxs) belong to the Trx superfamily of proteins. Grxs were first described as glutathione-dependent reductases three decades ago (86). They play an important role in cellular redox-dependent processes, mainly through deglutathionylation of proteins. In addition to the early-discovered dithiol Grxs, another group of Grxs has been identified. These monothiol Grxs lack the C-terminal active-site cysteine but contain all structural and functional elements to bind and use GSH. Therefore, two distinct mechanisms are known, the dithiol reaction in which the two cysteines in the active center take part in reducing both low-molecular-weight and protein disulfides and the monothiol mechanism in which only one cysteine in the active center is present [for review, see (123)]. In humans, four Grx isoforms have been identified: the dithiol isoforms Grx1 and Grx2 and the monothiol isoforms Grx3 (PICOT/TXNL-2) and Grx5 (94, 131, 249, 250). Grx1 is considered mainly to be a cytosolic protein, although some studies show a nuclear localization (133, 219) and a presence in the intermembrane space of mitochondria (171). Several studies have shown that Grx1 is able to deglutathionylate a variety of proteins, like actin, protein tyrosine phosphatase-1B, glyceraldehyde-3-phosphate dehydrogenase, Ras, and caspase 3 (3, 19, 126, 173, 240) and thereby influences several important cellular functions, including actin polymerization, hypertrophy, and apoptosis (3, 173, 240, 242). Furthermore, Grx1 regulates transcription-factor activity directly as well as indirectly, which is discussed later in this review in the respective transcription-factor sections. Grx2 is known to exist in at least three different splicing variants, Grx2a, Grx2b, and the newly discovered Grx2c. Grx2a is considered a mitochondrial protein, and Grx2b was detected in nuclear fractions (62, 131). A recent screening of diverse tissues showed a ubiquitous expression of Grx2a, whereas Grx2b and Grx2c were found exclusively in testis and some cancer cell lines (129). Grx3, also termed PICOT (protein kinase C–interacting cousin of thioredoxin), was first identified in 2000 as an interaction partner of protein kinase C θ (PKCθ) and described to exist in the cytosol (250). Grx5 is a monothiol enzyme with a mitochondrial localization signal and therefore resides within the mitochondria (190). Thus, only Grx1 and Grx2b are localized in the nucleus, and regulation of transcription factor activity in the nucleus has been demonstrated only for Grx1.

Peroxiredoxins (Prxs) are members of a superfamily of Se-independent peroxidases. Six members have been identified and characterized in mammals. Prxs execute enzymatic degradation of H_2O_2 and organic hydroperoxides by using electrons donated by Trx-1 (Prx1 to Prx5), cyclophilin A (Prx1 to Prx4), or glutathione (Prx6). Prxs are divided into three classes: typical 2-Cys Prxs; atypical 2-Cys Prxs; and 1-Cys Prxs [for review (102,187)]. These enzymes share the same basic catalytic mechanism, in which the single, redox-active cysteine is oxidized to a sulfenic acid by the peroxide substrate (38). The recycling of the sulfenic acid back to a thiol distinguishes the three enzyme classes: 2-Cys Prxs are reduced by thiols, particularly thioredoxin (188), whereas the 1-Cys enzymes are reduced by glutathione (136) and ascorbic acid (152). By using crystal structures, a detailed catalytic cycle has been derived for typical 2-Cys Prxs, including a model for the redox-regulated oligomeric state proposed to control enzyme activity (251). Prxs have a wide tissue distribution and specific subcellular localization. Nuclear localization has been demonstrated for Prx1, Prx2, Prx4, Prx5, and Prx6, at least in tumor cells (109). However, only for Prx5 has a nuclear function been demonstrated. Nuclear Prx5 significantly reduced nuclear DNA damage induced by H_2O_2 (15).

E. APEX nuclease (multifunctional DNA-repair enzyme)

APEX nuclease 1 (APEX1) is a multifunctional protein. Its first abbreviation, human apurinic/apyrimidinic (AP) endonuclease, or Ape1 (also called HAP1 or APEX) derived from one of its functions as an essential enzyme in the base-excision repair (BER) pathway. This pathway is responsible for repair of apurinic/apyrimidinic (AP) sites in DNA, which are a major end product of ROS damage. Unrepaired AP sites can halt mRNA and DNA synthesis or act as noncoding lesions resulting in the increased generation of DNA mutations (248). To initiate repair, APEX1 cleaves 5′ to the baseless site, which leads to generation of a 3′-hydroxyl group and an abasic deoxyribose-5-phosphate. Subsequently, DNA polymerase β and DNA ligase I are recruited to the abasic site to complete the repair process (223).

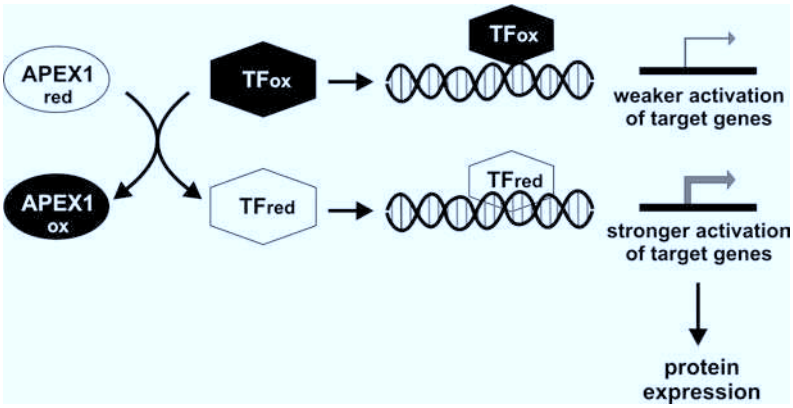

FIG. 4. APEX1. General model of APEX nuclease (multifunctional DNA-repair enzyme) 1 (APEX1) redox regulator function. APEX1 converts a transcription factor (TF) from an oxidized, less DNA-binding competent state to its reduced state that can bind more avidly to the regulatory regions of a variety of genes. This leads to activation of these downstream targets.

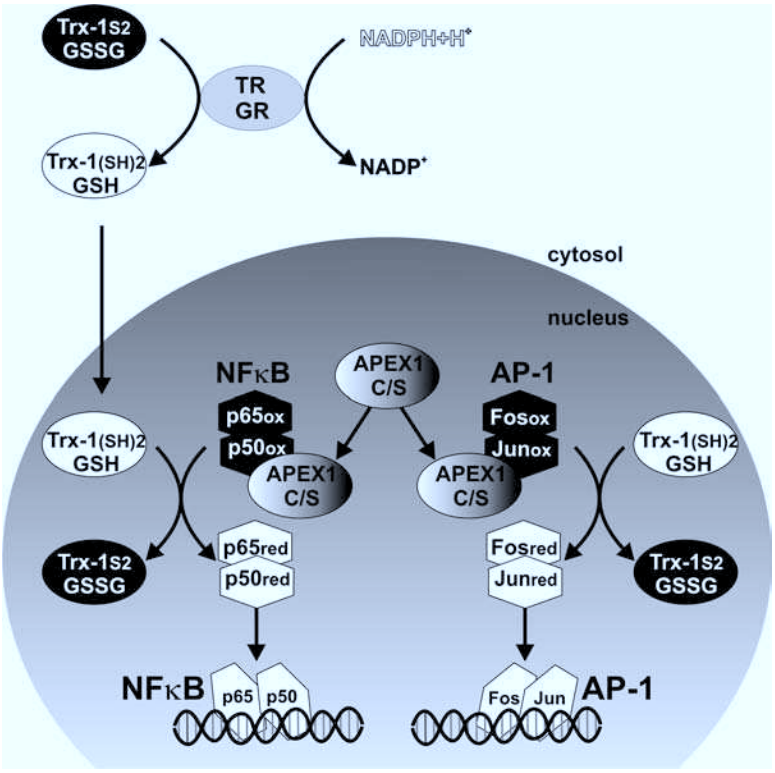

FIG. 5. APEX1 as a redox chaperone: model for the redox chaperone function of APEX1. A mutant of APEX nuclease (multifunctional DNA-repair enzyme) 1, in which all seven cysteine residues are replaced with serine (APEX C/S), can bind to the p50 and Jun subunits of the transcription-factor complexes nuclear factor-kappa B (NF-κB) and activator protein 1 (AP-1), respectively. In this complex, APEX C/S can recruit thioredoxin-1 (Trx-1) or glutathione (GSH), which subsequently reduce both subunits of the heterodimeric transcription factors. Reduction enables them to bind their cognate response elements and activate transcription of their target genes.

The second abbreviation, Ref-1, stands for redox effector factor-1 and reflects its function as a redox regulator of transcription factors (Fig. 4). Through its redox function, APEX1 maintains transcription factors in an active, reduced state required for DNA binding and transcriptional activation. The two functions of APEX1, repair and redox regulation, are independent and located in separate domains of the protein, which was demonstrated by deletion analysis. The N-terminal portion of APEX1 that is not present in functionally related proteins from other organisms is required for the redox activity, whereas the DNA-repair activity requires conserved C-terminal sequences. Chemical alkylation or oxidation of cysteines inhibits the redox activity of APEX1 without affecting its DNA-repair activity (255). In addition, mutation of cysteine residue 65 (Cys 65), which is unique to mammalian APEX1, abrogates the redox function of the human protein. The wild-type zebrafish APEX has a threonine residue in the corresponding position (Thr 58) and is redox inactive, but can be converted to a redox enzyme by conversion of Thr 58 to cysteine (59). Conversely, when Cys 65 in human APEX1 is converted to alanine, the resulting protein is redox deficient (132).

Two reports uncovered a third function of APEX1, the stimulation of transcription-factor reduction independent of its intrinsic redox activity. *In vivo* analysis of mouse APEX1 mutated at the cysteine previously identified as the redox catalytic site revealed a surprising result. Unlike APEX1-null mice, which die very early in embryonic development, homozygous APEX1(C64A) mice were viable with no overt phenotype. Although APEX1 is the major redox regulator of activator protein 1 (AP-1) in murine cells, AP-1 DNA-binding activity and reduction of Fos and Jun were unaltered compared with wild-type mice, demonstrating that the redox activity of APEX1 is not required for redox regulation of AP-1 (169). A more-robust proof was obtained by the analysis of human APEX1, in which all seven cysteine residues were substituted to serine (APEX1 C/S; Fig. 5). It was demonstrated that APEX1 can directly reduce *in vitro* oxidized recombinant p50, a subunit of nuclear factor-kappa B (NF-κB), at relatively high concentrations (*i.e.*, at concentrations more than 50-fold higher than that of p50). However, when Trx-1 or GSH was included in the reaction, a much lower concentration of APEX1 was sufficient to stimulate p50 DNA-binding activity, indicating that APEX1 might facilitate reduction of transcription factors by other reducing molecules such as Trx-1. Surprisingly, unlike the direct reduction of p50, this effect was not dependent on the redox activity of APEX1 because APEX1 C/S increased p50 DNA binding as efficiently as APEX1 wt in the presence of GSH or Trx-1. This activity of APEX1 was not restricted to the NF-κB transcription-factor complex, as the DNA-binding activity of AP-1 was stimulated by APEX1 C/S. Interestingly, a physical interaction of APEX1 and the C/S mutant occurs only with one subunit of these heterodimeric transcription factors (with p50, p52, c-Rel, and c-Jun, but not with p65 and c-Fos) (8).

Thus, APEX1 binding to certain subunits of heteromeric transcription factors leads to reduction of the transcription-factor subunits by other reductive systems. Therefore, APEX1 can function as a redox chaperone. This activity may be important for the modulation of the activity of a subset of transcription factors.

F. Trx-1/APEX1 interactions

A direct interaction between overexpressed APEX1 and Trx-1 has been demonstrated in the nucleus. Detailed analysis

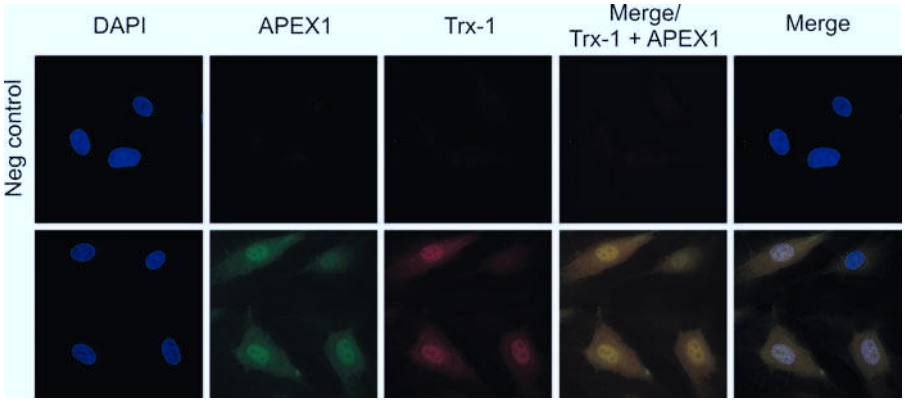

FIG. 6. Interaction of endogenous APEX1 and Trx-1 in human endothelial cells. Nuclear association of endogenous thioredoxin-1 (Trx-1) and APEX nuclease (multifunctional DNA-repair enzyme) 1 (APEX1). Representative immunostainings of human umbilical vein endothelial cells are shown. Nuclear staining with DAPI is shown in blue, APEX1 staining in green, and Trx-1 staining in red. The second panel from the right shows the merging of APEX1 and Trx-1 staining; the rightmost panel, the merging of DAPI, APEX1, and Trx-1. Cells were fixed and permeabilized. For coimmunostaining, cells were first incubated with an antibody against human Trx-1 (mouse, 1:50, overnight, 4°C; BD Pharmingen); as a secondary antibody, anti-mouse rhodamine RedX (1:100, 30 min, 37°C; Invitrogen) was used. Because both the Trx-1 and the APEX1 antibodies are from the same host species, a blocking step with an excess of mouse IgG was performed. After that, the cells were incubated with an antibody against human APEX1 (mouse, 1:200, overnight, 4°C; Novus Biologicals) followed by a secondary anti-mouse Alexa 488 (1:200, 30 min, 37°C; Invitrogen). Nuclei were stained with DAPI (0.2 μg/ml). Cells were visualized with fluorescence microscopy (Zeiss Axiovert 100, magnification 1:40, oil). The top row represents the negative control without primary antibodies.

4C ▶

of the cysteines in Trx-1 in a yeast two-hybrid system revealed that cysteines 32 and 35 are required for direct interaction with APEX1 (79). Conversely, cysteines in APEX1 were not mapped for Trx-1 interaction. We found that colocalization of the two endogenous proteins, APEX1 and Trx-1, is detectable mainly in the nucleus in human endothelial cells (Fig. 6). Mapping of the interaction domain within APEX1 is under further investigation. It must be noted that, under certain conditions, Trx-1 and APEX1 act in concert to regulate transcriptional activation (8, 244), whereas in other cases, APEX1 or Trx-1 alone is sufficient to control DNA binding of transcription factors. This is discussed in closer detail for the redox-regulated transcription factors in the following sections of this review.

F6 ▶

Table 1. Redox-regulated Transcription Factors

Transcription factor	*Critical amino acid*	*Affected function*	*Compartment of modification*
AP-1	Cys 269 (c-Fos) Cys 154 (c-Jun)	DNA binding (1)	Nucleus (79)
BPV E2	Cys 340	DNA binding (146)	n.d.
CBP/PEBP2	Cys 115, Cys 124	DNA binding (5)	n.d.
c-Myb	Cys 130	DNA binding (67, 155)	Nucleus (67)
CREB	Cys 300, Cys 310	DNA binding (64)	n.d.
Egr-1	n.d	DNA binding (91)	Nucleus (91)
estrogen receptor	n.d. (DBD)	DNA binding (122)	n.d.
glucocorticoid receptor	n.d. (DBD) Cys 481	DNA binding (93, 229) Nuclear import (167)	Nucleus (135) Cytoplasm (167)
HIF-1α	Cys 800	CBP interaction (51)	n.d.
HLF	Cys 28 Cys 844	DNA binding (116) CBP interaction (51)	n.d.
HoxB5	Cys-232	Cooperative DNA binding (58)	n.d.
MyoD	Cys 135	DNA binding (218)	n.d.
NFI/CTF	Cys 3 Cys 427	DNA binding (13) Transcriptional activation (154)	n.d.
NF-κB	Cys 62 (p50) Tyr 66, Tyr 152 (p65)	DNA binding (81, 142) Stability, nuclear retention (176)	Nucleus n.d.
NF-Y	Cys 85, Cys 89	DNA binding (156)	n.d
Nrf-2	Cys 506	DNA binding (26)	Nucleus (76)
p53	Cys 173, Cys 235, Cys 239	DNA binding (73, 185)	n.d.
Pax-5	n.d.	DNA binding (226, 227)	Nucleus (226, 227)
Pax-8	n.d.	DNA binding (103, 226)	Nucleus (226)
Sp1	n.d.	DNA binding (6, 7)	Nucleus (36)
TTF-1	Cys 87	DNA binding (11, 103, 225)	n.d.

III. Redox-Regulated Transcription Factors and Chromatin Modifiers

Transcription factors regulate cellular functions through altering the gene-expression profile. A number of transcription factors have been shown to be redox regulated through modulation of their DNA-binding capacity. Additional layers of regulation are on the level of transcriptional activation by changing cofactor interactions, oligomerization, or subcellular localization. Thereby cells can modulate their transcriptome to adjust to physiologic and pathophysiologic changes in ROS levels and exogenous noxae. Table 1 provides an overview over the mammalian transcriptional regulatory proteins for which a redox regulation has been shown. In the following sections, we review the molecules for which a more-detailed knowledge of their redox regulation is available.

T1 ▶

A. Activator protein-1

Activator protein-1 (AP-1) is one of the first mammalian transcription factors that were identified (119). AP-1 is not a single protein, but represents various homo- or heterodimers formed between the proteins of the basic region-leucine zipper (bZIP) family. They belong to the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, and Fra-2), Maf (c-Maf, MafB, MafA, MafG/F/K, and Nrl), and ATF (ATF2, ATF3/LRF1, B-ATF, JDP1, JDP2) subfamilies of the bZIP proteins. Their complexes bind to a canonic AP-1 site, originally described as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response element, or variants thereof. Recruitment of other transcription factors, coactivators, and chromatin-remodeling proteins generates a plethora of regulatory complexes with cell- and stimulus-specific transcriptional activities [for review, see (35)]. Therefore, AP-1 is involved in a wide range of physiologic functions, including proliferation and survival, differentiation, growth, apoptosis, cell migration, transformation, and carcinogenesis. AP-1 itself is target of a variety of upstream kinases like c-Jun NH_2-terminal kinases (JNKs), extracellular regulated kinases (ERKs), and p38 mitogen-activated protein kinases (MAPKs) through phosphorylation of Jun and Fos proteins.

DNA binding of AP-1 is regulated by the redox state of a cysteine residue within the DNA-binding domains of both proteins. Oxidation of Cys 154 in human Fos and Cys 269 in human c-Jun inhibits DNA binding of AP-1 *in vitro* (1). This was ascribed to an intermolecular disulfide bridge formed between the two cysteines. Oxidation of these residues could be achieved enzymatically, by using GPx, and DNA binding protected them from oxidation *in vitro* (16). In the viral homologue of c-Jun, the transforming oncogene v-Jun, the corresponding cysteine residue is replaced by a serine, which results in an insensitivity to oxidation, leading to constitutive DNA binding and aberrant transcriptional regulation. Similarly, Fos can escape regulation and is converted to a transforming protein when the critical cysteine is mutated (168). Shortly after uncovering the redox sensitivity of AP-1 DNA binding, a nuclear protein was identified that copurified with AP-1 and stimulated DNA binding of oxidized Fos-Jun heterodimers, Jun-Jun homodimers, and AP-1 complexes purified from HeLa cells (253, 254). This protein turned out to be APEX1. Besides a direct redox function, APEX1 can act as redox chaperone for AP-1, as discussed earlier.

AU1 ▶

B. Cyclic AMP response element–binding protein

The transcription factor CREB binds the cAMP response element (CRE) and functions in glucose homeostasis, growth factor–dependent cell survival, and has been implicated in learning and memory. CREB is activated in response to, among other signals, cAMP. The accumulation of cAMP triggered by extracellular signals induces most cellular responses through protein kinase A (PKA). An increase in cellular cAMP levels liberates the catalytic from the regulatory subunits of PKA, which then translocate into the nucleus and induce cellular gene expression by phosphorylating CREB at serine residue 133. Together with the related activating transcription factor 1 (ATF1) and the CRE modulator (CREM), CREB comprises another family of bZIP transcription factors [for review of the CREB/ATF family, see (145, 175, 197)]. Originally, the Fos/Jun and ATF/CREB protein families were regarded as distinct sets of transcription factors that recognize closely related, but different DNA-binding sites and form intrafamily dimers. However, it was shown that members of these two families can form selective cross-family heterodimers. These display DNA-binding specificities distinguishable from each other and from their parental homodimers (21, 72). Analogously to AP-1, DNA binding of CREB is regulated by its oxidation status. The reduction of two cysteine residues (Cys 300 and Cys 310) located in the DNA-binding domain, enhances the binding efficiency of CREB to DNA and regulates CRE-mediated gene expression. Substitution of these residues to serine renders CREB insensitive to reduction. These substitutions, which do not alter the secondary structure of the protein (189), enhance the binding of CREB to its cognate DNA sites under oxidative conditions and the CREB-dependent gene expression during normoxia (64). Interestingly, this redox regulation is bypassed by the Tax protein of the human T-cell leukemia virus type 1 (HTLV1), which recruits CREB independent of phosphorylation and redox status to the HTLV1 promoter (61, 64) to promote viral transcription.

