Rolle von neuen und bekannten Interaktionspartnern von Thioredoxin-1 für die Endothelzellfunktion

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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aus Remscheid

Düsseldorf, Mai 2012

aus dem IUF – Leibniz-Institut für umweltmedizinische Forschung

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Referent: PD Dr. Judith Haendeler
Korreferent: Prof. Dr. William Martin

Tag der mündlichen Prüfung:

Erklärung

Hiermit erkläre ich, die vorliegende Dissertation eigenständig und ohne unerlaubte Hilfe verfasst und diese in der vorgelegten oder ähnlichen Form noch bei keiner anderen Institution eingereicht zu haben. Alle Angaben, die veröffentlichten Schriften entnommen sind, wurden als solche gekennzeichnet.

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(Tim-Christian Zschauer)

Düsseldorf, Mai 2012

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Die Weltbevölkerung, insbesondere die der hoch entwickelten Industrienationen, unterliegt einer ungünstigen demographischen Entwicklung, welche sich in einer zunehmenden Überalterung der Gesellschaft bemerkbar macht. Dies stellt eine große wirtschaftliche Belastung der sozialen Sicherungssysteme und zukünftiger Generation dar. Der Alterungsprozess kann allgemein zusammengefasst werden als fortschreitender Funktionsverlust des Organismus und die zunehmende Schwäche, auf Umweltbedingungen zu reagieren. Dabei ist das Altern begleitet und beeinflusst durch viele verschiedene Einflüsse, wie z.B. Ernährung und physische Aktivität. Einhergehend mit dem Alterungsprozess treten vermehrt Krankheiten in den Vordergrund, welche als Risikofaktor auch die Alterung selbst beinhalten. So sind kardiovaskuläre Erkrankungen seit Beginn des 20. Jahrhunderts in epidemischem Maßstab angestiegen und gelten mittlerweile als die Haupttodesursache sowohl in der westlichen Welt als auch in großen Teilen der Entwicklungsländer. Ein Drittel aller Todesfälle pro Jahr, unabhängig von Geschlecht und ethnischer Zugehörigkeit, lassen sich auf kardiovaskuläre Erkrankungen zurückführen, wobei der Hauptgrund die sogenannte "westliche Lebensart" ist: Die Kombination von physischer Inaktivität und einer fettreichen Ernährung (Gaziano et al., 2006; Murray and Lopez, 1997). Ein wichtiger Bestandteil des Herz-Kreislauf Systems ist das Endothel, die innerste Auskleidung der Blutgefäße. Es bildet eine einschichtige, schützende Barriere aus Endothelzellen, welche das zirkulierende Blut von dem umgebenden Gewebe trennt. Stabilisiert wird das Blutgefäß von einer an die Endothelzellen grenzenden Schicht aus glatten Muskelzellen, die wiederum von einer Bindegewebsschicht umgeben ist. Das Endothel hat mehrere wichtige Aufgaben, zum einen bildet es eine nicht-adhäsive Oberfläche, die eine Anheftung von Leukozyten und die Aktivierung von Thrombozyten verhindert. Es stellt zudem eine selektiv permeable Barriere dar, die eine Versorgung der umgebenden Gewebe mit Nährstoffen und Sauerstoff gewährleistet. Des Weiteren beeinflusst das Endothel den Gefäßtonus durch Bereitstellung verschiedener vasoaktiver Substanzen (Vita, 2011). Die Integrität der endothelialen Zellschicht ist somit eine der Voraussetzungen für ein funktionierendes kardiovaskuläres System. Das Alter wird als unabhängiger Risikofaktor für kardiovaskuläre Erkrankungen angesehen, da mit zunehmendem Alter die Erkrankungen des Gefäßsystems zunehmen. Es konnte z.B. in einer Studie mit Langschwanzmakaken (*Macaca fascicularis*) als Primatenmodel in alten Affen gegenüber jungen Affen eine wesentlich höhere endotheliale Dysfunktion festgestellt werden, welche auf eine erhöhte endotheliale Apoptoserate sowie eine verringerte endotheliale Zelldichte zurückgeführt wird (Asai et al., 2000). Für die Alterung des Endothels

werden vor allem drei wichtige Mechanismen diskutiert, auf die ich in den folgenden Abschnitten näher eingehen werde: Eine verringerte Bioverfügbarkeit von Stickstoffmonoxid (NO), eine Erhöhung reaktiver Sauerstoffspezies (ROS) und der Verlust an Telomeraseaktivität bzw. Telomerase Reverse Transkriptase (TERT) Proteinmenge.