C. Specificity protein 1

Specificity protein 1 (Sp1) is one of the best-characterized zinc-finger transcription factors. It is a member of an extended family of DNA-binding proteins, harboring three Cys_2-His_2 zinc-finger motifs, which bind to GC-rich DNA recognition elements. By regulating the expression of a large number of genes that have GC-rich promoters, Sp1-like transcription factors are involved in the regulation of many cellular processes, including proliferation, apoptosis, differentiation, and neoplastic transformation. Individual members of the Sp1 family can function as activators or repressors, depending on which promoter they bind and the co-regulators with which they interact [for review of the Sp1 family, see (100, 193)].

A first hint that DNA binding by Sp1 is regulated by the cellular redox status came from the analysis of nuclear extracts from 30-month-old rat tissues, in which the DNA-binding efficiency of Sp1 was greatly decreased, although the protein was present in levels comparable to those in younger tissues (6). This was attributed to increased levels of reactive oxygen intermediates in the aged animals because high concentrations of DTT, added to the aged tissue extracts, fully restored Sp1 DNA binding. Conversely, H_2O_2 treatment of extracts from young tissues strongly decreased the Sp1 DNA-

binding activity, which again could be restored with DTT. The same results were obtained with purified Sp1, clearly demonstrating that oxidation directly affected Sp1 and not a cofactor (7). A similar phenomenon was observed during the transition of thymocytes from the resting to the proliferating state, in which production of ROS on priming with phorbol 12-myristate 13-acetate (PMA) is nearly abolished. In the proliferating state, Sp1 DNA-binding activity increased and could be compromised by the addition of H_2O_2 to extracts from these cells, whereas the binding activity in nuclear extracts from resting cells could be fully restored with DTT (199). This susceptibility to oxidation is conferred by thiol groups, depends on zinc coordination, and is prevented by DNA binding, suggesting that the DNA-binding domain is the target for oxidation and is protected when in contact with DNA. This was corroborated *in vivo* by findings that arsenic treatment of promyelocytic leukemia cells prevented DNA binding of Sp1 to specific promoters. Of note, the nuclear levels of Sp1 did not change with arsenic treatment, suggesting that the oxidation occurs in the nucleus (36). In addition, Sp1 DNA binding was inversely correlated with the GSSG/GSH ratio added to nuclear extracts *in vitro* (110). Moreover, Trx-1 alone or in conjunction with the full thioredoxin system (Trx-1/TR and NADPH) was able to increase the DNA-binding activity of recombinant Sp1 produced in *Escherichia coli* and of the protein from a mammalian cell line (27). Interestingly, not only DNA binding of Sp1 might be affected by oxidation, but also its transactivation properties; however, this phenomenon has not been investigated in detail (153).

D. Nuclear receptors

Nuclear receptors constitute to a large superfamily of ligand-activated transcription factors [for review, see (147)]. These intracellular receptors are activated by lipophilic ligands and play crucial roles in development, differentiation, metabolic homeostasis, and reproduction. The prototypic glucocorticoid and estrogen receptor (ER) consist of an N-terminal transactivation domain (TAD), a central DNA-binding domain (DBD) containing zinc-finger motifs, and a C-terminal ligand-binding (LBD) domain. In the cytosol, they are complexed with various chaperones of the heat-shock protein family. On steroid binding, glucocorticoid and estrogen receptors are released from these cytosolic complexes and translocate to the nucleus, where they interact as homodimers with their cognate DNA-binding sites, the glucocorticoid response element (GRE), and estrogen response element (ERE), respectively, from where they activate transcription.

Early studies with biochemically purified glucocorticoid receptor showed that it changes its conformation under oxidizing conditions, when it seemingly can form intra- and intermolecular disulfide bonds. This conformational change was completely reversible with DTT, and only the reduced form of the receptor was capable of binding DNA (93, 212, 229), which was later confirmed in intact cells (52). A role for Trx-1 in this process was first suggested by experiments that showed that suppression of Trx-1 expression decreases glucocorticoid-inducible gene expression (134). Mammalian two-hybrid and pull-down assays finally demonstrated a direct interaction between the two proteins involving the DNA-binding domain of the glucocorticoid receptor. Analysis of the subcellular localization demonstrated that this interaction most likely takes place in the nucleus under oxidative conditions (135). Besides DNA binding, the nuclear import of the glucocorticoid receptor also is under redox control, which affects a cysteine residue in its nuclear-localization signal (167). Similar observations were made for the estrogen receptor, whose DNA-binding activity is also sensitive to oxidation, which alters the conformation of the DNA-binding domain (122). As for the glucocorticoid receptor, the transcription of endogenous and transfected synthetic ER target genes was shown to depend on Trx-1 when cells were placed under oxidative stress (78).

E. Nuclear factor-kappa B

NF-κB is a collective name for inducible dimeric transcription factors composed of members of the Rel family of DNA-binding proteins that recognize a common sequence motif, the κB site. NF-κB is found in essentially all cell types and is involved in activation of an exceptionally large number of genes in response to infections, inflammation, and other stressful situations requiring rapid reprogramming of gene expression. NF-κB was originally identified as a nuclear protein binding to the immunoglobulin kappa light-chain enhancer (208). Shortly thereafter, it was demonstrated by the same investigators that its DNA-binding activity in pre-B cells can be induced by bacterial lipopolysaccharide (LPS) with a superinduction on cycloheximide treatment (207), which then provided the first evidence that the activity of transcription factors can be regulated posttranslationally. Later it was shown that NF-κB represents a protein complex composed of hetero- or homodimeric combinations of five different members of the NF-κB/Rel family: NF-κB1 encoding p50 and p105, NF-κB2 encoding the p52 precursor p100, RelA or p65, RelB, and c-Rel. All subunits contain a conserved Rel-homology domain important for nuclear localization, dimerization, and DNA binding [for review see (161)]. However, only the three Rel proteins contain transactivation domains required for the transcriptional activation of target genes. Therefore, and because each subunit has distinct biologic activities, different dimer combinations regulate specific sets of genes [for review, see (157)]. This combinatorial mode of action results in diverse effects on cell fate and function.

In unstimulated cells, NF-κB is sequestered in an inactive form in the cytosol. It can be released from these cytosolic pools by two main pathways [for review, see (201)], resulting in nuclear translocation of NF-κB complexes. The canonic pathway, which is triggered by several proinflammatory cytokines, pathogen-associated molecules, and antigen receptors, depends on phosphorylation of the inhibitor of NF-κB (IκB) by an IκB kinase (IKK) complex consisting of the catalytic subunits IKKα, IKKβ, and a regulatory IKKγ subunit. Phosphorylated IκB is then subject to proteasomal degradation. This pathway leads primarily to the activation of p50/RelA and p50/c-Rel dimers. The noncanonic pathway engaged by various members of the tumor necrosis factor (TNF)-receptor family selectively requires IKKα activated by the upstream kinase NF-κB–inducing kinase (NIK). IKKα in turn phosphorylates p100, leading to proteasomal degradation of its C-terminus to generate p52, which then forms heterodimers predominantly with RelB. Besides these two major pathways, other mechanisms can release cytosolically

sequestered NF-κB components [*e.g.*, the phosphorylation of p105 by TPL-2, accelerating proteasome-mediated removal of the carboxy terminus yielding p50 (20)].

The observation that increased levels of the oxidoreductase Trx-1 are found in lymphocytes under conditions in which the transcription factor NF-κB is active [*e.g.*, after lymphocyte activation or in EBV- or HTLV-1–infected cells (238)], led to the suspicion that NF-κB might be under redox control. It could be shown that *in vitro* DNA-binding activity of NF-κB is inhibited by agents modifying free sulfhydryls (141, 142, 230). Mutagenesis of conserved cysteine residues in the p50 subunit revealed that Cys 62 is critical for high-affinity binding to the κB motif. DNA-binding activity of wild-type p50, but not a mutant, in which Cys 62 was exchanged to serine (C62S), was stimulated by Trx-1. Detection of disulfide cross-linked dimers in p50 wild type but not C62S suggested that Trx-1 stimulates DNA binding by reduction of a disulfide bond involving Cys 62 of the NF-κB subunit p50. Cotransfection of a plasmid expressing human Trx-1 and an NF-κB–dependent reporter construct demonstrated that Trx-1 also can regulate DNA binding and transcriptional activation by NF-κB in living cells (142). Interestingly, depending on its subcellular localization, Trx-1 can have opposing effects on NF-κB. In the cytosol, it interferes with signals to IKKs and thereby blocks the degradation of IκB, resulting in cytosolic retention of NF-κB, which prevents the activation of target genes. In contrast, nuclear Trx-1 has a positive effect on NF-κB transcriptional activity by enhancing its ability to bind to DNA (81). Molecular-modeling studies finally provided a clue to this dual role of Trx-1. Oxidized, disulfide-bridged NF-κB is more compact than the reduced form, which might facilitate its nuclear translocation. However, the inter-subunit disulfide blocks DNA from entering the active site of the oxidized dimer, explaining why reduction in the nucleus is essential for DNA binding and transcriptional activation to occur (32). Similar to Trx-1, Prx1 has the same dual role in the regulation of NF-κB activity (75). Besides disulfide cross-linked dimer formation, another oxidation product of NF-κB subunits has been detected. Both p50 and p65 can be glutathionylated, which leads to reduced NF-κB DNA binding and transactivation (181, 182). In the case of p65, glutathionylation was detected under hypoxic conditions, when the intracellular GSH levels were increased. The modification of p65 required Grx1, and the authors speculated that p65-SSG formation takes place in the cytosol and that modified p65 is still transported to the nucleus (182). However, as Grx1 can be detected in the nucleus, it also is possible that glutathionylation of this transcription-factor subunit takes place there.

In addition to cysteine oxidation, the modification of specific tyrosine residues in p65 has been reported. Peroxynitrite inhibited NF-κB activity through nitration of p65 at Tyr 66 and Tyr 152, leading to p65 destabilization and nuclear export (176).

An interesting observation was made concerning the role of NF-κB redox regulation in the action of thalidomide, which causes severe malformations, especially of the extremities, in children when taken by the mothers during pregnancy. Thalidomide increases the production of free radicals and elicits oxidative stress. Oxidative stress, as marked by GSH depletion, occurs preferentially in limbs of thalidomide-sensitive rabbits. Activation of an NF-κB–dependent reporter gene is attenuated in limb bud cells of treated rabbits and can be restored on addition of *N*-acetylcysteine and a free radical spin-trapping agent (74). NF-κB is a key factor in limb development, because it regulates expression of Twist and fibroblast growth factor 10 (FGF10), two genes necessary for proper limb outgrowth. Therefore, its oxidative inactivation triggered by thalidomide might explain the malformations occurring in children.

F. p53

The protein p53 is one of the best-characterized tumor suppressors and is constitutively expressed in nearly all cells and tissues. It functions as a tetrameric transcription factor found at very low levels in normal cells. Several genome-wide surveys have been undertaken to map genomic binding sites of p53 and thus to identify its target genes (83, 104, 213), yielding a plethora of genes potentially regulated by p53. Various kinds of cellular stress that alter normal cell-cycle progression or induce mutations in the genome lead to stabilization and thus accumulation of the protein. Depending on the cell type and tissue and the extent of damage, p53 now either leads to cell-cycle arrest to repair the lesions or forces the cell into apoptosis. Therefore, p53 has been termed "guardian of the genome" (117). Somatic mutations in the *p53* gene are critical events in a wide variety of malignancies, and *p53* is the gene most frequently mutated in human cancers. The majority of the mutations are missense mutations, and a hotspot is the region of the gene coding for the DNA-binding domain [for review of *p53* and its mutations, see (28, 84, 217, 239)].

Because of its prominent role in human cancers, the regulation of *p53* has been at the focus of intensive studies. With respect to redox regulation, it has to be noted that all 10 cysteine residues in *p53* are within its DNA-binding domain. This prompted very early studies on redox regulation. It was shown that oxidation of *in vitro* translated and recombinant, baculovirus-produced p53 disrupted its DNA binding, which was attributed to a change in conformation that could be reversed by reduction (44, 73). Interestingly, the redox state of p53 seemingly regulates only sequence-specific DNA binding and activation of p53-dependent genes. In contrast, no difference is found in the binding of oxidized p53 and reduced p53 to double-stranded nonspecific DNA (177).

That APEX1 can modulate the redox-dependent properties of p53 was demonstrated by the stimulation of sequence-specific DNA binding of oxidized p53 *in vitro* (97). The expression of reporter genes driven by p53-responsive promoters and endogenous p53 target genes, like p21 and cyclin G, was equally stimulated on overexpression of APEX1 (57, 97). Importantly, downregulation of APEX1 caused a marked reduction in p53-dependent induction of p21 and diminished the transcriptional activation of p21 and Bax by p53. In addition, the same authors demonstrated a physical interaction between APEX1 and p53, although only a small portion of both proteins was present in the same complex (57). Interestingly, APEX1 modulates p53 DNA binding, not only as a redox regulator, but also to facilitate formation of p53 tetramers, the most active form in terms of DNA binding (245), independent of its redox activity (77). This is substantiated by the fact that the cysteine residues, which are exclusively found in the DNA-binding domain of p53 and are the targets for oxidation/reduction, have no influence on tetramerization

(185). Collectively, these data suggest that APEX1 stimulates p53 by both redox-dependent and -independent means and imply a key role for it in p53 regulation.

G. Nuclear factor I/CAAT transcription factor

NFI/CTF was originally described as being required for the replication of adenovirus DNA [for review, see (42)]. Later it was shown that NFI can regulate the transcription of a large number of cellular and viral genes. NFI represents a family of four genes in vertebrates (NFI-A, NFI-B, NFI-C, and NFI-X), which are expressed in overlapping patterns. The transcripts of all four genes can be spliced differentially, yielding distinct proteins. NFI proteins have been associated with changes in the growth state of cells and a number of malignancies [for review of NFI proteins, see (65)].

As for other transcription factors, it has been shown for NFI that its DNA-binding activity is redox sensitive (164). A single cysteine residue (Cys 3) in the DNA-binding domain of the NFI-family proteins, which is conserved from *Caenorrhabditis elegans* to humans is the target for this regulation, as was shown by site-directed mutagenesis (13). Oxidized, inactive NFI can be reduced to a DNA-binding form by Grx1 *in vitro*. This requires the GSH/GR system to regenerate reduced Grx1. The *in vivo* relevance has been shown by treatment of HeLa cells with buthionine sulfoximine, an agent that inhibits GSH synthesis. This GSH depletion potentiated the inactivation of NFI by the oxidizing agent diamide. Similarly, a stronger restoration of NFI activity after oxidation with diamide was observed, when the cells were treated with *N*-acetylcysteine, an agent that can replenish intracellular GSH (14).

In the case of NFI, not only DNA binding is subject to redox control. By using fusions with a heterologous DNA-binding domain from the Gal4 protein, it was demonstrated that transcriptional activation by NFI also is regulated through oxidation of a cysteine residue (Cys 427) in the transcription-activation domain (TAD) (153) (Fig. 7). These findings were corroborated in living cells subjected to various stress conditions that induce cellular ROS formation, including inflammatory cytokine treatment, GSH depletion, heat and osmotic shocks, and chemical stress. In all cases, suppression of a reporter gene was specific for the NFI TAD, as no effects were observed with the transactivation domains of activator protein 2 (AP-2) and octamer transcription factor 2 (Oct-2). A common target for all these stressors was again Cys 427, leading to the suggestion that the NFI TAD might be a negative sensor of cellular stress (154). Based on the concentrations of exogenously applied H_2O_2 required to block transcriptional activation and DNA binding by NFI, it was calculated that a 100-fold difference in sensitivity to oxidation exists between the DNA-binding domain of NFI and its TAD (153). During this investigation, TADs from other transcription factors (Sp1 and Oct-2) were analyzed to show that redox regulation of TADs is not a general effect. Whereas the Oct-2 TAD was not sensitive to oxidative stress, the analogous domain of Sp1 showed some responsiveness, indicating that other transcription factors could be subject to similar control mechanisms, which might have been overlooked during the characterization of the redox dependency of their DNA-binding capacity.

◀F7

It is not clear whether the thiol moiety of Cys 427 of NFI undergoes an oxidation with a gain of oxygen atoms or if it forms an intra- or intermolecular disulfide bridge, although mutations of a cysteine in the neighborhood (Cys 405) indicate that the formation of an intramolecular disulfide bridge within the TAD is not the mechanism most likely to trigger the effect. The oxidation of Cys 427 could affect the conformation of the TAD, which is the interface for interactions with the TATA-box–binding protein TBP, the coactivator CBP/p300, and histones H1 and H3.

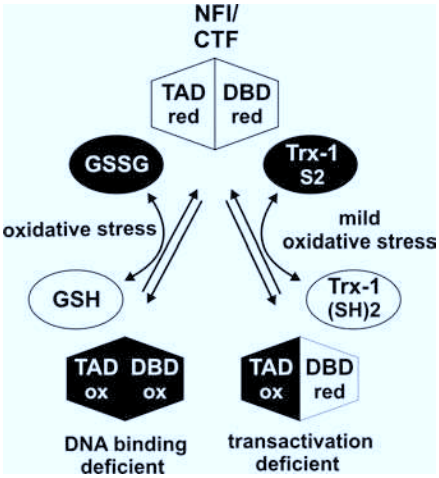

FIG. 7. Redox regulation of NFI/CTF: dual regulation of NFI/CTF transcriptional activity. Nuclear factor I/CAAT transcription factor (NFI/CTF) must be reduced to activate its target genes. Oxidative stress leads to oxidation of its transactivation domain (TAD) and DNA-binding domain (DBD), which prevents DNA binding. Much lower concentrations of ROS only oxidize the TAD in a thioredoxin-1 (Trx-1)-dependent manner. This partially oxidized molecule can still bind to DNA, but is incapable of activating transcription.

H. Hypoxia-inducible factor 1

HIF-1 is the most prominent regulator of genes induced by hypoxia. It is a transcription factor that binds to the hypoxia-responsive element (HRE) in the promoters and enhancers of various hypoxia-inducible genes. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β, which is identical to the aryl hydrocarbon-receptor nuclear translocator (ARNT). Both proteins contain a basic helix–loop–helix (bHLH) and a PAS domain at their N-terminus, the latter being an acronym for Per, ARNT, and Sim, the first three members of the protein family characterized. Whereas the basic domain is essential for DNA binding, the HLH domain and the N-terminal half of the PAS domain are required for heterodimerization and DNA binding. HIF-1β contains a single C-terminal transactivation domain, whereas in HIF-1α, two such domains are found, termed NAD and CAD, according to their location closer to the N- or C-terminus. The NAD is embedded in a region controlling protein stability, the oxygen-dependent degradation domain (ODD). Both subunits of HIF-1 are expressed constitutively, but the α-subunit has an extremely short half-life (less than 5 min) under normoxic conditions, because of continuous proteolysis by the ubiquitin-proteasome pathway targeting the ODD. As both subunits are required for the activation of

HIF-1 target genes, these are not or only weakly expressed at normal oxygen tension. During hypoxia, HIF-1α is instantaneously stabilized, resulting in a functional heterodimer and expression of hypoxia-inducible genes. HIF-1α is hydroxylated at proline 564 (Pro 564) in the ODD in an oxygen-dependent fashion. This hydroxylated HIF-1α is bound by the von Hippel–Lindau (VHL) protein, which acts as an E3-ubiquitin ligase, tagging HIF-1α for proteasomal degradation. The critical molecules for the rapid turnover of HIF-1α under normoxia are members of a family of prolyl-4-hydroxylases (PHs). These enzymes require oxygen and 2-oxoglutarate as cosubstrates and contain iron liganded by two histidine and one aspartic acid residues. Oxygen binding requires the vitamin C–dependent maintenance of iron in its ferrous state. When oxygen is low, Pro 564 in HIF-1α remains unmodified, resulting in stabilization of the protein, which is no longer bound by VHL. Similarly, iron chelation or replacement by transition metal ions (Co^{2+}, Ni^{2+}, Mn^{2+}) can mimic hypoxia-inducible stabilization, explaining the apparent "upregulation" of HIF-1α by these metals [for review of HIF-1, see (205, 206, 246, 247)].

In addition to HIF-1α, two other family members are known, HIF-2α/HLF (HIF-1α–like factor) and HIF-3α. They show a more-restricted expression pattern, but contain domains similar to HIF-1α and have comparable biochemical properties.

In 1996, it was shown that pretreatment of cells with hydrogen peroxide has an inhibitory effect on transcriptional activation by HIF-1, which suggested an additional redox control mechanism. Sulfhydryl alkylation or oxidation, the latter being reversible, indicated an involvement of cysteine residues in this process. These findings were supported by the fact that purified, oxidized Trx-1 in combination with DTT could stimulate HIF-1 DNA binding in hypoxic extracts. In addition, overexpression of Trx-1 or APEX1 enhanced the hypoxic induction of an HIF-1–dependent reporter gene (90). Interestingly, DNA binding of HLF-, but not HIF-1α–containing complexes was redox dependent. This is explained by the fact that the critical cysteine residue in the basic domain of HLF (Cys 28), which seems to be a target for APEX1, is replaced by a serine residue at the corresponding position (Ser 25) in HIF-1α. However, APEX1 also is important for transcriptional activation by HIF-1α (116). The last finding is in agreement with a previous report that the CADs of HIF-1α and HLF interact with the transcriptional coactivator CBP/p300 and that APEX1 and Trx-1 further enhanced the activity of a luciferase reporter activated by a fusion protein between the Gal4 DNA-binding domain and CAD under hypoxic conditions. This potentiation required the catalytic activity of Trx-1, suggesting that a redox reaction is involved. Interestingly, a single cysteine residue is conserved in the 49-amino-acid CAD between HLF (Cys 844) and HIF-1α (Cys 800). Mutation of this cysteine abolished the hypoxia-inducible transcriptional activation by Gal4-CAD and interaction with CBP. The importance of this cysteine residue was confirmed by mutation in the context of full-length HIF-1α and HLF, which markedly reduced the transcription-enhancing activity (51).

I. Nuclear factor erythroid 2–related factor 2/NF-E2 related factor 2

The NF-E2–related factor 2 (Nrf-2), not to be confused with the GA-binding protein nuclear respiratory factor 2, which is also called Nrf-2, is a transcription factor implicated in the cellular responses to oxidative stress and to chemical compounds that are metabolically transformed to reactive or electrophilic intermediates. Nrf-2–deficient mice show a lower expression of xenobiotic enzymes and are predisposed to tumors induced by carcinogens (186). Nrf-2 is a member of the so-called cap 'n' collar basic region leucine zipper (CNC-bZIP) family of transcription factors, the bZIP region of which is distinct from other bZIP families, such as the Jun/Fos family. Nrf-2 heterodimerizes with other bZIP transcription factors, including the small Maf (sMaf) proteins. These heterodimers bind to antioxidant-response elements (AREs) and thereby upregulate numerous genes coding for detoxification enzymes, antioxidants, and the enzymes required for *de novo* GSH synthesis (148). However, Nrf-2 controls not only inducible, but also low-level gene expression under nonstressed conditions, suggesting that its activity is tightly controlled. It has been shown that Nrf-2 is tethered by the Kelch-like ECH-associated protein 1 (Keap1), and in this complex, is not available as a transcriptional activator. Keap1 serves not only simply to sequester Nrf-2, but also functions as an adaptor for a Cul3-dependent E3 ubiquitin ligase modifying Nrf-2 for proteasomal degradation, which explains the short half-life of Nrf-2 ($\sim$ 15 min). It is still a matter of debate whether Keap1 transiently enters the nucleus and targets Nrf-2 for ubiquitinylation there or is capable of engaging in a nucleocytosolic shuttling of Nrf-2 dependent on CRM-1 (235). Nevertheless, the generally accepted scenario involves a cytosolic retention and degradation of Nrf-2 by Keap1.

Keap1 contains an N-terminal BTB/POZ domain (for broad-complex, Tramtrack and Bric-a-brac; also known as a Poxvirus and zinc finger domain) potentially serving as an interface for homomeric or heteromeric interactions. The C-terminus comprises six Kelch repeats, each of which forms a four-stranded β-sheet resulting in a propeller-like structure binding Nrf-2. Keap1 contains 25 cysteine residues, the most reactive of which are found in the intervening region between the BTB/POZ and Kelch repeat domains (47). Inducers of ARE-dependent genes disrupt the Keap1/Nrf-2 interactions by modifying two of these residues (Cys 273 and Cys 288). Transfection of Keap1- and Nrf-2–deficient mouse embryonic fibroblasts with constructs expressing cysteine-to-alanine mutants of these two amino acids in Keap1 demonstrated that release of Nrf-2 is the consequence of the formation of an intermolecular, disulfide-linked Keap1 dimer. In this dimer, the disulfide bridges most likely are formed crosswise between Cys 273 and Cys 288 (237). On release from Keap1, Nrf-2 escapes degradation and can bind to AREs in a heteromeric complex with an sMaf to activate gene expression. Several kinases have been shown to phosphorylate Nrf-2, but the molecular consequences of these phosphorylation events have not been elaborated [for review of Nrf-2 and its regulation by Keap1, see (107, 158, 159)].

Besides the cytosolic retention of Nrf-2 by Keap1 and its release on formation of an intermolecular Keap1 dimer, a second layer of Nrf-2 activity regulation exists. Like other transcription factors, Nrf-2 must be in a reduced state for efficient DNA binding. The critical residue is Cys 506, whose oxidation reduced its affinity for the ARE, leading to decreased expression and antioxidant induction of NAD(P)H/quinine oxidoreductase 1 (NQO1). However, mutation of this residue to serine did not affect the retention of Nrf-2 by Keap1

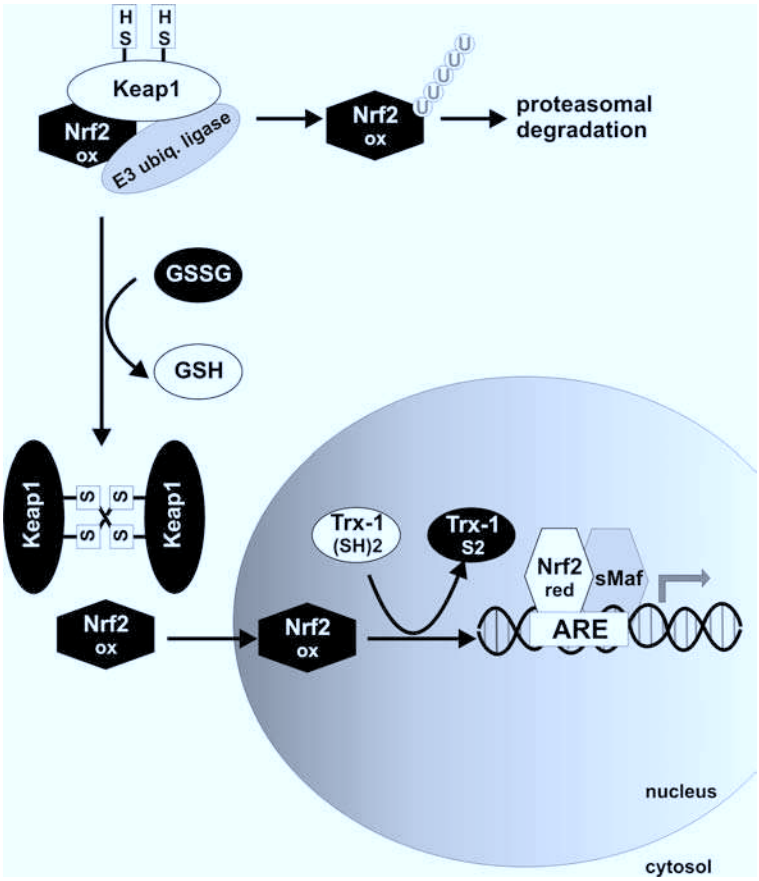

FIG. 8. Activation of Nrf-2: general model of gene induction by the Keap1/Nrf-2 pathway. Nuclear factor erythroid 2–related factor 2/NF-E2–related factor 2 (Nrf-2) is sequestered in the cytosol by Kelch-like ECH associated protein 1 (Keap1). In addition to binding Nrf-2, Keap1 functions as an adaptor for an E3 ubiquitin ligase, which ubiquitinates Nrf-2, thereby tagging it for proteasomal degradation. After induction, Keap1 is oxidized to an intermolecular, disulfide-linked dimer involving reciprocal cysteine residues of both monomers. This oxidation requires GSSG. Released Nrf-2 translocates to the nucleus, where it is reduced by thioredoxin-1 (Trx-1). Nrf-2 must be in a reduced state for efficient DNA binding as a heterodimer with a small Maf protein (sMaf). These heterodimers induce transcription of genes, whose promoters contain antioxidant responsive elements (AREs).

in the cytosol or its release in response to antioxidants (26). It has been shown that this two-layered, compartmentalized regulation of Nrf-2–dependent gene expression involves two cellular redox systems. Whereas cytosolic retention is controlled by GSH/GSSG ratios, the Nrf-2/DNA interactions F8 ▶ depend on nuclear Trx-1 (76) (Fig. 8).

J. Homeobox B5

To our knowledge, only one case exists in which oxidation of a cysteine residue in a transcription factor can enhance DNA binding, homeobox B5 (HoxB5). It is a homeodomain (HD) protein of the antennapedia family and functions as a sequence-specific transcription factor that is involved in lung and gut development. In the cardiovascular system, HoxB5 is an upstream transcriptional switch for differentiation of the vascular endothelium from precursor cells (252). It was shown *in vitro* that the cooperative stabilization of HoxB5 DNA binding, but not sequence-specific DNA binding, is under redox regulation. Cooperative binding and redox regulation were found to require the presence of a cysteine residue (Cys 232) in the turn between homeodomain helices 2 and 3 and that oxidation of this cysteine is necessary for cooperative binding of the protein to tandem binding sites. This was shown by DTT treatment of purified, recombinant HoxB5, which resulted in loss of cooperativity (58). Multiple clustered HD protein-binding sites are found in the promoters of *Drosophila* and vertebrate genes whose expression is regulated by antennapedia-type HD proteins. Thus, cooperative interactions could have a large influence on the DNA binding of HD proteins to these sites.

K. Other redox-regulated transcription factors

Besides the transcription factors discussed in detail, DNA binding of several others is inhibited by oxidation: CBP/PEBP2 (5, 101), c-Myb (67, 155), Egr-1 (91), MyoD (218), NF-Y (156), Pax-5 (226, 227), Pax-8 (103, 224), TTF-1 (11, 103, 225), and bovine papilloma virus E2 (BPV E2) (146). In some of these cases, oxidation sensitivity has been mapped to the DNA-binding domain; in others, it has been shown that Trx-1 or APEX1 can restore the DNA-binding capacity after oxidation.

L. Histone deacetylase 2

Interestingly, not only transcription factors as *bona fide* DNA-binding proteins are affected by modification of cysteine residues. Recently, it was shown that the chromatin modifier histone deacetylase 2 (HDAC 2) becomes nitrosylated on treatment of rat cortical neurons with neurotrophins. This modification occurs on two cysteine residues (Cys 262 and Cys 274) and is dependent on neuronal nitric oxide synthase (nNOS), as shown in neurons from nNOS-deficient mice and nonneuronal cells expressing nNOS. Intriguingly, nitrosylation of the two cysteines did not change the enzymatic activity of HDAC 2 but rather induced its release from chromatin. This dissociation of HDAC 2 leads to acetylation of histones H3 and H4, activation of BDNF target

Table 2. Nuclear Kinases and Phosphatases

	Critical amino acid	*Molecular consequence*	*Cellular consequence*	*Compartment of modification*	*Ref.*
Nuclear kinase					
PKCδ	Tyr 512	Kinase activation	Apoptosis	Cytosol	(46, 222)
PKA	n.d	Kinase activation	Cell survival	Nucleus	(17)
JNKs	n.d	Kinase activation	Apoptosis	Nucleus	(24)
Akt	Ser 473	Kinase activation	Apoptosis inhibition, delayed	Nucleus	(68, 70)
ERK2	Thr 183, Tyr 185	Kinase activation	*De novo* GSH synthesis	Nucleus	(108, 263)
Src	Tyr 416	Kinase activation	Senescence, apoptosis	Cytosol	(68, 69)
	Cys 277	Kinase inactivation	n.d.	Cytosol	(106)
Yes	Tyr 426	Kinase activation	Senescence, apoptosis	Cytosol	(68, 69, 96)
	n.d. (Cys)	Kinase inactivation	n.d.	Cytosol	(106)
Nuclear phosphatase					
Shp-2	Cys 459	Reduced phosphatase activity	ROS induction, apoptosis	n.d.	(37, 96)
	Cys 331, Cys 367	Backdoor cysteines, protection of Cys 459	n.d.	n.d.	(33)
TC-PTP	n.d.	Dephosphorylation of transcription factors	n.d.	Nucleus	(228, 257)
Cdc25C	Cys 330	Reduced phosphatase activity	Cell-cycle progression	Nucleus	(179, 198)
	Cys 377	Backdoor cysteine, protection of Cys 330	Cell-cycle progression		

genes, and dendritic growth (163). This mechanistic study could explain why HDAC 2 can negatively regulate memory formation and synaptic plasticity (66).

IV. Redox-Regulated Nuclear Kinases and Phosphatases

A. Nuclear-localized kinases

Protein kinases are involved in many different cellular signaling pathways. Therefore, a strict regulation of these kinases is necessary for the survival of the cell. The most important regulatory mechanism is their activation by phosphorylation [for review, see (98, 99)]. Kinases are phosphorylated under physiologic and pathophysiologic conditions. Recently a direct oxidation of Src and the fibroblast growth factor type 1 was demonstrated, leading to their inactivation. This mechanism seemingly only works for kinases containing a cysteine in the Gly loop capable of forming disulfide homodimers and therefore applies only to a small number of human protein tyrosine kinases (106).

In the context of this review, we restrict the detailed discussion to kinases, for which a nuclear localization has been demonstrated, even if the redox regulation takes place outside the nucleus. Nuclear localization has been shown for protein kinase C δ (PKCδ), PKA, JNKs, Akt, ERKs, and some members of the Src kinase family. The following (188) paragraphs discuss the kinases mentioned earlier in more detail; a brief overview is given in Table 2. T2 ▶

1. Protein kinase Cδ (PKCδ). The protein kinase C (PKC) family of serine/threonine kinases is involved in many cellular signaling pathways such as growth, proliferation, and cell death [for review, see (259)]. This family consists of three groups: (a) the conventional PKCs (cPKCs: α, βI, βII γ); (b) the novel PKCs (nPKCs: δ, ε, θ, μ); and (c) the atypical PKCs (aPKCs: ζ, λ). PKCδ belongs to the novel PKCs and is redox regulated in several cell types. One well-described role for PKCδ is in mitochondria-dependent apoptosis induction. Overexpression of PKCδ in keratinocytes leads to translocation of PKCδ to mitochondria, alterations in mitochondrial functions, and induction of cell death (121). But studies also show translocation of PKCδ into the nucleus in various cell types. In response to cellular stresses, like oxidative stress, PKCδ is activated by tyrosine phosphorylation, and nuclear translocation occurs. PKCδ possesses its own nuclear-localization sequence (NLS) (46) and has several tyrosine phosphorylation sites, which regulate its kinase activity. One of these sites, tyrosine 512, is phosphorylated by the proapoptotic tyrosine kinase c-Abl in response to oxidative stress. Phosphorylation results in activation of PKCδ followed by its nuclear translocation (222, 260). After nuclear translocation, activated PKCδ initiates a sequence of events that activates caspase 3, which in turn cleaves PKCδ (25, 46). This cleavage results in a 40-kDa catalytically active fragment and a 38-kDa regulatory fragment of PKCδ. The catalytically active fragment induces apoptosis by phosphorylation of the apoptosis-related protein DNA-dependent protein kinase (DNA-PK) (22, 60). DNA-PK is essential for repair of DNA double-strand breaks (214). Phosphorylation by PKCδ and also its catalytically active fragment induces the dissociation of DNA-PK from DNA, resulting in an inhibition of DNA repair and enhanced DNA fragmentation (22) DeVries *et al.* (46) showed that caspase 3–dependent cleavage of PKCδ increases the rate of nuclear translocation of the 40-kDa PKCδ cleavage fragment, which results in an amplification of the apoptotic signal (46) (Fig. 9). ◀ F9

PKCδ also interacts with and activates IKKα in response to oxidative stress. Active IKKα translocates into the nucleus

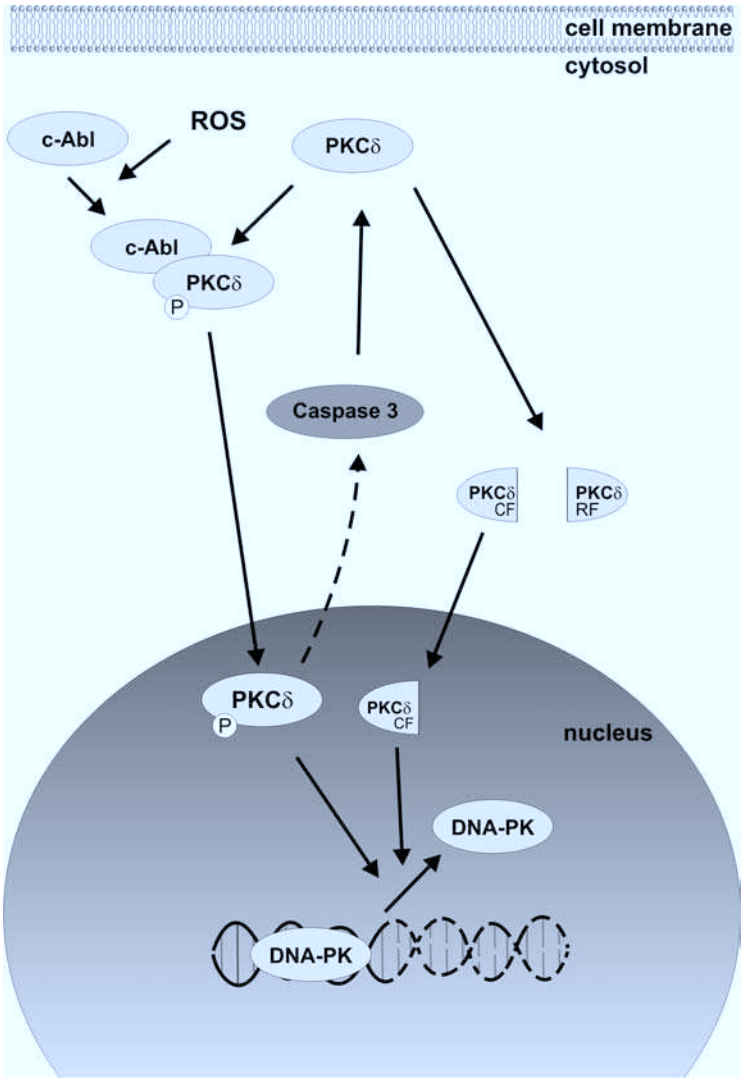

FIG. 9. Role of nuclear PKC*δ*. ROS induce association of protein kinase C *δ* (PKC*δ*) and c-Abl. This leads to phosphorylation, activation, and nuclear import of PKC*δ*. In a direct or indirect way, phosphorylated PKC*δ* activates caspase 3, which in turn leads to cleavage of PKC*δ* into a catalytically active (PKC*δ* CF) and a regulatory fragment (PKC*δ* RF). PKC*δ* CF translocates into the nucleus and, like PKC*δ*, induces the dissociation of DNA protein kinase (DNA-PK) from the DNA, which leads to fragmentation of DNA.

and regulates the transcriptional activity of the tumor-suppressor p53 by phosphorylation at serine 20 (256). This is a recently described mechanism for ROS-induced p53 activation.

2. Protein kinase A. Cyclic AMP formed by adenylyl cyclases binds the regulatory subunits (R) of the tetrameric PKA holoenzyme and promotes dissociation of the catalytic subunits (C-PKA). A fraction of C-PKA translocates to the nucleus and stimulates cAMP-dependent gene expression in different cell types (174). The best-characterized target of nuclear C-PKA is CREB, which is phosphorylated at serine 133 by C-PKA (71). H_2O_2 has been shown to initiate an increase in CREB phosphorylation at serine 133 in a non-transformed murine alveolar type II epithelial cell line (18), leading to transcriptional activation (145). The increased phosphorylation of CREB was due to an H_2O_2-induced increase in nuclear accumulation of C-PKA (17). This was confirmed by pharmacologic inhibition of PKA with H89, which reduced H_2O_2-mediated phosphorylation of CREB. Because the downregulation of CREB by siRNA increased the sensitivity of cells to H_2O_2-induced apoptosis and reduced transcription of the antiapoptotic gene B-cell lymphoma protein 2 (Bcl-2), CREB seems to play a role in cell survival in response to oxidative stress (17).

3. c-Jun NH_2-terminal kinases. JNKs are involved in the regulation of cell proliferation and apoptosis. The activation of these pathways is dependent on the actual stress stimulus and cell type (125, 128). Sustained activation of JNKs leads to apoptosis, whereas the acute and transient activation induces survival pathways and cell proliferation (196). The sustained stress-induced activation of JNKs by phosphorylation through upstream MAP-kinases, ASK1, and MAP kinase kinase (MKK) 4/7 leads to an induction of apoptosis [for review, see (127)]. JNKs translocate into the nucleus and phosphorylate c-Jun and activating transcription factor 2 (ATF2) [for review, see (41)], leading to the formation of an AP-1 complex and to the transcription of proapoptotic genes [*e.g.*, tumor necrosis factor α (TNF-α), Fas-L, and Bak] (53). However, JNKs also regulate physiologic and homeostatic processes. One attractive explanation for these differences is the existence of three isoforms of JNK and the subcellular pools of the JNKs. Only combined siRNA knockdown of all JNKs 1, 2, and 3 provides substantial protection from cell death. In contrast, knockdown or knockout of individual

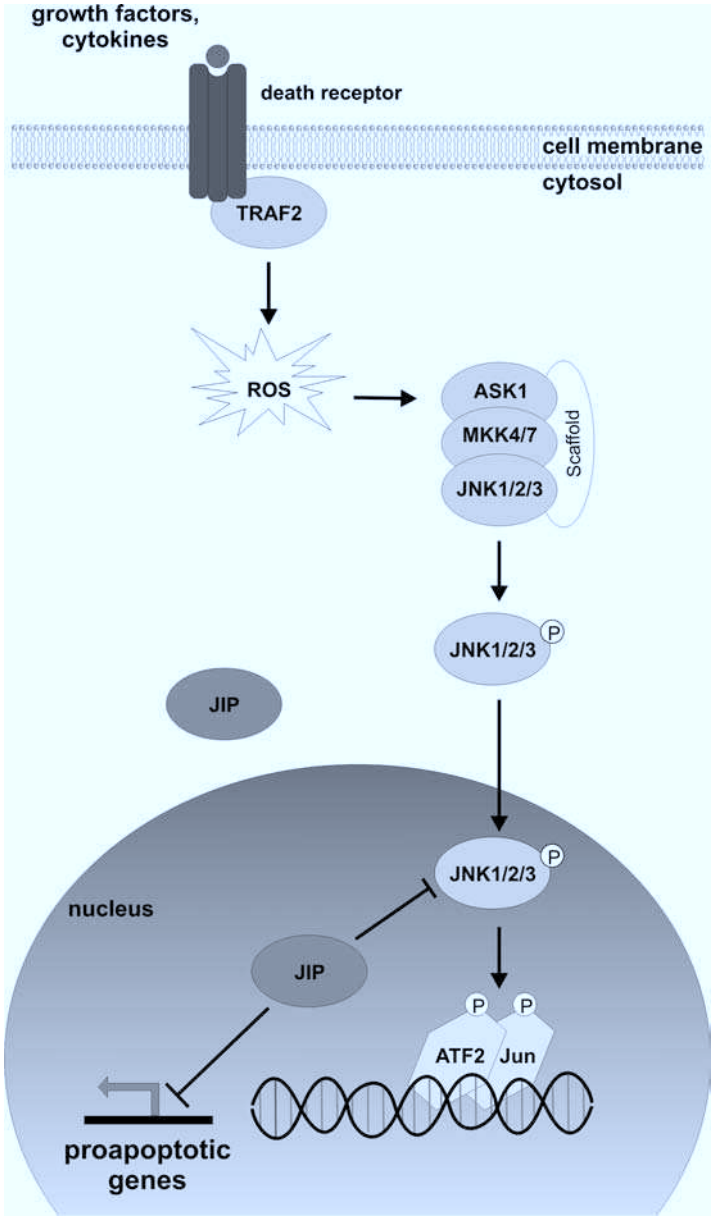

FIG. 10. Nuclear targeting and signaling of JNKs in the regulation of apoptosis. Growth factors and cytokines elevate ROS levels mediated by death receptor–coupled Traf2. This induces the activation of c-Jun NH_2-terminal kinases (JNKs) through upstream kinases apoptosis-signaling kinase 1 (ASK1) and MAP kinase kinase (MKK) 4/7. Activated JNK1, 2, and 3 translocate into the nucleus and phosphorylate c-Jun and activating transcription factor 2 (ATF2), which leads to formation of an activator protein 1 (AP-1) complex and to transcription of proapoptotic genes. Inhibition of nuclear active JNK1, 2, and 3 by JNK inhibitor protein (JIP) results in complete apoptosis inhibition.

JNKs or two JNKs together does not protect. Moreover, to determine whether cytosolic or nuclear JNKs are responsible for JNK-dependent cell death, compartment-specific inhibitors for JNKs were generated. Therefore, a nuclear-exclusion sequence (NES) from MKK1 or three NLSs from SV40 large-T antigen was fused in tandem upstream of the JNK inhibitor protein JIP. These targeted proteins localized to cytosolic and nuclear compartments, respectively (23). Overexpression of these compartment-specific JNK inhibitors revealed that cell death requires nuclear but not cytosolic JNK activity, as the nuclear dominant-negative inhibitor of JNK protected against apoptosis, whereas the cytosolic form only blocked physiologic JNK function (24). Thus, any one of the three JNKs is capable of mediating apoptosis, and this is dependent on their nuclear localization (Fig. 10).

4. Protein kinase B. The activity of the serine/threonine kinase Akt is redox regulated by Grx. By keeping Akt in its reduced form, Grx enables the activation and stimulation of the endothelial NO-synthase/NO signaling pathway (241). However, it is unclear whether this has to be attributed only to cytosolic Akt or also to the nuclear Akt.

We previously reported that nuclear Akt is required for the maintenance of telomerase activity and inhibition of apoptosis in human umbilical vein endothelial cells (70). Mechanistically, after the first assembly of the telomerase holoenzyme, which requires Hsp90 and p23 (89), Akt is recruited into this complex in the nucleus. On its recruitment, Akt phosphorylates TERT at serine 823, which increases nuclear telomerase activity (29, 70). Furthermore, the complex formation of TERT with HSP90 and Akt protects Akt from being dephosphorylated by the protein phosphatase 2A and thereby keeps Akt and TERT in their active forms (70, 105). Independent of its telomere-elongation function, nuclear TERT also has antiapoptotic effects. Inhibition of telomerase activity or overexpression of nuclear TERT induces or inhibits

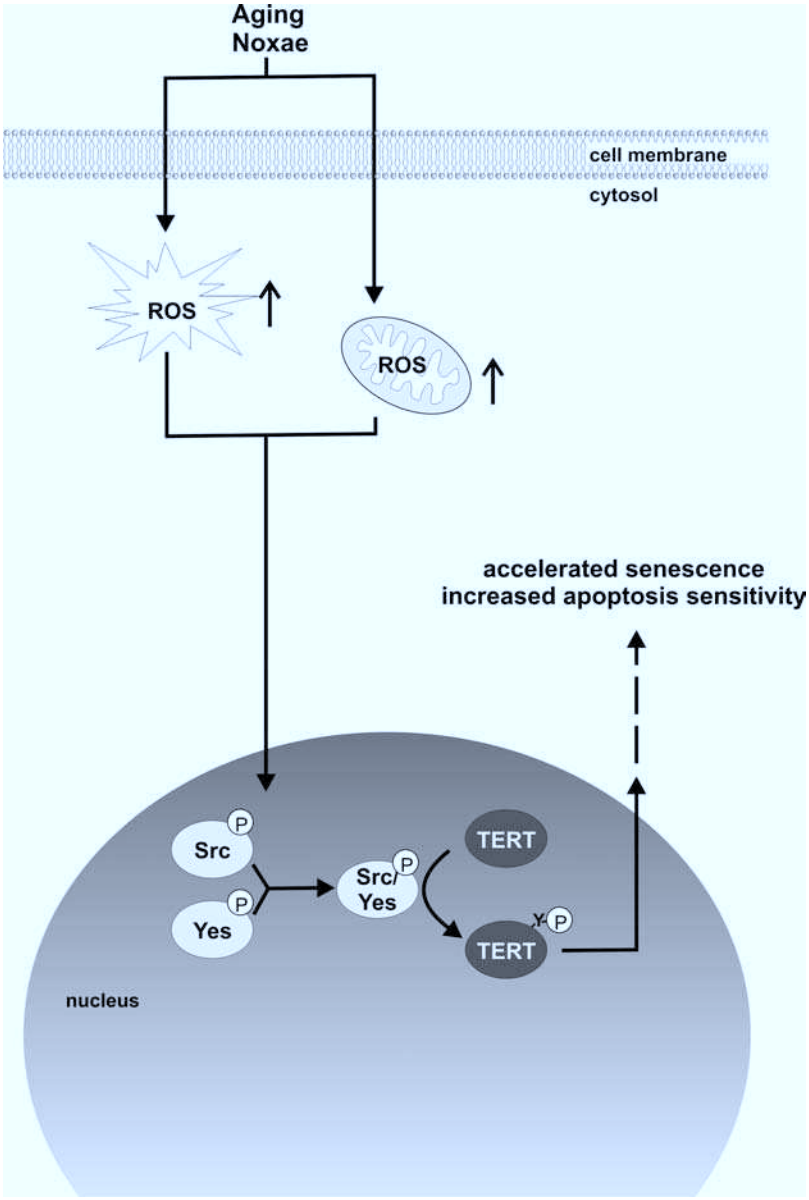

FIG. 11. Nuclear Src and Yes induce TERT export under conditions of oxidative stress. Cytosolic and mitochondrial ROS induce increased activity of nuclear Src and Yes. This leads to tyrosine phosphorylation of telomerase reverse transcriptase (TERT) and its nuclear export. The functional consequences are increased apoptosis sensitivity and accelerated senescence.

apoptosis, respectively (68, 166, 192, 262). Thus, maintaining TERT in its active state in the nucleus by Akt protects cells against apoptosis.

It has been demonstrated that, on its activation, myocardial Akt accumulates in the nucleus (30). Therefore, important studies investigated the specific function of nuclear Akt in cardiomyocytes by using an adenovirus expressing Akt with nuclear-localization signals. Nuclear-targeted Akt inhibited cardiomyocyte hypertrophy and apoptosis (211, 232). Moreover, transgenic mice with cardiac-specific expression of nuclear-targeted Akt were protected from ischemia/reperfusion injury (211). Given the fact that constitutive active Akt causes hypertrophy in cardiomyocytes, specifically, nuclear-targeted Akt provides a new opportunity for therapeutic applications.

5. Extracellular regulated kinases 1/2. ERK1/2s are a subfamily of the MAP kinases and are involved in many important cellular processes like cell proliferation, survival, apoptosis, and metabolism (165). Their stimulation is triggered by growth factors and different environmental and oxidative stresses. The cytokine epidermal growth factor (EGF) is one of the activators of ERK1/2 through phosphorylation of the EGF receptor (EGFR) (31), whereas H_2O_2 activates ERK1/2 by EGFR-dependent and -independent pathways (63, 243). The phosphorylation state of ERK2 is more important for its nuclear retention than is the activity of ERK2. This was confirmed with catalytically inactive ERK2, in which lysine 52 was mutated to arginine [ERK2(K52R)]. Like the wild-type protein, microinjected ERK2(K52R) transiently translocated to the nucleus after stimulation. To reduce the susceptibility to dephosphorylation, the two activating phosphorylation sites were thiophosphorylated *in vitro*. The stably phosphorylated wt and K52R mutant directly moved into and remained in the nucleus, demonstrating that ERK2 activity is not necessary for nuclear accumulation (108).

Besides phosphorylation of ERK2, homodimerization is necessary for its nuclear accumulation (108). The dimerization

of ERK2 induces a change of conformation and exposes a binding site for an NLS-containing protein. One of the potential candidates for such a NLS protein is growth factor–receptor bound protein 2-associated protein 1 (Gab1), which associates with ERK2. It was suggested that binding of ERK2 and Gab1 regulates the nuclear import of phosphorylated ERK2 (170). Another important role for ERK1 and ERK2 is their involvement in the nuclear localization of Nrf-2, which, among others, activates genes coding for enzymes required for *de novo* GSH synthesis (263). Thus, ERK1/2 or mainly ERK2 plays a role in ensuring an appropriate GSH supply for the cell.

6. Src kinase family. The Src kinase family consist of at least nine members (178). The most prominent members are the kinases Src, Fyn, and Yes. In the nucleus, only the kinases Src and Yes can be found, but not Fyn (96). In response to oxidative stress or growth factors, Src is phosphorylated at tyrosine 416, and Yes, at tyrosine 426, which increases their enzymatic activity. Under conditions of oxidative stress or during the process of aging, these nuclear kinases are involved in tyrosine phosphorylation of TERT, which then leads to nuclear export of TERT. This has been demonstrated by mutation of tyrosine 707 within TERT, resulting in nuclear retention of the enzyme and by pharmacologic inhibition of Src kinase family activation, which inhibits tyrosine phosphorylation of TERT and its subsequent export from the nucleus (70, 96) (Fig. 11). The underlying export mechanism is mediated by a complex formation between TERT, the well-described export receptor CRM-1, and the nuclear GTPase Ran (70). Functional consequences of this Src kinase family–dependent nuclear tyrosine phosphorylation of TERT are increased apoptosis sensitivity and accelerated senescence (68, 69). This is in agreement with other studies, demonstrating that nuclear TERT acts as an inhibitor of apoptosis in several cell types (56, 144, 166). Therefore, an imbalance in the redox status seems to enhance active nuclear Src and Yes, which importantly contribute to apoptosis and accelerated senescence.

Recently a new mechanism for inactivation of the Src kinase was discovered. Src is active only in its reduced form, and oxidation results in complete inactivation. This inactivation is caused by a specific, reversible oxidation of Cys 277, located in the catalytic domain of Src, which results in homodimerization of Src linked by a disulfide bridge. This cysteine residue is conserved only in three of the Src family members, Src, Yes, and Egr, and could therefore be a specific mechanism for regulating a specific subset of Src kinases (106). Interestingly, only Src and Yes could be found in the nucleus, leading to the speculation that the formation of disulfide homodimers might be a prerequisite for nuclear import.

B. Redox-regulated phosphatases

Protein phosphatases can be divided into serine/threonine phosphatases and tyrosine phosphatases (PTPs). Both are important regulators in the activation and inactivation of cell-signaling pathways. Serine/threonine phosphatases are generally regulated by association with regulatory subunits and their own phosphorylation (49, 209). In the last few years, the role of oxidative stress in the regulation of PTPs has received more attention. PTPs have been shown to be redox sensitive and to be inhibited reversibly or irreversibly, depending on the degree and mechanism of oxidation (120, 150, 180, 231). Oxidation of the essential cysteine in the active site by H_2O_2 inactivates phosphatase activity (45). The reversible oxidation of this cysteine residue to sulfenic acid (Cys-SO^-) has been identified as a key mechanism for the regulation of many pathways. Higher oxidation to sulfinic (Cys-SO_2^-) or sulfonic (Cys-SO_3^-) acid leads to an irreversible inactivation of the phosphatase (Fig. 12A). Many PTPs prevent a higher oxidation by formation of intracellular disulfide bonds between the active-site cysteine and nearby so-called backdoor cysteines [for example, Cdc25 phosphatases (216) or the phosphatase and tensin homologue (PTEN) (113)]. These intramolecular disulfides can then rapidly and effectively be reduced by

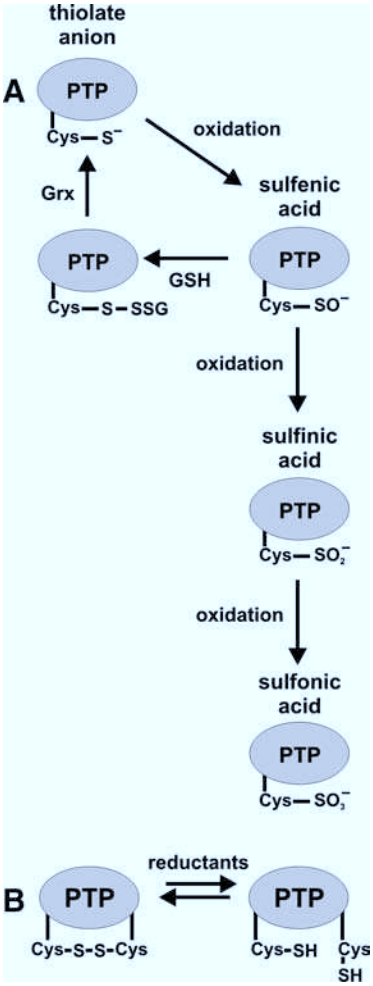

FIG. 12. Model for the regulation of PTP activity. (**A**) Under physiologic conditions, the catalytic cysteine of active protein tyrosine phosphatases (PTPs) is in the thiolate anion form. Oxidation leads to reversible sulfenic acid formation. Further oxidation is irreversible and leads to sulfinic and sulfonic acid formation. To prevent this further oxidation, GSH can form a mixed disulfide with the sulfenic acid, which is then reduced by glutaredoxin (Grx). (**B**) To prevent PTPs from being irreversibly oxidized, the reversible inactive state is stabilized by formation of an intramolecular disulfide bond between the cysteine in the catalytic center and a backdoor cysteine. This intramolecular disulfide bond can be rapidly and effectively reduced by several reductants.

reductants like dithiothreitol (DTT), GSH, Trx-1, or a combination of these (113, 216) (Fig. 12B). The oxidation of PTPs is important for the regulation of many cellular signaling pathways in response to oxidative stress.

C. Nuclear-localized phosphatases

Recently a new concept emerged, which emphasizes an important role for nuclear tyrosine phosphatases and their signaling pathways in response to oxidative stress. We concentrate on the detailed discussion of PTPs for which a nuclear localization has been shown, even if the redox regulation takes place outside the nucleus: Shp-2, T-cell protein tyrosine phosphatase (TC-PTP), and Cdc25C (an overview is given in Table 2).

1. Protein tyrosine phosphatase Shp-2. The Src homology 2 (SH2) domain containing protein tyrosine phosphatase Shp-2 is involved in many signal-transduction processes induced by cytokines and growth factors (2, 4, 54, 55). Further findings indicated a regulation of Shp-2 by ROS (150). Stimulation of Rat-1 cells with platelet-derived growth factor (PDGF) induced production of intracellular ROS, which leads to oxidation and inactivation of Shp-2. This oxidation requires complex formation between Shp-2 and the activated PDGF receptor (PDGFR). In its basal state, the active site of Shp-2 is covered by the N-terminal SH2 domain (N-SH2). The binding of a specific phosphotyrosyl ligand opens the active site of Shp-2 (82). Meng *et al.* (150) speculated that binding of PDGFR to Shp-2 promotes an open, active conformation of Shp-2, and that not only substrates but also ROS can interact with this site.

Like other PTPs, Shp-2 is oxidized by ROS. Recently, two backdoor cysteines were found to be involved in the redox regulation of Shp-2. Instead of forming a disulfide bond between the active-site cysteine and a backdoor cysteine to prevent the phosphatase from further oxidation, the reduced catalytic cysteine in Shp-2 (and also in Shp-1) is shielded by a disulfide bond between two backdoor cysteines (33).

In 2002, Chughtai *et al.* (37) reported a nuclear localization of Shp-2 in connection with an association of the signal transducer and activator of transcription 5 (STAT5) and Shp-2. This nuclear translocation of Shp-2 in a complex with Stat5 is induced by the stimulation with prolactin in mammary cells. Formation of this complex requires the carboxy-terminal SH2 domain and the catalytic activity of Shp-2 and correlates with the tyrosine phosphorylation of STAT5 by Janus kinase 2 (JAK2) on the tyrosine residue 694 (Fig. 13). The authors speculated that the nuclear Shp-2/STAT5 complex binds to DNA and regulates transcription of milk-protein genes (37). In endothelial cells, Shp-2 is localized in nuclear and cytosolic fractions under basal conditions (96). Our findings identified nuclear Shp-2 acting as a counterplayer for the nuclear export of TERT. Under conditions of oxidative stress, the nuclear export of TERT is blocked by Shp-2 overexpression. This is dependent on the activity of Shp-2 because the dominant-negative Shp-2(C459S) reduces nuclear TERT protein and telomerase activity. Ablation of endogenous Shp-2 leads to an increased tyrosine phosphorylation of TERT. Tyrosine 707 within TERT (which has previously been shown, once phosphorylated, to be essential for nuclear export of TERT) seems to be the target tyrosine for Shp-2. Thus, Shp-2 inhibits the nuclear export of TERT by regulating the tyrosine 707 phosphorylation (Fig. 14). This implies a new role for nuclear Shp-2 in protecting nuclear TERT, and therefore, nuclear Shp-2 may delay cellular senescence.

2. T-cell protein tyrosine phosphatase. T-cell protein tyrosine phosphatase (TC-PTP) is a ubiquitously expressed PTP. As a result of alternative splicing, two isoforms of TC-PTP are generated, a 45-kDa isoform located in the nucleus and a 48-kDa isoform targeted to the endoplasmic reticulum (130). Specific cellular stresses cause a reversible cytosolic accumulation of the 45-kDa isoform of TC-PTP (114). For example, oxidative stress or hyperosmolarity induces the nuclear export of TC-PTP, whereas other stresses like heat shock have no effect on the localization of TC-PTP. This

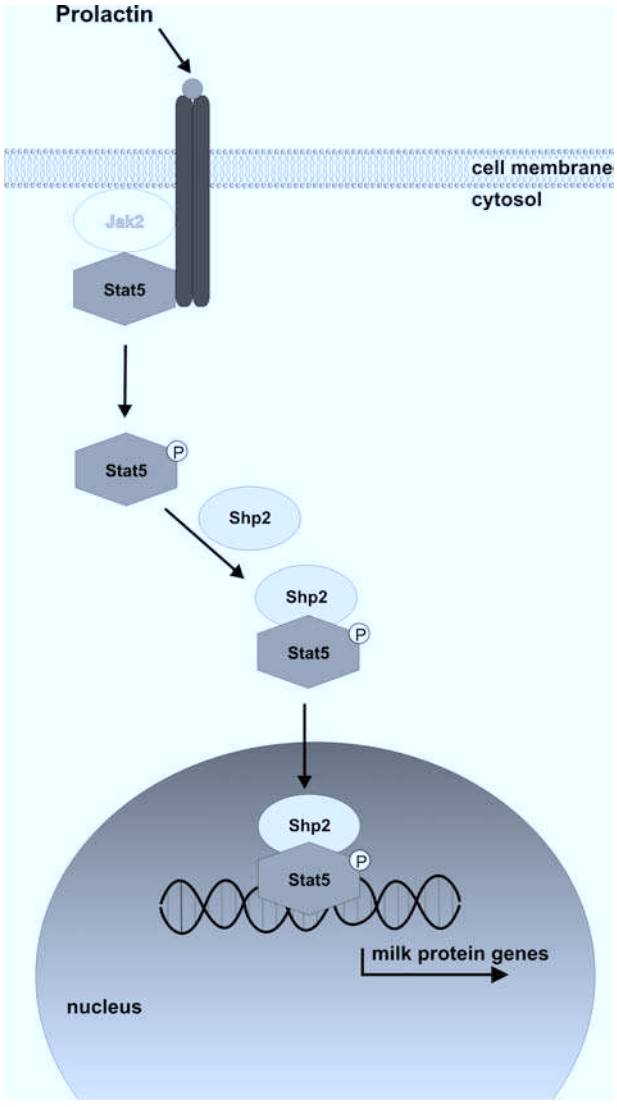

FIG. 13. Nuclear translocation of Shp-2 induces STAT5-dependent gene transcription. Prolactin induces tyrosine phosphorylation of STAT5 at tyrosine 694 by the Janus kinase 2 (JAK2), which leads to complex formation of Shp-2 and STAT5. This complex translocates into the nucleus, binds to DNA via STAT5, and induces milk-protein gene transcription.

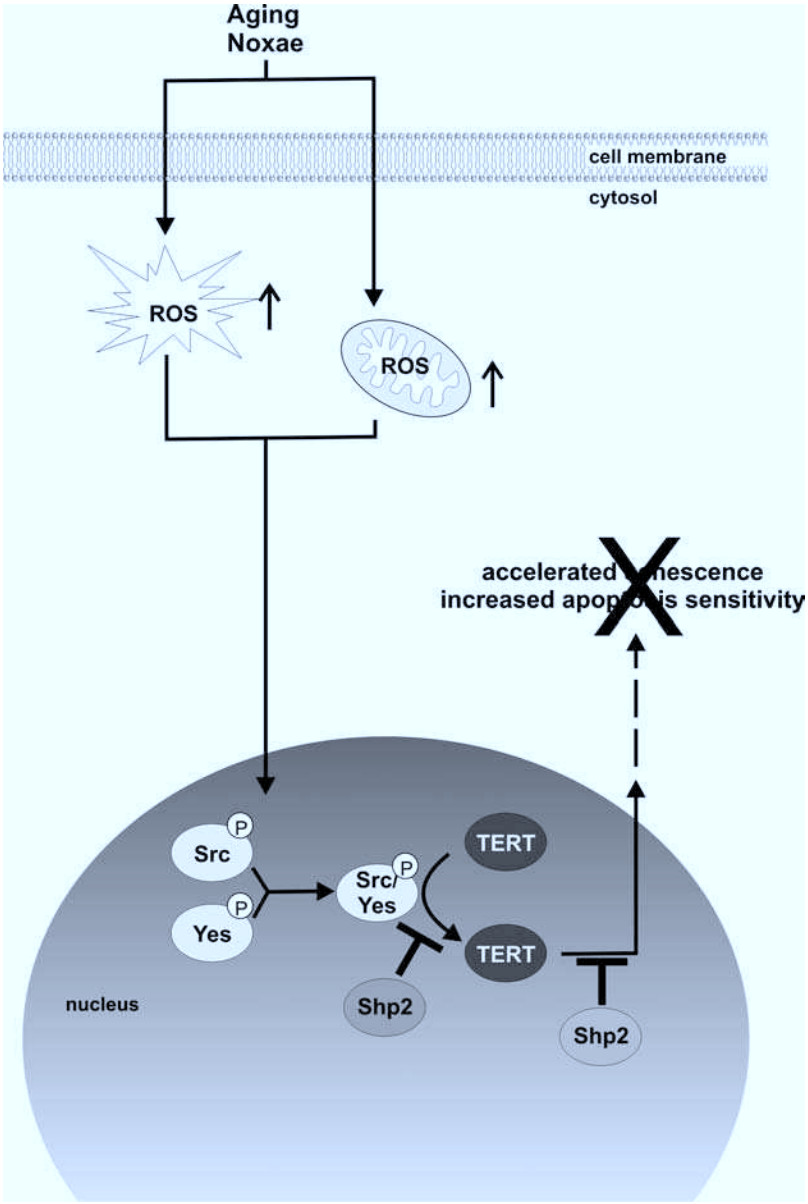

FIG. 14. Shp-2 inhibits TERT nuclear export induced by oxidative stress. ROS induce increased activity of nuclear Src and Yes. This leads to tyrosine phosphorylation of telomerase reverse transcriptase (TERT) and to its nuclear export. Nuclear Shp-2 prevents this export.

change in localization could be observed in different cell types. Nonnuclear TC-PTP is involved in the regulation of cell growth and cell differentiation in endothelial cells (143). There, TC-PTP binds to vascular endothelial growth factor receptor 2 (VEGFR2) and dephosphorylates specific tyrosine residues. Dephosphorylation of tyrosines 1045 and 1059 delays VEGF-induced VEGFR2 internalization and thus reduces sprouting angiogenesis.

Another target tyrosine of TC-PTP is tyrosine 1214, which has been implicated in VEGF-induced actin remodeling and thereby plays a role in endothelial cell migration (115). Together, these data suggest an important role for cytosolic TC-PTP in endothelial cell growth and differentiation.

Several other groups have investigated the functions of nuclear TC-PTP. The 45-kDa form of TC-PTP was first identified as a nuclear STAT1 tyrosine phosphatase. On stimulation, STAT1 becomes tyrosine phosphorylated by the family of JAKs and translocates into the nucleus. There it binds to DNA and activates transcription (40). To terminate this transcription, STAT1 must be dephosphorylated to dissociate from DNA. Ten Hoeve *et al.* (228) were the first to identify the 45-kDa form of TC-PTP to be the tyrosine phosphatase of STAT1. They also investigated the dephosphorylation of other STATs (STAT3, STAT5, and STAT6) and found that, in TC-PTP–deficient mouse embryonic fibroblasts, only the dephosphorylation of STAT1 and STAT3 is affected on interferon stimulation, but not that of STAT5 and STAT6 (228). This is in agreement with findings of Yamamoto *et al.* (257), who demonstrated dephosphorylated STAT3 after interleukin 6 treatment and showed a direct interaction between STAT3 and nuclear TC-PTP. Similarly, Aoki and Matsuda (10) found that in epithelial cells, stably expressing mouse TC-PTP STAT5a and STAT5b are dephosphorylated after prolactin stimulation (10). TC-PTP has different regulatory roles in diverse pathways, dependent on its localization. Because nuclear export of TC-PTP occurs under specific stresses, one can

imagine stress-specific functions of TC-PTP. This opens up an interesting field for further studies to understand why specific stress inducers like heat shock do not induce the nuclear export of TC-PTP.

3. Cdc25C. In mammalian cells, cell-cycle progression is tightly regulated by the cyclin-dependent protein kinases (CDKs). CDK1 (also named Cdc2) is the key component of the checkpoint pathway, which delays mitotic entry after DNA damage or stalled replication. CDK1 forms complexes with cyclin B1 that, in their phosphorylated, inactive form, are retained in the cytosol. During prophase, CDK1/cyclin B1 complexes accumulate in the nucleus and are activated through the phosphatase Cdc25C by dephosphorylation of threonine 14 and tyrosine 15 of CDK1 (48, 112). As mentioned earlier, phosphatases can protect themselves from irreversible oxidation by forming a disulfide bond between their catalytic cysteine and a backdoor cysteine. This protection has also been demonstrated for Cdc25C. Its catalytic cysteine is cysteine 330, and the backdoor is cysteine 377. Mutation of cysteine 377 in Cdc25C leads to irreversible oxidation of cysteine 330, which results in degradation of Cdc25C (198). During interphase of the cell cycle, a formation of the disulfide bond between cysteine 330 and 377 in Cdc25C is induced, and Cdc25C is phosphorylated at serine 216. This phosphorylation leads to binding of Cdc25C to 14-3-3 proteins and results in the nuclear export of Cdc25C (179). Mutation of serine 216 in Cdc25C perturbs mitotic timing and allows cells to escape the G_2-checkpoint arrest (179, 195).

For the fate of the cytosolic Cdc25C, different hypotheses exist. One possible mechanism is the degradation of Cdc25C, according to the destruction hypothesis of Savitsky and Finkel (198). It has been demonstrated that vitamin C induces formation of ROS, which leads to decreased Cdc25C levels. Conversely, the formation of cytosolic Cdc25C/14-3-3 complexes occurs without reduction in the Cdc25C levels. Thus, one may speculate that, dependent on the levels of ROS, cells decide to induce cell death or G_2-checkpoint arrest. High ROS levels would lead to irreversible oxidation of Cdc25C, its degradation, and finally to cell death. Minor damage, conversely, could induce formation of the disulfide bond in Cdc25C, its phosphorylation, and cytosolic sequestration by 14-3-3 proteins. Thus, Cdc25C would be immediately available for cell-cycle progression after damage repair.

V. Conclusions and Outlook

Redox regulation plays an important role in intracellular signal transduction. Numerous proteins have been described to be redox regulated. However, it must be noted that, for the nuclear import and export receptors, only initial studies in yeast have shown that these receptors change their localization from the nucleus to the cytosol and can be oxidized on oxidative stress, which could importantly contribute to gene regulation (111, 183, 184). Thus, further studies in higher eukaryotes are required to investigate the redox regulation of the nuclear import and export machinery. Furthermore, many studies do not distinguish between the intracellular compartments in which the redox modification of the protein of interest takes place. This is of special importance, because many antioxidative systems exist in different cellular compartments like the cytosol, the mitochondria, and the nucleus. However, not all antioxidative systems are equally distributed throughout the cell. The distribution strongly depends on the cell type and on the stimulus used. Therefore, it is noteworthy that cellular functions regulated by redox modifications of proteins are intimately associated with their cellular localization. Unfortunately, many previous investigations did not accurately discriminate between the compartments where redox regulation occurs and the corresponding cellular function. Without this differentiation, pharmacologic interventions may exhibit undesirable and unanticipated side effects because the compartment-specific effects of the proteins have been disregarded in the past. Therefore, compartment-specific investigations will help us to determine protein functions in more detail and to uncover misunderstood protein functions.

Finally, compartment-specific delivery of inhibitors and activators will open a new field of drug design to regulate protein actions more precisely and to reduce unwanted side effects.

Acknowledgments

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Author Disclosure Statement

The authors have nothing to disclose.

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Abbreviations Used

AP-1 = activator protein 1
APEX1 = APEX nuclease (multifunctional DNA-repair enzyme) 1
ATF = activating transcription factor
bZIP = basic region-leucine zipper
cAMP = cyclic adenosine monophosphate
CDK = cyclin-dependent kinase
CRE = cAMP response element
CREB = cAMP response element–binding protein
CREM = CRE modulator
DBD = DNA-binding domain
DNA-PK = DNA protein kinase
DTT = dithiothreitol
Egr-1 = early growth-response factor 1
ER = estrogen receptor
ERE = estrogen response element
ERK = extracellular regulated kinase
GPx = glutathione peroxidase
GR = glutathione reductase
GRE = glucocorticoid response element
Grx = glutaredoxin
GSH = glutathione
GST = glutathione *S*-transferase
HIF = hypoxia-inducible factor
HLF = HIF-1α–like factor
HRE = hypoxia-response element
Hsp = heat-shock protein
HTLV = human T-lymphotropic virus
IκB = inhibitor of nuclear factor-κB
IKK = IκB kinase
JAK = Janus kinase
JIP = JNK-inhibitor protein
JNK = c-Jun NH_2-terminal kinase
Keap1 = Kelch-like ECH-associated protein 1
MAP kinase = mitogen-activated protein kinase
MKK = MAP kinase kinase
NFI/CTF = nuclear factor I/CAAT transcription factor
NF-κB = nuclear factor-κB
NIK = NF-κB–inducing kinase
NLS = nuclear localization signal
Nrf-2 = nuclear factor erythroid 2–related factor 2/NF-E2 related factor 2
ODD = oxygen-dependent degradation domain
PKA = protein kinase A
PKC = protein kinase C
Prx = peroxiredoxin
PTP = protein tyrosine phosphatase
ROS = reactive oxygen species
SOD = superoxide dismutase
Sp1 = specificity protein 1
STAT = signal transducer and activator of transcription
TAD = transcription-activation domain
TC-PTP = T-cell protein tyrosine phosphatase
TERT = telomerase transcriptase
TR1 = thioredoxin-1 reductase
Trx-1 = thioredoxin-1

Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage

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Arterioscler. Thromb. Vasc. Biol., 29, 929-935, 2009

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Mitochondrial Telomerase Reverse Transcriptase Binds to and Protects Mitochondrial DNA and Function From Damage

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Mitochondrial Telomerase Reverse Transcriptase Binds to and Protects Mitochondrial DNA and Function From Damage

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Objective—The enzyme telomerase and its catalytic subunit the telomerase reverse transcriptase (TERT) are important for maintenance of telomere length in the nucleus. Recent studies provided evidence for a mitochondrial localization of TERT. Therefore, we investigated the exact localization of TERT within the mitochondria and its function.

Methods and Results—Here, we demonstrate that TERT is localized in the matrix of the mitochondria. TERT binds to mitochondrial DNA at the coding regions for ND1 and ND2. Binding of TERT to mitochondrial DNA protects against ethidium bromide–induced damage. TERT increases overall respiratory chain activity, which is most pronounced at complex I and dependent on the reverse transcriptase activity of the enzyme. Moreover, mitochondrial reactive oxygen species are increased after genetic ablation of TERT by shRNA. Mitochondrially targeted TERT and not wild-type TERT revealed the most prominent protective effect on H_2O_2-induced apoptosis. Lung fibroblasts from 6-month-old $TERT^{-/-}$ mice (F2 generation) showed increased sensitivity toward UVB radiation and heart mitochondria exhibited significantly reduced respiratory chain activity already under basal conditions, demonstrating the protective function of TERT in vivo.

Conclusion—Mitochondrial TERT exerts a novel protective function by binding to mitochondrial DNA, increasing respiratory chain activity and protecting against oxidative stress–induced damage. **(*Arterioscler Thromb Vasc Biol.* 2009;29:929-935.)**

Key Words: aging ■ apoptosis ■ mitochondrial functions ■ mitochondrial DNA ■ reactive oxygen species ■ telomerase reverse transcriptase

To date several theories exist to explain the phenomenon of normal and pathological aging. The free radical theory of aging[1] proposes that reactive oxygen species (ROS) in biological systems attack molecules and thereby cause functional decline of organ systems that eventually leads to death. This damage accumulates over time and may contribute to diseases associated with aging like atherosclerosis, neurodegeneration, or cataracts.[2] Recently, Schriner et al produced transgenic mice that overexpressed human catalase localized to the peroxisome, the nucleus, and the mitochondria. Only mice overexpressing mitochondrially targeted catalase showed a significant increase in life span and a reduction in oxidative damage to DNA and consequently in apoptosis.[3] Thus, these data define the mitochondria as compartment of ROS formation, which contributes to aging processes. Further evidence supporting the importance of mitochondria and formation of ROS in the mitochondria comes from findings that overexpression of mitochondrially localized antioxidant enzymes lengthens lifespan of *Drosophila*[4,5] and that deletion of manganese superoxide dismutase results in the age-related decline of mitochondrial function, culminating in increased apoptosis.[6] Recent studies using isolated complex I of the respiratory chain clearly demonstrated that superoxide production into the mitochondrial matrix is predominantly dependent on flavine-mononucleotide within complex I.[7,8]

The enzyme telomerase counteracts the shortening of the physical ends of chromosomes and, thereby, prevents the onset of replicative senescence and genetic instability.[9–12] The catalytic subunit, which antagonizes telomere-shortening, is the telomerase reverse transcriptase (TERT).[9] Several studies suggested that TERT exerts functions independently of its net telomere lengthening (for review see[13]). TERT increased cell survival and resistance against oxidative stress after short term incubation of cells with different stimuli, which did not affect telomere shortening.[14–18] TERT has been shown to have a genuine mitochondrial leader sequence which targets this protein to the mitochondria.[19,20] However the exact role of mitochondrial TERT remains

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controversial, with 1 group showing that it exacerbates oxidative injury[19,20] and another group demonstrating a protective effect after oxidative stress.[21] Taking into account that aging processes are associated with dysfunctional mitochondria, the goal of the present study was to elucidate the localization of TERT within the mitochondria and its functional role in the mitochondria with specific respect to respiratory chain activity, apoptosis, and formation of ROS.

Methods

Please see http://atvb.ahajournals.org for expanded Methods section.

Cell Culture

Human umbilical vein endothelial cells (HUVECs; Lonza, Cologne, Germany) were transfected with Superfect as previously described.[22] After detachment with trypsin, cells were grown for at least 18 hour prior to all further manipulations.

Isolation of Primary Mouse Lung Fibroblasts

A small piece of lung tissue was minced with 2 scalpels in a culture dish and incubated in HEK 293 medium at 37°C. After 5 days the fibroblasts grown out from tissue fragments were passaged to culture flasks.

Proteinase K Digestion of Mitochondria

To determine whether TERT is localized in the mitochondrial matrix or the intermembrane space, 700 μg of mitochondria was distributed in 3 equal aliquots. Mitochondria were pelleted and incubated at 4°C on a shaker in 40 μL of 3 different buffers for 20 minutes. Buffer 1 (isotonic buffer): 250 mmol/L sucrose, 1 mmol/L EGTA, 10 mmol/L HEPES, pH7; Buffer 2 (hypotonic buffer): 1 mmol/L EGTA, 10 mmol/L HEPES, pH7, 25 μg/mL proteinase K; Buffer 3 (hypotonic buffer with detergent): 1 mmol/L EGTA, 10 mmol/L HEPES, pH7, 1% (v/v) Triton-X100, 25 μg/mL proteinase K. After 20 minutes, digestion was stopped by adding phenylmethylsulfonyl fluoride to a final concentration of 2 mmol/L and incubated for further 5 minutes with shaking. Aliquot 3 was boiled for 5 minutes in Laemmli-buffer. Aliquot 1 and 2 were washed once with Buffer 1 and resuspended in 40 μL RIPA-buffer (50 mmol/L Tris/HCl pH8, 1% [v/v] Nonidet, 150 mmol/L NaCl, 0.1% [wt/vol] SDS, 0.5% [wt/vol] Desoxycholate) and boiled for 5 minutes in Laemmli-buffer. Western blot analyses were performed using antibodies against endogenous TERT (Epitomics, 1:500), TOM40 (Santa Cruz, 1:250), and TIM23 (BD Transduction laboratories 1:1000).

Results

TERT Is Localized in Mitochondria

It has been demonstrated that mitochondria contain measurable telomerase enzymatic activity,[19] suggesting that a fraction of cellular TERT is localized in these organelles. After having previously shown that telomerase activity is found in mitochondria of HUVECs,[23] we confirmed that this is also true for HEK cells. As shown in Figure 1A, telomerase enzyme activity was detectable in all 3 compartments under basal conditions. Because commercially available antibodies against endogenous TERT could not be used for immunoprecipitation, we next tested whether overexpressed TERT wild-type protein containing a myc-tag at the C terminus (TERTwt-myc) is detectable in mitochondria. From our previous studies, we knew that TERTwt-myc is exported from the nucleus on stress and thereby behaved identical to endogenous TERT in HEK cells and HUVECs.[17] Overexpressed TERTwt-myc was detected in the mitochondria by immunoblot (supplemental Figure IA). The biochemical fractionation was always controlled by proteins, which are known to be located in the nucleus or in the mitochondria.

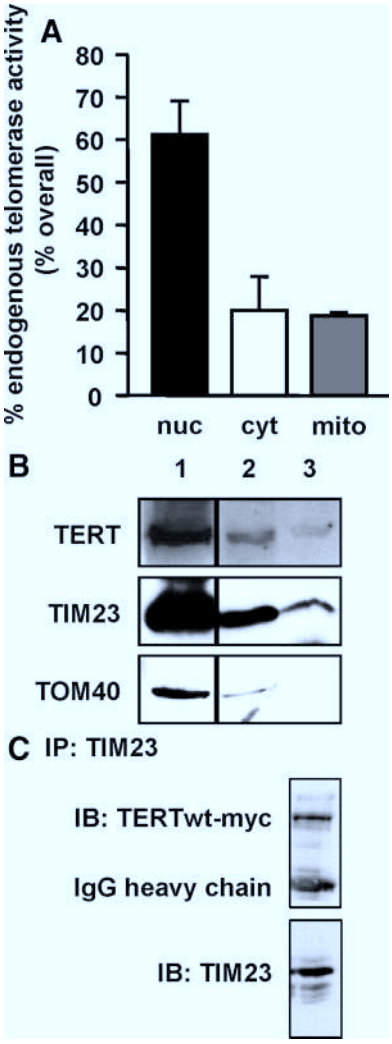

Figure 1. TERT is localized in mitochondria. A, Endogenous telomerase enzyme activity was measured in the nuclear (nuc), cytosolic (cyt), and mitochondrial (mito) fraction of HEK cells. B, Total mitochondrial proteins (1), matrix proteins (2), and proteinase K–treated matrix proteins (3) were assayed for the presence of TERT, TIM23, and TOM40. C, TIM23 was immunoprecipitated from the mitochondrial fraction of the TERTwt-myc transfected cells and the precipitate analyzed for the presence of TERTwt-myc (upper panel) and as a control for TIM23 (lower panel).

To determine the exact localization of TERT, which has a genuine mitochondrial leader sequence at the N terminus, within these organelles, we digested the outer mitochondrial membrane with proteinase K. With this approach we could demonstrate that a large portion of endogenous TERT is localized in the mitochondrial matrix as indicated by its presence in the proteinase K resistant fraction (Figure 1B). This requires an import into the mitochondria, as the protein is encoded by the nuclear genome and thus translated in the cytoplasm. The best-characterized pathway to import a protein into the mitochondria of higher eukaryotes is by binding to a complex containing the translocases of the outer membrane 20 and 40 (TOM20 and TOM40) and the translocase of the inner membrane 23 (TIM23). We therefore investigated

whether TERT can associate with this complex by coimmunoprecipitation studies. As stated above, TERTwt-myc was detected in the mitochondria, and all antibodies directed against endogenous TERT could not be used for immunoprecipitation. Therefore, we performed all further experiments with overexpressed TERTwt-myc. The fact that TERTwt-myc physically interacts with the TOM20/TOM40 complex (supplemental Figure IB) and with TIM23 (Figure 1C) strongly suggests a transport mechanism involving these proteins. However, these experiments do not exclude participation of other translocases, like for instance TIM22.

TERT Binds to mtDNA

One important component of the mitochondrial matrix is the mtDNA. This prompted us to test whether TERTwt-myc can associate with the genome of the organelle. Therefore, we performed a modified chromatin-immunoprecipitation assay, using primers amplifying segments of mtDNA. After careful examination of the different regions of the circular mitochondrial genome, we identified 2 regions containing the coding sequences for NADH:ubiquinone oxidoreductase (complex I) subunit 1 and 2 (ND1, ND2) as interaction sites for overexpressed TERTwt-myc protein. In contrast, another region of the mtDNA containing the coding sequence for ATP synthase subunit 6 (ATP6) did not show interaction with TERTwt-myc (supplemental Figure II).

TERT Protects mtDNA From Damage

It has previously been shown that telomerase can protect mitochondrial function under oxidative stress.[21] As a number of mitochondrial proteins are encoded by the mitochondrial genome it was suggestive to analyze whether TERT bound to mtDNA could protect it against damage. Therefore we first assessed in vitro whether TERT can prevent UV-induced destruction of mtDNA. We treated isolated total cellular DNA (containing nuclear and mtDNA) in vitro in the presence or absence of TERTwt-myc for 3 minutes with short-wave UV light (254 nm) and analyzed intactness of mtDNA by long-range PCR. Indeed, preincubation of the DNA with in vitro translated TERTwt-myc effectively protected mtDNA against UV-induced degradation, whereas immediate irradiation showed only weak protection. Preincubation with β-galactosidase, a protein of comparable size, had no effect (Figure 2A).

To assess whether this protection of mtDNA is of relevance in living cells, we irradiated primary lung fibroblasts from homozygous TERT knockout (TERT $^{-/-}$) mice and their wild-type littermates with different doses of UVB irradiation and determined MTT conversion as a measure for mitochondrial activity. We demonstrated that TERT$^{-/-}$ fibroblasts are more sensitive to UV-irradiation with regard to their mitochondrial activity (Figure 2B), indicating that TERT has a protective function for mitochondria in vivo. In addition we could show that TERTwt-myc directly protects mtDNA in HEK cells (Figure 5A).

TERTwt Protects the Respiratory Chain and Specifically Complex I in Cells and in Mice

To further investigate whether TERT binding to mtDNA has an impact on mitochondrial function, we next measured

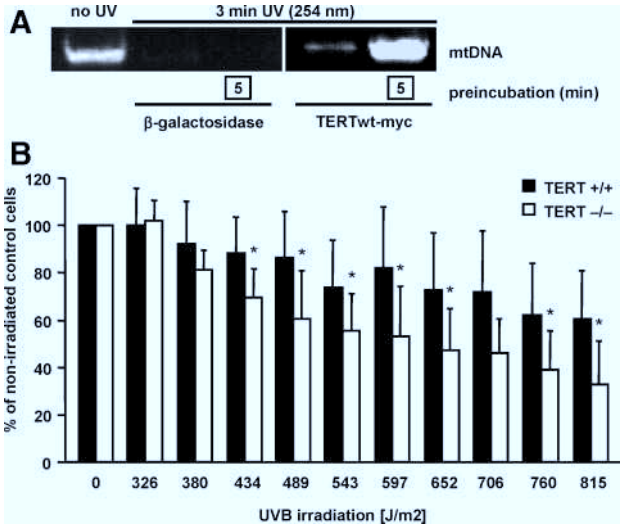

Figure 2. TERT protects mtDNA and function from UV-induced damage. A, Isolated total cellular DNA containing nuclear and mtDNA was irradiated with short wave UV light (254 nm). In vitro translated TERTwt-myc or β-galactosidase were added as indicated. Integrity of mtDNA was assessed and the products analyzed by agarose gel electrophoresis. B, Isolated primary lung fibroblasts from homozygous TERT knockout mice (TERT$^{-/-}$) and their wild-type littermates (TERT$^{+/+}$) were irradiated with different doses of UVB and mitochondrial function was measured. Data are presented as mean±SEM, n=3 with 8 replicates in each experiment, *$P<0.05$ vs TERT$^{+/+}$ cells.

respiratory chain activity in TERT-transfected cells. We included a TERT mutant (TERTD868A-myc) lacking reverse transcriptase activity,[24] from which we know that it can still bind to mtDNA (data not shown), to elucidate whether catalytic activity of the protein is required for maintaining mitochondrial functions. Measuring the respiratory chain activity in general, we discovered a 30% reduction in succinate-dependent respiration in cells overexpressing TERTD868A-myc (Figure 3A and supplemental Figure III), suggesting that not only the mtDNA binding but also the activity of TERT plays a role for mitochondrial functions. Besides the overall decrease found in respiratory chain activity, we wanted to determine whether the effect was most pronounced for complex I. To emphasize the latter, we normalized all other values to the same rate of succinate-respiration that was set to 1 (Figure 3A) and found a significant reduction in complex I activity. To further underscore these findings, we used a second method to measure complex I activity. Indeed, a significant increase in complex I activity was only found in cells overexpressing TERTwt-myc. (Figure 3B). Of note, the data shown in Figure 4b demonstrate that although all respiratory rates were decreased in TERTD868A, the mitochondria showed no differences in respiratory chain control factors ie, in the ratios of state 4/state 3 or state 4/FCCP-uncoupled rates, which essentially also excludes changes in membrane permeability. To determine whether our results obtained by overexpression of TERT are of relevance in vivo, we next analyzed the respiratory chain activity of intact heart and liver mitochondria isolated from TERT$^{-/-}$ mice and wild-type animals. Indeed, we found that heart mitochondria from TERT$^{-/-}$

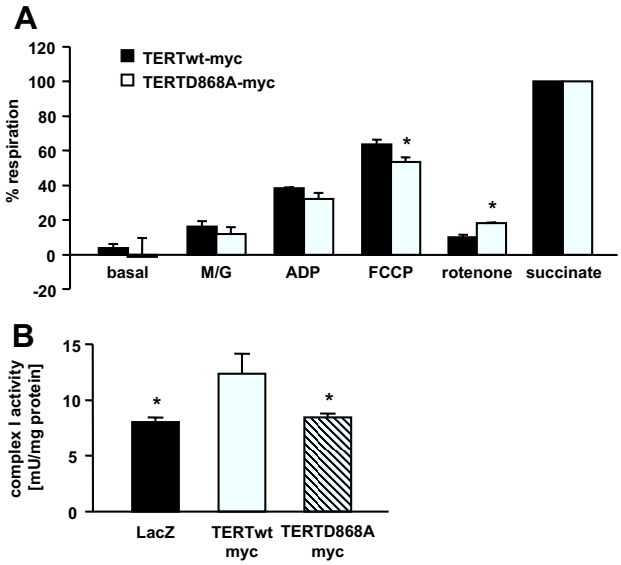

Figure 3. TERT alters respiratory chain activity in mitochondria. A, Respiratory chain activity was measured in transfected HEK cells as indicated. Rates were normalized to succinate-linked respiration that in absolute terms was on average 30% lower for TERTD868A-myc than for TERTwt-myc. Data are presented as mean±SEM, n=4, *$P<0.05$ vs TERTwt-myc. B, NADH:decylubiquinone oxidoreductase activities were determined in transfected HEK cells as indicated. Data are presented as mean±SD, n=6 *$P<0.05$ vs TERTwt-myc.

mice showed a significant reduction of the complex I–dependent respiration with the NADH-generating substrates malate and glutamate, which was most obvious in state 3 (ADP-stimulated) respiration (Figure 4a). In contrast, no difference between $TERT^{-/-}$ mice and their wild-type littermates was observed in liver mitochondria. This indicated that the protective effect of TERT in vivo might be more important in tissues with a high respiratory rate like the myocardium and with no or weak regenerative capacity.

TERT Reduces Formation of ROS in Mitochondria

Recent studies from Santos et al using a retroviral approach showed that reintroduction of human TERT into human fibroblasts increased oxidative stress, mtDNA lesions, and apoptosis.[19,20] In contrast, Ahmed et al showed that TERT protects mitochondria from damage and reduces oxidative stress.[21] Therefore, we investigated the effect of TERT under basal and EtBr-induced conditions on ROS formation. Overexpression of TERTwt-myc reduced ROS formation compared to LacZ- and TERTD868A-myc–overexpressing cells (Figure 4B). This is in contrast to Santos[19] but in agreement with Ahmed et al.[21] Moreover, ablation of endogenous TERT by shRNA (Figure 4C and supplemental Figure IVA) revealed increased mitochondrial ROS formation as measured with Mitosox, a specific compound, which is imported into the mitochondria for ROS detection (Figure 4D and supplemental Figure IVB), suggesting that the presence of TERT in the mitochondria protects from respiratory chain dysfunction.

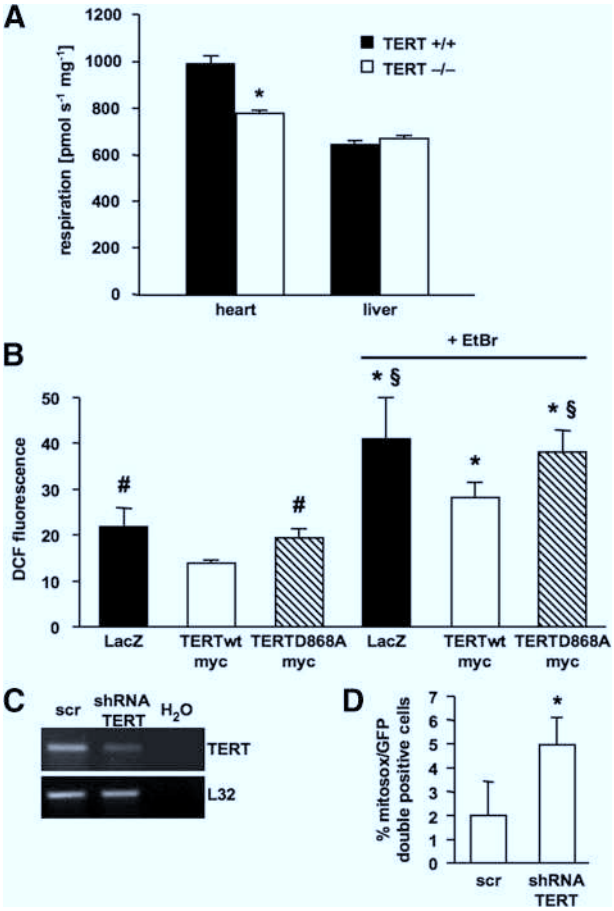

Figure 4. TERT protects respiratory chain activity in mice and inhibits mitochondrial derived ROS. A, State 3 respiration with the NADH-generating substrates malate and glutamate was measured in intact heart and liver mitochondria isolated from $TERT^{-/-}$ or $TERT^{+/+}$ mice. Data are presented as mean±SD, n=5 for each group, *$P<0.05$ vs their wild-type littermates. B, Formation of endogenous ROS (-/+EtBr) was measured with 2′,7′ dichlorodihydrofluorescein diacetate (H_2DCF-DA). n=4, *$P<0.05$ vs without EtBr, #$P<0.05$ vs TERTwt-myc, §<0.05 vs TERTwt-myc with EtBr. C, HEK cells were transfected with vectors expressing GFP and a TERT-specific shRNA (shRNA TERT) or a scrambled control sequence (scr). The transfection efficiency (=GFP-positive cells) was 54±9%. PCR analyses were performed out of whole cell lysates. D, The transfected living HEK cells from (C) were incubated with Mitosox. GFP/Mitosox double positive cells were measured by FACS analyses. Data are presented as±SEM, n=3 to 5, *$P<0.05$ vs scr.

Mitochondrially Targeted TERT Is More Protective Than TERTwt Against mtDNA Depletion and Oxidative Stress–Induced Apoptosis

Because mitochondrial damage importantly contributes to apoptosis induction, we cloned TERT into a mitochondrial target vector (mitoTERT-myc) to specifically investigate the effect of the mitochondrial localization of TERT on mtDNA protection and apoptosis inhibition.

After having shown that TERT binds to mtDNA and protects cells against UVB-induced decrease of mitochondrial function, we first assessed the effect of mitoTERT-myc on mtDNA protection in living cells. Therefore we reduced mtDNA content in HEK cells transfected with an empty control vector, TERTwt-myc, or mitoTERT-myc by treating

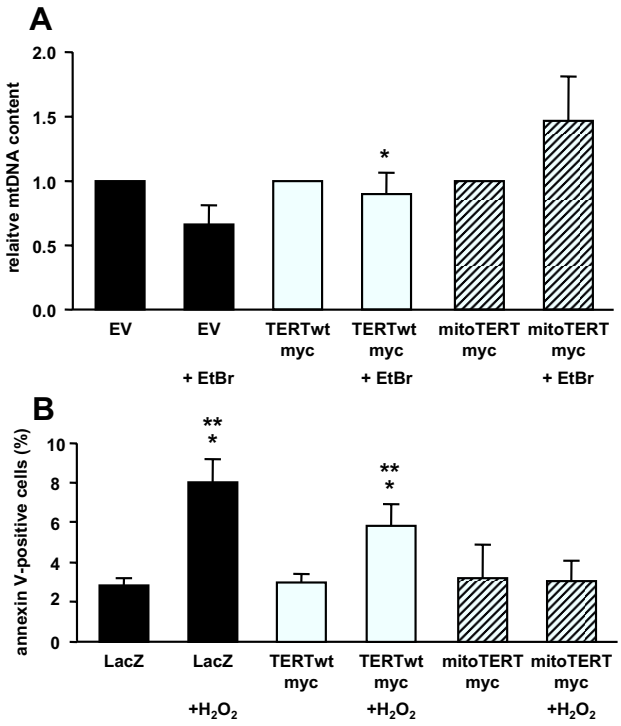

Figure 5. Mitochondrially targeted TERT rescues cells from ethidium bromide–induced mtDNA depletion and oxidative stress–induced apoptosis. A, HEK cells were transfected with an empty control vector (EV), TERTwt-myc, or mitoTERT-myc and treated with EtBr. mtDNA content was measured by quantitative real-time PCR and normalized to a unique nuclear DNA sequence using the ΔΔCt method. Values are mean±SEM and are shown relative to untreated cells. n=2 to 6 with 2 duplicates in each experiment, $^{*}P<0.05$ vs EV + EtBr. B, HUVECs were transfected with the constructs indicated and treated with 200 μmol/L H_2O_2. Apoptosis was measured with annexin V using FACS analysis. Data are mean±SEM, n=4, $^{*}P<0.05$ vs mitoTERT-myc + H_2O_2, $^{**}P<0.05$ vs mitoTERT-myc+H_2O_2.

cells with low doses of ethidium bromide (EtBr). Expression of TERTwt-myc completely rescued the loss of mtDNA content observed in control transfected cells. This effect was even more pronounced in cells expressing mitoTERT-myc (Figure 5A).

We previously described that H_2O_2 induces apoptosis in endothelial cells.[22] Overexpression of TERTwt-myc in HUVECs showed a slight but significant inhibition of H_2O_2-induced apoptosis, whereas mitoTERT-myc completely abrogated H_2O_2-triggered apoptosis (Figure 5B). These data suggest that protection of mtDNA integrity, mitochondrial function, and apoptosis protection by TERT can be mainly ascribed to the mitochondrial function of this protein.

Discussion

Our results presented here demonstrate that a large portion of endogenous mitochondrial TERT is localized in the mitochondrial matrix. TERT binds to mtDNA and thereby protects mtDNA from damage. Concomitantly we observe a reduction in the formation of mitochondrially produced ROS. Overexpression of mitochondrially targeted TERT is more protective than TERT wild-type against EtBr-induced DNA damage and ROS-induced apoptosis. Strikingly, the protective function of TERT was also evident in vivo from the fact that heart mitochondria from $TERT^{-/-}$ mice exhibited significantly reduced respiratory chain activity.

Previous studies have shown that TERT contributes significantly to processes of aging by protecting telomeres from critical shortening and by inhibiting apoptosis and decreasing oxidative stress. At the same time, oxidative stress and mitochondrial dysfunction have been demonstrated to be important players of aging processes. Our study now shows that mitochondrial TERT inhibits formation of mitochondrially produced ROS and can contribute to increased energy metabolism. Thus, it is tempting to speculate that not only nuclear TERT has an impact on aging processes, but also mitochondrially localized TERT. A reduction in mitochondrially produced ROS has recently been demonstrated to increase the lifespan of mice. Schriner et al produced mice that overexpressed mitochondrially targeted catalase as well as nuclear targeted catalase and showed that in both mice strains catalase was able to reduce H_2O_2, but only mice which overexpress mitochondrially targeted catalase exhibited an increased lifespan compared to their wild-type littermates.[3] Likewise, overexpression of manganese superoxide dismutase, a mitochondrially localized antioxidative enzyme reducing $O_2^{-\cdot}$ to H_2O_2, increased the lifespan of adult *Drosophila melanogaster*.[25] However, recent studies on mice deficient for the mitochondrially localized DNA polymerase γ (Pol-γ) did not find an increase in oxidatively damaged proteins or a reduction in mitochondrial aconitase activity, a classic marker for oxidative damage of proteins. Nevertheless, an accumulation of mitochondrial mutations and the development of many of the phenotypes characteristic of human premature aging were reported.[26,27] Thus, it was concluded that the Pol-γ–deficient mice exhibited an aging phenotype in the absence of enhanced ROS production. However, it was also demonstrated that embryonic fibroblasts of the Pol-γ–deficient mice show a 95% reduction in oxygen consumption,[27] which would suggest that the extensive mutations lead to a complete breakdown of the respiratory chain. This would exclude the formation of mitochondrially produced ROS in these mice. Therefore, these data do not prove that mitochondrially produced ROS are not involved in aging processes.

Recently, it has been reported that flavine-mononucleotide in complex I of the respiratory chain is the major site where ROS are produced.[7,8] Our data now demonstrate that TERT protects the genes for subunits ND1 and ND2 in the mtDNA specifically. Thus, it is tempting to speculate that an increase in ROS production by complex I leads to more damaged mtDNA spurring a vicious cycle finally resulting in dysfunctional respiratory chain and mitochondria. Mitochondrial TERT may counteract ROS production by complex I by binding to the ND1 and ND2 genes, thereby increasing the synthesis of functional complex I subunits which in turn can reduce the formation of damaged complex I that leaks electrons onto oxygen. Unfortunately, too little is still known about the structure and function of complex I.[28] Therefore, we do not know whether protection of ND1 and ND2 subunit genes by TERT is a key prerequisite for reducing complex I produced ROS.

Several studies discuss an important role for apoptosis in the process of aging and age-related diseases. Here, we demonstrate that TERT protects mtDNA from damage and that mitochondrially targeted TERT is protective against oxidative stress induced damage. Our data could implicate that mitochondrial TERT also plays an important role in apoptotic processes leading to pathological aging. Key processes during the onset of apoptosis are outer mitochondrial membrane permeabilization, release of cytochrome c into the cytosol, and activation of caspases. Of note, Bayir et al demonstrated that a mitochondrial pool of cytochrome c exists which acts as a cardiolipin oxygenase.[29] This cytochrome c/cardiolipin oxygenase is activated during apoptosis, uses ROS, and causes selective oxidation of cardiolipin. The oxidized cardiolipin is required for the release of proapoptotic factors from mitochondria to the cytosol. Thus, a reduction of ROS by mitochondrial TERT could result in a reduced activation of the cytochrome c/cardiolipin oxygenase, reduced oxidized cardiolipin, and therefore inhibition of the release of proapoptotic factors into the cytosol. Taking this into account, it is not surprising that nuclear as well as mitochondrial TERT can inhibit apoptotic processes, because one may speculate that mitochondrial TERT inhibits the formation of ROS by complex I, blocks cardiolipin oxidation and release of proapoptotic factors from the mitochondria, whereas nuclear TERT protects telomeres from shortening, interacts with the antiapoptotic proteins PARP and Akt and is regulated by p53, a known gatekeeper of apoptotic processes.[30–32]

Moreover, it has been shown that cells depleted of mtDNA ($\rho 0$ cells) are resistant to apoptosis induction by several stimuli.[33] Dissipation of mitochondrial potential and release of cytochrome c into the cytosol was absent in these cells.[33] These data clearly demonstrate that mitochondria are necessary for the onset of apoptosis. Similar to these findings, it has been demonstrated that mitochondrial dysfunction is a prerequisite for the onset of aging processes. Therefore, it is tempting to speculate that apoptosis is also a prerequisite for aging processes or that apoptosis and aging processes are tied together. This hypothesis is strengthened by data from Kujoth et al who found that many tissues of Pol-γ–deficient mice show at early age a phenotype of accelerated aging with increased levels of caspase-3 activity and TUNEL-positive cells. Similar to this study in mice, patients who carry a high load of mitochondrial mutations show an increase in TUNEL-positive muscle fibers.[34] We show here that protection of mtDNA by TERT is also associated with a higher protection from apoptosis.

Conclusion/Perspectives

Therefore, one may consider revising the old dogma that only the nuclear function of TERT is important for aging and apoptotic processes. Mitochondrial TERT may also exert a significant contribution to the antiaging and antiapoptosis function of TERT.

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Disclosures

None.

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Forum Review

Well-known signalling proteins exert new functions in the nucleus and mitochondria

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Abstract

One distinguishing feature of eukaryotic cells is their compartmentalization into organelles, which all have a unique structural and functional identity. Some proteins are exclusively localized in a single organelle, whereas others are found in more than one. A few proteins, whose function was thought to be completely understood, were only recently found to be present in the mitochondria. Although these proteins come from diverse functional classes their common new denominator is the regulation of respiratory chain activity. Therefore, this review focuses on new functions of the Signal Transducer and Activator of Transcription 3, originally described as a transcription factor, the most prominent Src kinase family members, Src, Fyn and Yes, which were so far known as plasma membrane-associated molecular effectors of a variety of extracellular stimuli, the tyrosine phosphatase Shp-2 previously characterized as a modulator of cytosolic signal transduction involved in cell growth, development, inflammation and chemotaxis, and Telomerase Reverse Transcriptase, the key enzyme preventing telomere erosion in the nucleus. Their unexpected localization in other organelles and regulation of mitochondrial and/or nuclear functions by them adds a new layer of regulatory complexity. This extends the flexibility to cope with changing environmental demands using a limited number of genes and proteins.

Introduction

One distinguishing feature of eukaryotic cells in comparison to prokaryotes is their compartmentalization into organelles, which are obvious already at the microscopic level. Each compartment or organelle contains a characteristic set of proteins providing it with a unique structural and functional identity. Therefore, proteins, which - with the exception of a few respiratory chain components in the mitochondria – are translated in the cytoplasm, have to be targeted to their place of final destination. Eukaryotic cells have evolved highly specialized mechanisms to perform this task. Most commonly, specific topogenic sequences within proteins are used to target them to a distinct subcellular localization, like the nucleus, mitochondria, peroxisomes and the endoplasmic reticulum, from where they are transported through the Golgi apparatus to become secreted or membrane proteins. All these targeting sequences are characterized by conserved amino acids and are recognized by highly specialized transport complexes, which are required to carry their cargo to the respective organelle. Specific sequences of amino acids can easily be recognized by appropriate analysis software and therefore a large number of programs are available to predict the subcellular localization of a protein based on its primary structure (table 1). However, not all proteins contain such conserved targeting sequences despite a highly specific subcellular distribution (7,8,11,20,21).

Besides proteins that are exclusively localized in a single organelle, others exist, which are present in more than one compartment. One cellular strategy to achieve distribution to several or different locations is to produce different polypeptides possessing or lacking one or the other targeting sequence, either from separate genes or from a single gene by means of alternative transcription or translation initiation, differential splicing or post-translational modification. However, several

proteins possess two targeting signals leading to distribution between several organelles. In these cases, the desired and/or required localization can be achieved by different relative affinities to the transport machineries, accessibility of the targeting signals, incomplete translocation or redistribution via retrograde transport, leakage out of the organelle or active export (for review see (9,23)).

As the distribution of single translation products to more than one destination within the cell is less well understood than targeting to a single compartment, this review will be far from comprehensive, but rather a compilation of a few interesting proteins, for which a role in the nucleus and in the mitochondria has been shown just recently. The aim is not to describe the regulation of targeting of these proteins, but rather their function in different organelles. The major emphasis will be on proteins newly discovered in the mitochondria, which play a role in regulating the electron transfer chain. Mitochondria contain the most reducing compartment, have the highest rate of electron transfer and are highly sensitive to oxidation. They are the most redox-active compartment of mammalian cells, accounting for more than 90% of electron transfer to O_2 as the terminal electron acceptor. Therefore, proteins that have well described functions in other cellular compartments and were recently shown to be involved in the regulation of respiratory chain regulation are in the focus of this review. Specifically, we will discuss functions of 4 proteins in compartments, where they had not been suspected before: 1. the Signal Transducer and Activator of Transcription 3 (STAT3), originally described as a transcription factor 2. the most prominent Src kinase family members, Src, Fyn and Yes, which were so far known as plasma membrane-associated molecular effectors of a variety of extracellular stimuli 3. the tyrosine phosphatase Shp-2 previously characterized as a modulator of cytosolic signal transduction involved in cell growth, development, inflammation and

chemotaxis and 4. Telomerase Reverse Transcriptase (TERT), the key enzyme preventing telomere erosion in the nucleus.

1. Signal Transducer and Activator of Transcription 3 (STAT3)

STATs were originally described as key components of a direct signal transduction pathway from the cell surface to the nucleus in response to cytokines and growth factors. For a long time the tyrosine phosphorylation of STATs by ligand activated receptors has been thought to be an obligatory requirement for dimerization in an active conformation, nuclear import and transcriptional activation (10,25). More recently it has been shown that non-phosphorylated STATs shuttle between the cytoplasm and the nucleus at all times in a constitutive manner and that also these non-phosphorylated STATs can be transcriptionally active, either as homodimers or in a complex with other transcription factors. However, these non-phopshorylated STATs regulate a different set of target genes than their phosphorylated counterparts (36,42) (figure 1).

Lately, new functions for STAT3 outside the nucleus became evident. STAT3 was shown to be present in the mitochondria of cultured cells and primary tissue, although it does not contain a mitochondrial targeting sequence. Immunoprecipitations demonstrated an association with complex I and possibly with complex II of the electron transport chain. On the functional level an influence of STAT3 on the respiratory chain was demonstrated in STAT3 deficient pro-B cells, where the activities of complexes I and II were reduced by 40 and 85 % respectively, although the mitochondrial content in the STAT3 -/- cells was unaltered. These findings were confirmed in hearts of mice with cardiomyocyte specific ablation of STAT3. Reconstitution of STAT3 deficient cells with different STAT3 mutants

specifically targeted to the mitochondria revealed that mitochondrial STAT3 is sufficient to modulate respiratory chain activity and that phosphorylation on serine 727 and a monomeric conformation play a crucial role in this process. In addition, the effects of STAT3 on the respiratory chain were unrelated to its actions as a transcription factor (39). A second report described a function of mitochondrial STAT3 in cellular transformation by the non-tyrosine kinase oncogene Ras (14). Ras mediated transformation in vitro and tumor growth in mice were impaired in STAT3 deficient cells. Mutational analysis demonstrated that the N-terminal DNA binding domain, the Src homology 2 (SH2) domain, phosphorylation on tyrosine 705 and nuclear localization of STAT3 are dispensable for supporting malignant transformation by Ras. In contrast, tyrosine phosphorylation and presence in the nucleus are required for transformation by the tyrosine kinase oncogene v-Src. This newly discovered function of STAT3 was ascribed to its mitochondrial localization accompanied by augmentation of respiratory chain activity, particularly that of complex II and V, and a dependence on phosphorylation of serine 727.

In summary, these reports lead to the conclusion that mitochondrial STAT3 can modulate the activity of the electron transport chain and that the structural requirements are completely different than the ones for transcriptional activation in the nucleus (figure 2).

2. Src, Fyn and Yes kinases

The Src family of non-receptor protein tyrosine kinases consists of at least 9 members some of which like Src, Yes and Fyn are ubiquitously expressed, whereas others show more limited expression patterns (28). In this review we will focus on the most prominent kinases, Src, Fyn and Yes, because they can compensate for each

other. These three kinases are important for the regulation of cell proliferation by modulating cell metabolism, division, survival and migration. Their function as plasma membrane-associated molecular effectors of a variety of extracellular stimuli is well known. However, recent studies demonstrated that at least Src fulfills also important functions in the nucleus and mitochondria.

Changes in the chromatin structure indicative of active or inactive transcription are observed during cell cycle, tumorigenesis and senescence. Increased euchromatic hypocondensation and heterochromatic hypercondensation are detected upon growth factor stimulation. These processes depend on nuclear tyrosine phosphorylation by Src, Fyn and/or Yes, since they are not observed in Src, Fyn, Yes triple deficient mouse embryonic fibroblasts (MEFs) (38). Recently, our group revealed a different cellular function for nuclear Src and Yes in endothelial cells by demonstrating that they contribute to the hydrogen peroxide induced nuclear export of telomerase reverse transcriptase (TERT) (22), which will be discussed in more detail later in this review.

A mitochondrial localization of Src has been demonstrated by several groups (2,26,30). In the experiments described in these publications several complexes of the respiratory chain have been identified as substrates for Src. First, the cytochrome c oxidase, the terminal complex of the electron transport chain was shown to be activated by Src (26). Recently, it has been discovered that Src has also effects on other complexes of the respiratory chain. Arachiche et al. reported an increase of Src activity in response to ATP in rat brain mitochondria. ATP addition induced an autophosphorylation of Src at its catalytic site, which leads to its activation. This activated Src increased the activity of the complexes I, III and IV and decreased that

of complex V (2). Taken together, these data indicate that respiratory chain activity is partially dependent on tyrosine phosphorylation by Src.

3. Protein tyrosine phosphatase Shp-2

The ubiquitously expressed protein tyrosine phosphatase Shp-2 contains two N-terminal SH2 domains and a C-terminal protein tyrosine phosphatase domain. Shp-2 plays an important role in cytosolic signal transduction. It modulates different pathways involved in cell growth, cell development, tissue inflammation and cellular chemotaxis. These cytosolic functions of Shp-2 are well known and reviewed elsewhere (5). However, over the last years also nuclear and mitochondrial functions of Shp-2 have been identified.

In 2002 Chughtai et al. reported a nuclear localization of Shp-2 associated with the signal transducer and activator of transcription 5 (STAT5). After stimulation of mammary cells with prolactin, Shp-2 forms a complex with STAT5, which translocates into the nucleus. Formation of this complex is dependent on tyrosine phosphorylation of STAT5 in response to prolactin. Shp-2 binds to tyrosine phosphorylated STAT5 for which it requires the distal SH2 domain and an intact catalytic center. The nuclear Shp-2/STAT5 complex binds to DNA and regulates transcription of milk protein genes (6), demonstrating a transcriptional regulation by nuclear Shp-2. Given the fact that Shp-2 does not dephosphorylate STAT5 and that binding of Shp-2 to STAT5 is required for keeping STAT5 in its tyrosine phosphorylated and thereby active state, nuclear Shp-2 acts as an enhancer of transcription by binding to STAT5 and not as a phosphatase. Thus, these findings revealed a new phosphatase independent function of Shp-2. In contrast, an inhibition of the transcriptional activity of STAT1 by dephosphorylation of tyrosine and serine

residues of STAT1 in the nucleus by Shp-2 has been shown (41), demonstrating that nuclear Shp-2 can also function as a phosphatase. In line with these findings are data from our group. We demonstrated that hydrogen peroxide induced nuclear export of TERT is dependent on the tyrosine kinases Src and Yes. We identified nuclear Shp-2 as the counterplayer for this export, demonstrating again a phosphatase activity of Shp-2 in the nucleus (22). The maintenance of TERT in the nucleus by Shp-2 under conditions of oxidative stress will be discussed in more detail later in this review.

Recently, a tyrosine phosphatase activity was detected in the mitochondria of rat brains. The responsible phosphatase was identified as Shp-2. It was mainly located inside the mitochondria associated with cristae and intercristal space (31). Arachiche et al. also showed a mitochondrial localization of Shp-2 (2). As mentioned above, the same group also detected the tyrosine kinase Src in the mitochondria and suggested that Src is partially involved in the regulation of the respiratory chain activity. For cytosolic Src and Shp-2 it has long been demonstrated that the two proteins regulate each other in their activity. Therefore, it is tempting to speculate that mitochondrial Shp-2 acts as a phosphatase and inhibits Src activity and is thus also involved in the regulation of respiratory chain activity.

4. Telomerase Reverse Transcriptase (TERT)

Telomeres, the physical ends of the chromosomes, are necessary for their stability and integrity. They are shortened during each cell division. This shortening is counteracted by the enzyme telomerase. Telomerase is a large ribonucleoprotein complex and consists of the reverse transcriptase subunit TERT, which contains the catalytic activity of the enzyme, and the associated RNA component TERC, which

serves as the template for synthesis of the telomeric sequence (15,27). Several telomerase-associated proteins are involved in the assembly and activity of the holoenzyme (figure 3).

TERT is differentially regulated on transcriptional and post-translational levels but also via its localization. One major post-translational event is the phosphorylation of TERT by kinases like Src, Akt, PKC and ERK1/2. The kinase Akt has a dominant role in the activation of TERT (3). Upon phosphorylation of serine 823 in TERT by Akt telomerase activity is increased. Also the binding of HSP90 to TERT is essential for telomerase activity via stabilization of a TERT/Akt/HSP90 complex (19) (figure 4). The localization of TERT is highly regulated. Our group demonstrated that TERT is exported form the nucleus after treatment with tumor necrosis factor alpha in combination with cycloheximide as well as with hydrogen peroxide in a CRM1/ Ran-GTPase dependent manner. This export is mediated by phosphorylation of TERT on tyrosine 707 by Src kinases in several cell types, including endothelial cells (19). During induction of replicative senescence in the latter cell type an increase in active Src kinase phosphorylated on tyrosine 416 is observed, which induces nuclear export of TERT (18). Taking into account that cytosolic Shp-2 and Src kinases can regulate and antagonize each other under certain conditions, we hypothesized that a nuclear Shp-2 also exists in endothelial cells which may counteract the Src kinase dependent nuclear export of TERT. Indeed, ablation of endogenous Shp-2 resulted in increased tyrosine phosphorylation of nuclear TERT and a reduction of telomerase activity in the nucleus. Moreover, overexpression of Shp-2 inhibited hydrogen peroxide induced tyrosine phosphorylation and export of TERT from the nucleus. It has to be noted that this process requires the catalytic activity of Shp-2, since the catalytically inactive

mutant Shp-2(C459S) can not prevent nuclear export of TERT (22) suggesting that either nuclear Src or TERT or both are dephosphorylated by Shp-2 (figure 5).

Interestingly, nuclear TERT was shown to be not only involved in telomere elongation but also in the regulation of apoptosis (13,17,19). The anti-apoptotic capacity of TERT occured within few hours after transfection, which indicates a function independent of direct telomere elongation. Further studies supported telomere independent functions of TERT. In cell culture models the suppression of TERT or TERC in cancer and stem cells has been shown to reduce proliferation and render the cells more vulnerable to apoptosis in a largely telomere length independent fashion (12,34,43). Similar results were obtained by ectopic expression of TERT. Stewart et al. demonstrated that TERT enhances tumorigenesis independent of its telomeric function although the mechanism for this effect is not entirely clear (37). Importantly, Sarin et al. showed that conditional transgenic induction of TERT can activate epidermal stem cells independent of its catalytic function (35). This demonstrated for the first time that TERT has an important telomere independent function in stem and progenitor cells. In line with the emerging non-telomeric functions, Santos et al. showed that telomerase activity and TERT protein can be detected in mitochondria (32,33). Although this came as a surprise to the scientific community, it is supported by the finding that TERT has a N-terminal mitochondrial targeting sequence. In addition, we showed that TERT is imported by the translocases of outer and inner membrane (16) (figure 6). However, the functions that TERT fulfills in mitochondria are still controversial. Santos et al. associated the mitochondrial localization of TERT with an increased apoptosis induction and interpreted this as a potential selective mechanism for the elimination of damaged stem cells (33). Recently our laboratories have contradicted these findings by

demonstrating a beneficial role of TERT within mitochondria (1,16). Independent of each other we found an improved mitochondrial function, decreased apoptosis and reduced mitochondrial reactive oxygen species measured as a decrease in Mitosox fluorescence in cells expressing TERT (figure 6). Furthermore, we demonstrated that TERT directly or indirectly binds to mitochondrial DNA. Moreover, we showed that mouse lung fibroblasts from TERT knockout animals are more sensitive to ultraviolet B (UVB) induced decrease in proliferation and respiration than their wildtype counterparts. UVB radiation causes cell death and DNA damage. It induces the formation of cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts (44). Together with our finding that TERT associates with mitochondrial DNA one could speculate that TERT protects mitochondrial DNA against the deleterious effects of UVB. However, there is accumulating evidence that other mechanisms, such as free radical formation, play important roles in the cellular responses caused by UVB radiation (24). This would offer an additional explanation for the protective function of mitochondrial TERT, which reduces reactive oxygen species in this organelle. We also demonstrated that TERT overexpression activates respiratory chain activity and found that the respiration rate is decreased in heart, but not in liver from TERT knockout animals (16).

In accordance with TERT expressing cells having lower reactive oxidative species levels it has recently been demonstrated that cells and tissues from mice deficient for the RNA component of telomerase (TERC) have an imbalance in their redox systems resulting in higher levels of oxidative stress. Perez-Rivero and colleagues found increased MnSOD level in MEFs and tissues from first generation TERC knockout mice, which do not display telomere shortening, but a decrease in catalase accompanied by a higher oxidative stress and oxidative damage. Elevated reactive

oxygen species were shown by increased dichlorofluorescein diacetate and dihydroethidine fluorescence and oxidative damage of proteins was assessed by quantitation of 4-hydroxynonenal protein adducts. Most importantly, re-introduction of TERC restored the redox balance (29). This in vivo demonstration of a direct relationship between telomerase deficiency and oxidative stress is supported by data from our laboratories. We showed a reduced oxygen uptake in heart tissue from TERT knockout mice and a decreased UVB resistance in lung fibroblasts derived from these animals (16). These data are complemented by our in vitro findings demonstrating a decrease of reactive oxygen species and improvement of mitochondrial function in TERT overexpressing fibroblasts (1). In accordance with this, higher catalase protein levels were found in TERT overexpressing fibroblasts while there was no change in the levels of MnSOD (Saretzki, unpublished data). Moreover, the influence of TERT on heart function has been further investigated in a voluntary running mouse model (40). We showed that physical exercise can stimulate telomerase in the heart and has beneficial anti-aging effects measured by a decrease in senescence-associated markers such as p16, p53 and Chk2. In TERT deficient mice however, the effect of exercise was absent pointing to an important role of telomerase in this process. This leads to the conclusion that the running-induced upregulation of telomerase reduces oxidative stress and thereby may slow down senescence. Given the facts that serum levels of insulin-like growth factor 1 (IGF-1) are increased with voluntary running and that IGF-1 has been shown to activate Akt in cardiomyocytes, we wanted to prove whether increased IGF-1 levels serve as a mediator of increased telomerase activity. Therefore, mice were treated with IGF-1 and as expected, the IGF-1 treatment resulted in an activation of Akt in the heart and a substantial increase of telomerase (40). In addition, an increased

proliferation rate in cardiomyocytes was observed after voluntary running. One possible explanation might be a change in pro-proliferative transcriptional programs due to increased TERT levels, because changes in the cellular transcriptome have been observed upon overexpression of TERT (4). However, it is undeniable that mitochondrial function is required for cardiomyocyte proliferation suggesting that increased TERT levels, which result in enhanced respiratory chain activity, are one of the reasons for cardiomyocyte proliferation.

In conclusion, it is tempting to speculate that nuclear and mitochondrial TERT act in concert to improve cardiomyocyte and thereby heart function.

Conclusion

In this review we have summarized recent evidence for several proteins extending their functions to cellular compartments beyond the ones, which have been textbook knowledge for a long time. These proteins can have similar roles in different organelles or can perform completely different, so far unexpected tasks depending on their subcellular localization. These new functions are not restricted to a specific class of proteins, as they have been described for transcription factors, protein kinases and phosphatases and the only eukaryotic reverse transcriptase, TERT. Interestingly, all these proteins are involved in regulatory processes, which help cells to adapt to changing environmental situations. Thus, one may speculate that such additional functions in other cellular compartments, especially in the mitochondria, are not restricted to the few examples described here, but could be a more general phenomenon, which might have been overlooked in the past. Changing the subcellular distribution of a particular protein and thereby sometimes making use of other functional properties, adds a new layer of complexity in addition to the well

described regulatory processes on the transcriptional, translational or post-translational levels. Thereby cells, organs and whole organisms would extend their flexibility to cope with changing environmental demands using a limited number of genes and proteins.

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Abbreviations: Chk2: checkpoint kinase 2; CRM1: chromosome region maintenance 1; ERK: extracellular regulated kinase; Hsp90: heat shock protein 90; IGF-1: insulin-like groth factor 1; MEF: mouse embryonic fibroblast; MnSOD: manganese superoxide dismutase; PKC: Protein Kinase C; SH2: Src homology 2; STAT: signal transducer and activator of transcription; TERT: telomerase reverse transcriptase; UV: ultraviolet.

Author Disclosure Statement

The authors have nothing to disclose.

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Figure legends

Figure 1: Nuclear functions of STAT3

STAT3 is tyrosine phosphorylated by Janus kinase activation (Jak) in response to cytokine binding to the corresponding receptor. Phosphorylation of tyrosine 705 leads to nuclear translocation of the STAT3 protein. Dimerized, phosphorylated STAT3 causes transcriptional activation of a specific set of target genes (X). Non-phosphorylated STAT3 can shuttle between the cytoplasm and nucleus. Dimers of unphosphorylated STAT3 activate a different set of target genes (Y) than phosphorylated dimers.

Figure 2: Mitochondrial functions of STAT3

Phosphorylation on serine 727 is required for STAT3 translocation to the mitochondria. Here, monomeric STAT3 binds to complexes of the respiratory chain and thereby enhances their activities. This newly discovered function is unrelated to its actions as a transcription factor. Mitochondrial STAT3 is also required for malignant transformation induced by the proto-oncogene Ras.

Figure 3: Nuclear function of TERT

Telomerase is composed of the catalytical subunit telomerase reverse transcriptase (TERT) and the telomerase RNA component (TERC). TERC binds to the telomeric repeats ($TTAGGG_n$) and thus serves as a template for the reverse transcriptase. Several telomerase binding proteins are involved in the assembly and activity of the holoenzyme.

Figure 4: Regulation of nuclear TERT tyrosine phosphorylation by Src and Shp-2

Active Src, phosphorylated on tyrosine 416, phosphorylates TERT on tyrosine 707, leading to nuclear export of TERT. This is counteracted by catalytically active Shp-2 (C459), which either dephosphorylates TERT directly or indirectly through inactivation of Src by dephosphorylation.

Figure 5:

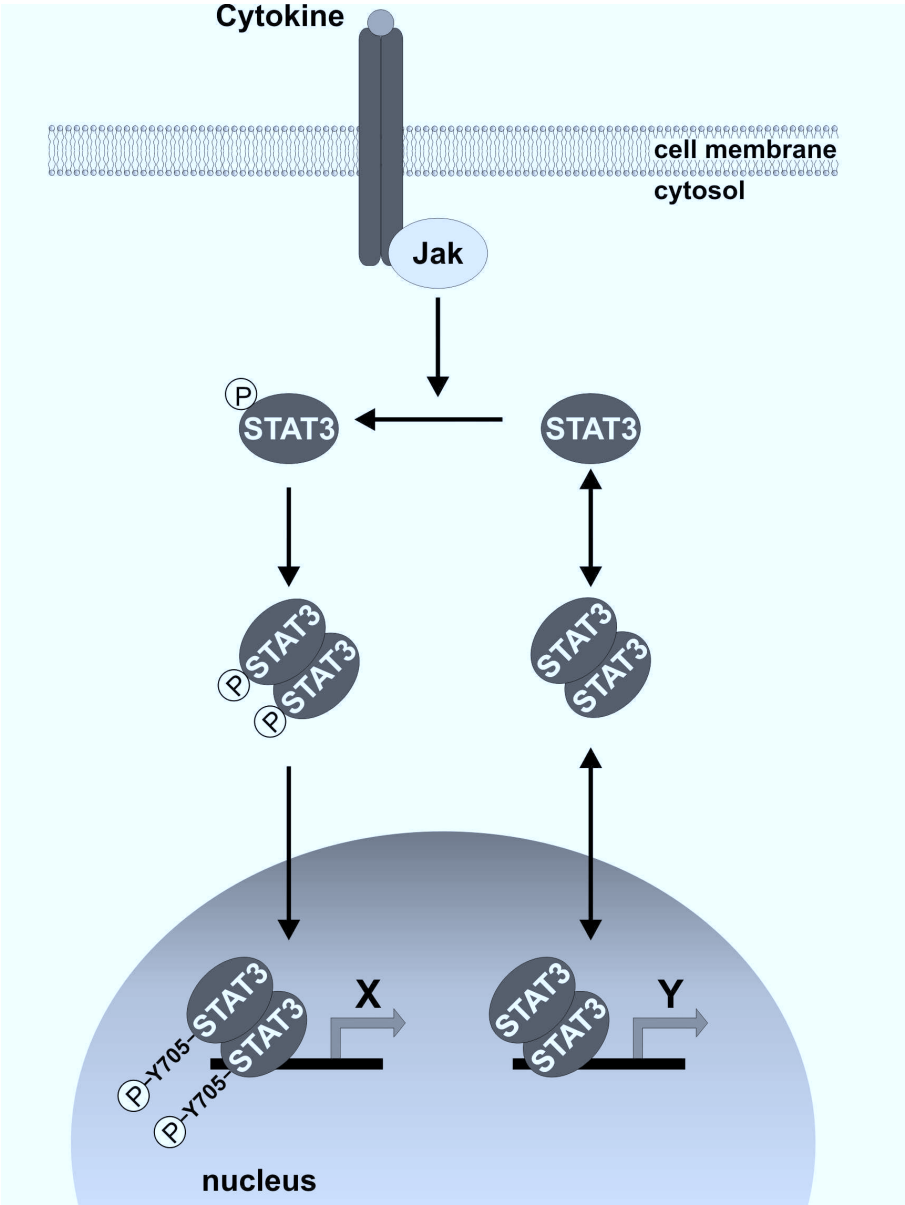
In the nucleus TERT forms a complex with Akt and Hsp90, which keeps TERT phosphorylated on serine 823 and therefore in its active state. Active nuclear TERT prevents telomere erosion and can inhibit apoptosis. Under conditions of oxidative stress Src kinases induce phosphorylation of nuclear TERT on tyrosine 707 resulting in nuclear TERT export via the nuclear pore in a CRM1/RanGTP dependent manner. Protein tyrosine phosphatase Shp-2 inhibits phosphorylation and TERT export.

Figure 6:

TERT is imported into the mitochondria via the translocases of outer and inner membrane (TOM, TIM). Here, TERT is located in the matrix and can bind mitochondrial DNA (mtDNA). Mitochondrial TERT improves mitochondrial function, decreases apoptosis and lowers ROS levels in the cell.

Table 1: Publicly available programs for the prediction of protein localization or specific targeting sequences

program	prediction	homepage	ref.
WoLF PSORT	subcellular localization	http://wolfpsort.org	(21)
TargetP	subcellular localization	http://www.cbs.dtu.dk/services/TargetP/	(11)
MultiLoc TargetLoc	subcellular localization	http://www-bs.informatik.uni-tuebingen.de/Services/MultiLoc/	(20)
Mitoprot	mitochondrial targeting sequence and cleavage site	http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html	(7)
SignalP	signal peptide	http://www.cbs.dtu.dk/services/SignalP/	(11)
PredictNLS	nuclear localization sequence	http://cubic.bioc.columbia.edu/services/predictNLS/	(8)


Cytokine
cell membrane
cytosol
Jak
P
STAT3
STAT3
P
STAT3
P
STAT3
STAT3
STAT3
P-Y705-
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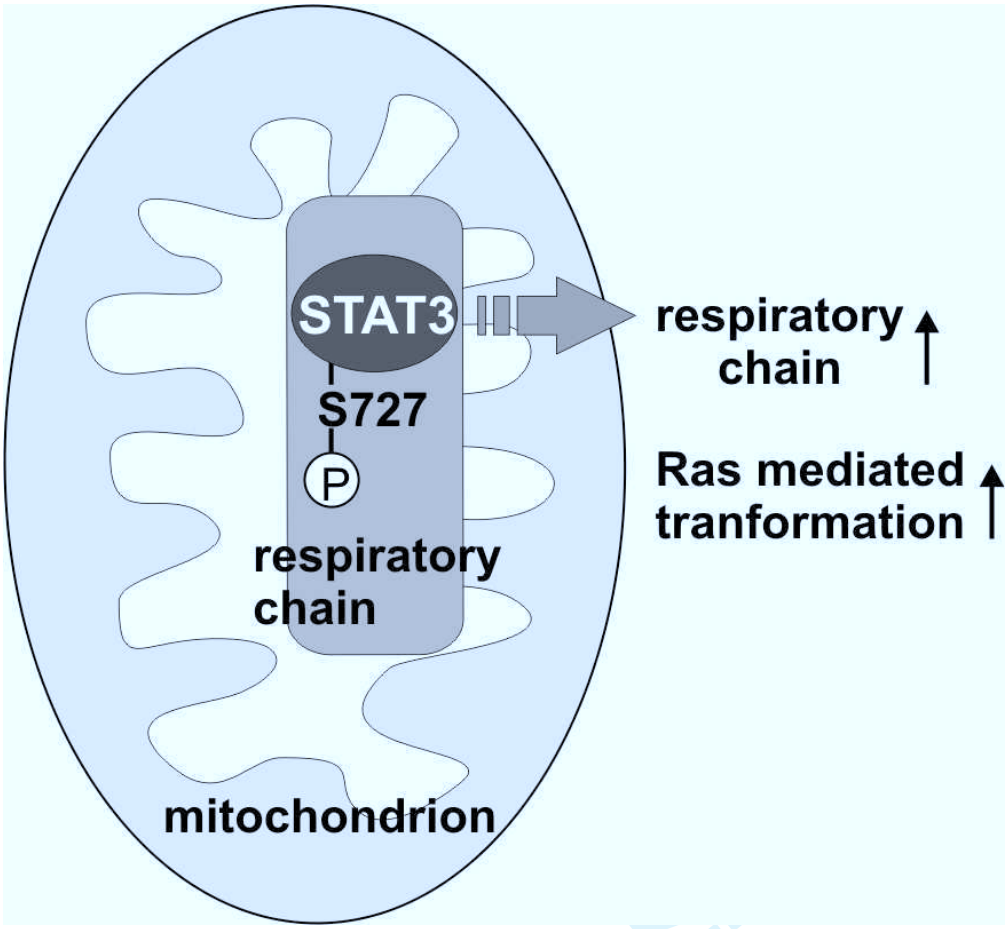
P-Y705-
STAT3
X
STAT3
STAT3
Y
nucleus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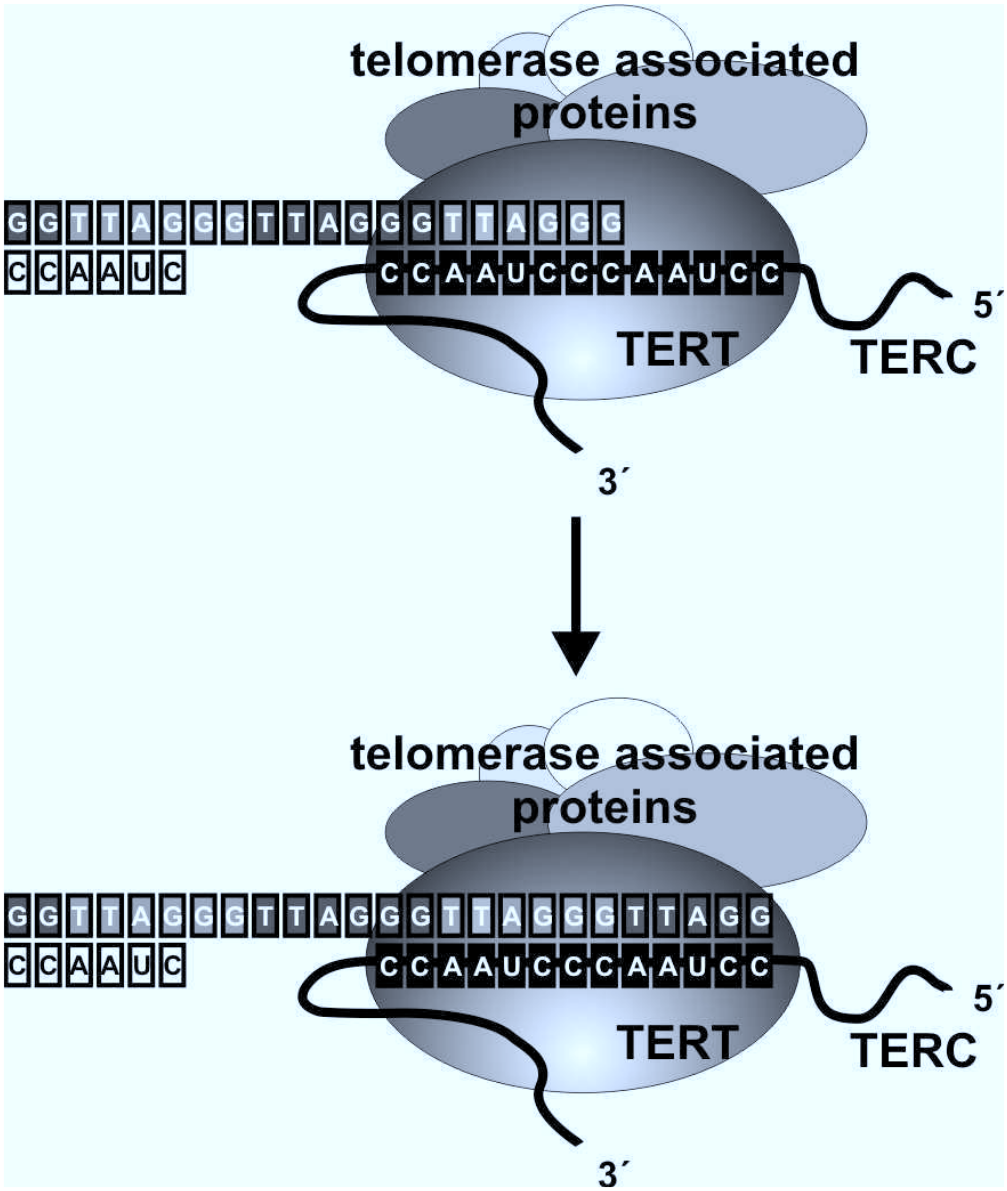
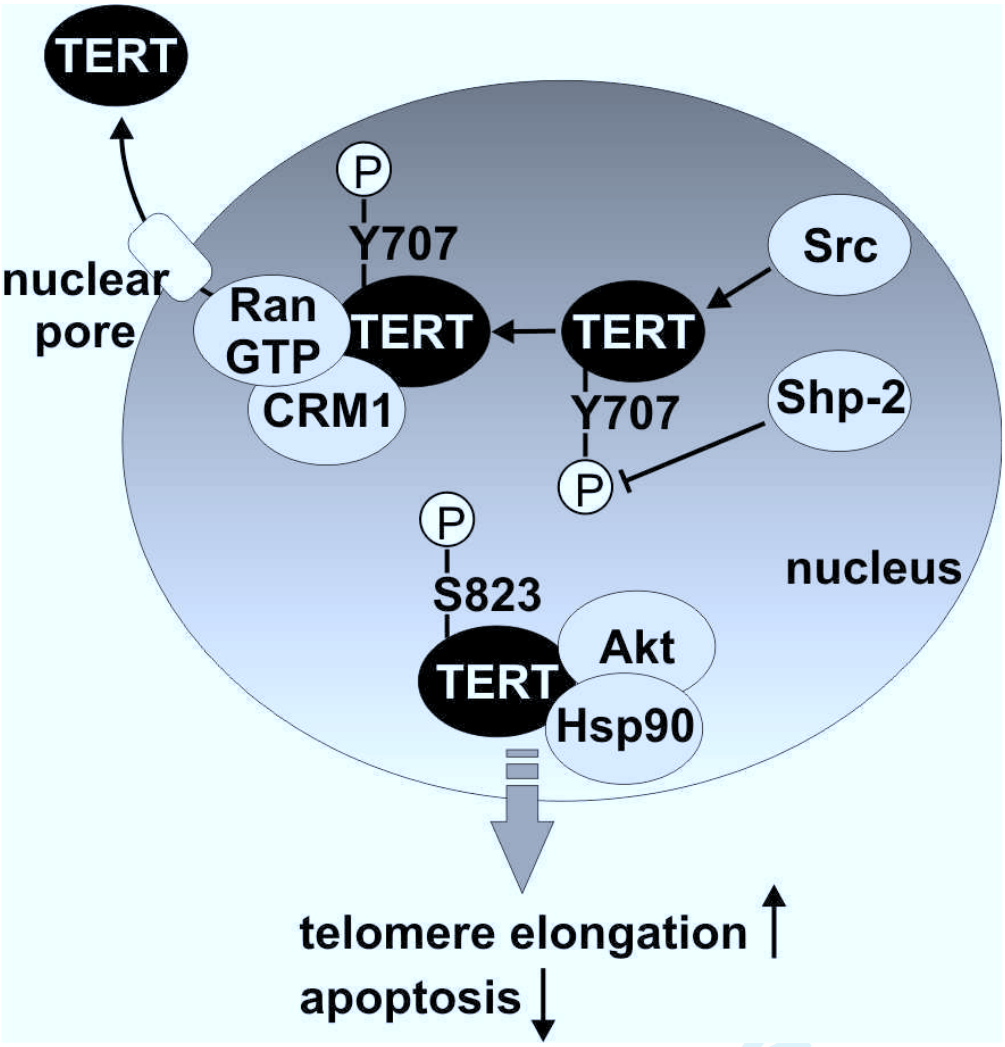
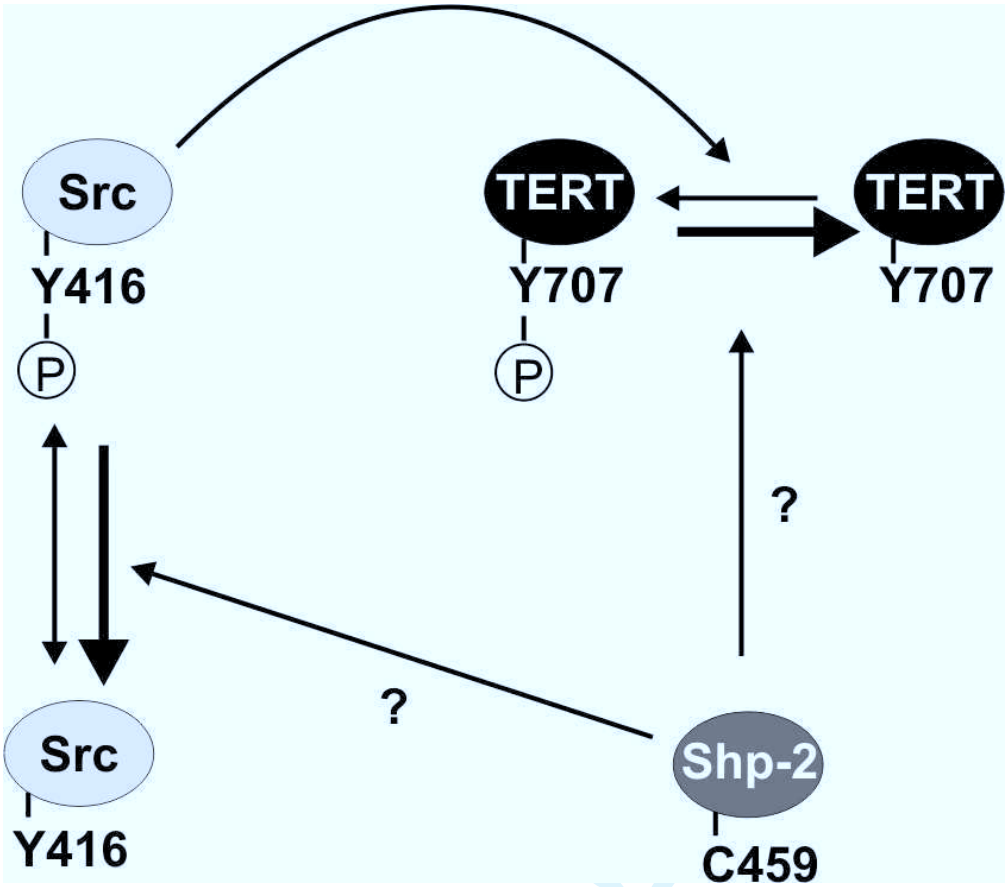
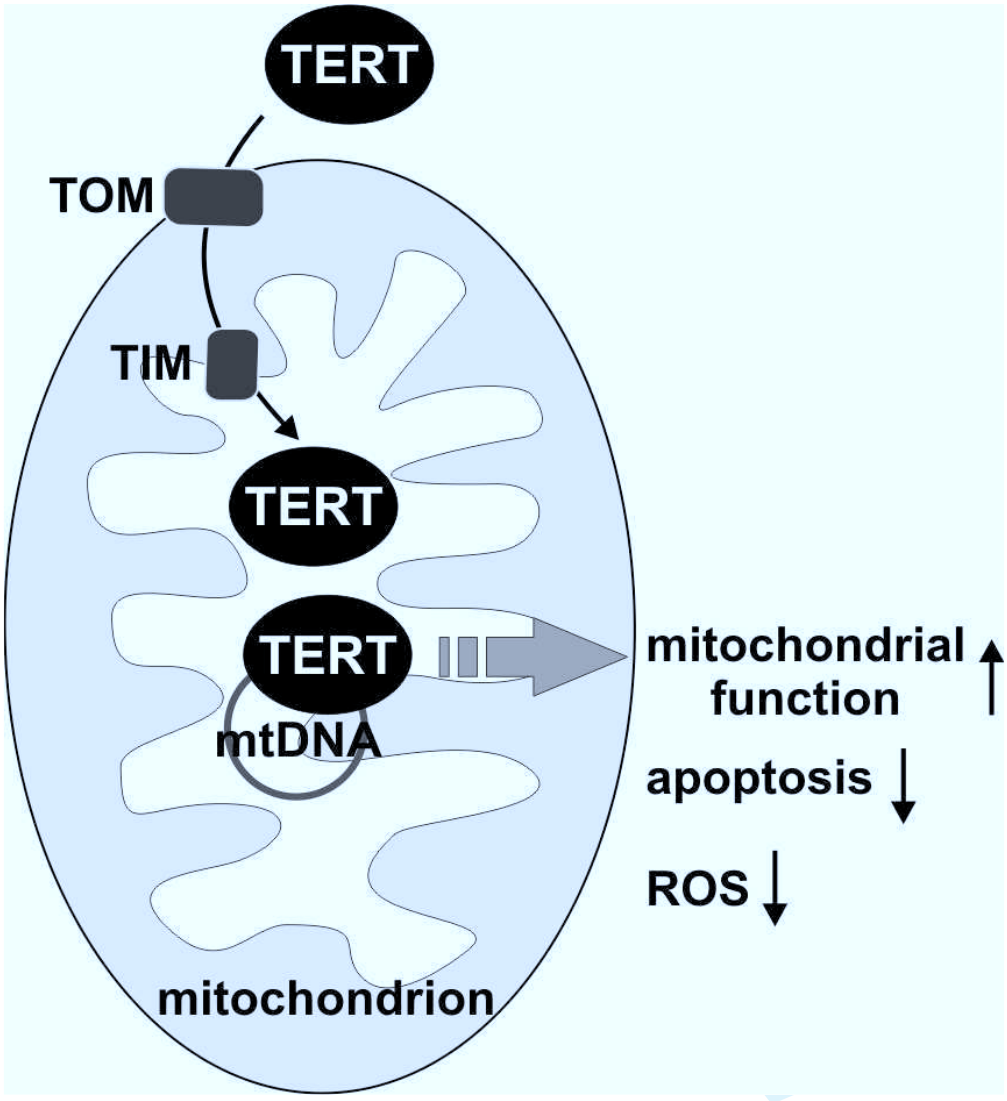
S727
P
respiratory chain
mitochondrion
respiratory chain
Ras mediated tranformation


telomerase associated proteins
GGTTAGGGTTAGGGTTAGGG
CCAAUC
CCAAUCCCAAUCC
TERT
TERC
5´
3´
telomerase associated proteins
GGTTAGGGTTAGGGTTAGGGTTAGG
CCAAUC
CCAAUCCCAAUCC
TERT
TERC
5´
3´


TERT
P
Y707
nuclear
pore
Ran
GTP
CRM1
TERT
TERT
Src
Y707
Shp-2
P
P
S823
nucleus
Akt
TERT
Hsp90
telomere elongation
apoptosis


Src
Y416
P
TERT
Y707
P
TERT
Y707
?
?
Src
Y416
Shp-2
C459


TERT
TOM
TIM
TERT
TERT
mtDNA
mitochondrial function
apoptosis
ROS
mitochondrion

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