Stickstoffmonoxid

Eine erhebliche Ursache für das Auftreten endothelialer Dysfunktion während des Alterungsprozesses und kardiovaskulärer Erkrankungen ist die verringerte Verfügbarkeit von Stickstoffmonoxid (NO). NO übt im vaskulären System viele verschiedene Funktionen aus, so verhindert es z.B. die Thrombozytenaggregation. Ebenso reduziert NO die Expression von Adhäsionsmolekülen auf der Endothelzelloberfläche und verringert somit die Adhäsion von Leukozyten. Auch die Proliferation der gefäßumgebenden glatten Muskelzellen wird durch NO inhibiert. Einen gefäßerweiternden Einfluss übernimmt NO als Aktivator der löslichen Guanylylzyklase in den benachbarten glatten Muskellzellen, wodurch eine Gefäßrelaxation hervorgerufen wird. Neben diesen zahlreichen Aufgaben, spielt NO noch eine zentrale anti-apoptotische Rolle in Endothelzellen (Hirst and Robson, 2011). NO wird von der Stickstoffmonoxid-Synthase (NOS) produziert. Hierbei katalysiert NOS die Oxidation der Aminosäure L-Arginin unter Verwendung von NADPH und molekularem Sauerstoff zu L-Zitrullin und NO. Es sind drei Isoformen des Enzyms bekannt, die induzierbare NOS (iNOS), die neuronale NOS (nNOS) und die endotheliale NOS (eNOS), welche alle im kardiovaskulären System exprimiert werden (Tsutsui et al., 2010). Sowohl die nNOS als auch die eNOS sind konstitutiv aktiv und produzieren NO in picomolaren Mengen, die iNOS hingegen produziert höhere NO Konzentrationen im nanomolaren Bereich in Makrophagen als Antwort auf Infektionen und Entzündungsreaktionen (Kroncke et al., 1998). In Endothelzellen stellt die endotheliale Isoform der NOS, die eNOS, die wichtigste Quelle des NO dar (Muller and Morawietz, 2009). Die bedeutende Schutzfunktion des NO wird, wie oben bereits angedeutet, zu einem Teil durch eine Inhibition von pro-apoptotischen Signalwegen und Molekülen gewährleistet. Dabei ist vor allem die Proteinfamilie der Caspasen (Cystein-Aspartat-Proteasen), die einen zentralen Bestandteil der Zellantwort auf apoptotische Stimuli darstellt und die Zellen nach Aktivierung irreversibel in den apoptotischen Zelltod führt, ein Ziel von NO. Es wird unterschieden zwischen Initiatorcaspasen und Effektorcaspasen. Initiatorcaspasen aktivieren proteolytisch die Effektorcaspasen, welche wiederum zelluläre Proteine proteolytisch degradieren (Riedl and Shi, 2004). Es konnte gezeigt werden, dass eine S-Nitrosierung von Cystein im katalytischen Zentrum der Caspasen durch NO-freisetzende Substanzen die Enzymaktivität inhibiert

(Mannick, 2007). Es stellte sich heraus, dass eine Aktivierung der Caspase 3 und Apoptoseinduktion in Endothelzellen eine Konsequenz der Inhibition der eNOS ist (Dimmeler et al., 1997; Haendeler et al., 1997). Ein weiterer Faktor, der mit einer endothelialen Dysfunktion im Alterungsprozess in Verbindung steht, ist, das Zytokin Tumornekrosefaktor α (TNFα). So ist die Alterung assoziiert mit einer veränderten Zytokinproduktion und einer erhöhten Menge TNFα im Blut von älteren Menschen (Bruunsgaard et al., 2000). Auch sind gealterte humane Endothelzellen sensitiver gegenüber apoptotischen Stimuli wie TNFα. Hierbei spielt wiederum eine geringere Verfügbarkeit von NO eine bedeutende Rolle. So zeigen gealterte Endothelzellen eine verringerte Menge S-nitrosierter Proteine sowie eine erhöhte Caspaseaktivität einhergehend mit einer verstärkten Apoptoseinduktion durch TNFα (Hoffmann et al., 2001). Es konnte nachgewiesen werden, dass TNFα eine Phosphorylierung an Serin 1179 und somit Aktivierung der eNOS partiell inhibieren kann und somit zu einer geringeren NO-Verfügbarkeit beiträgt (Kim et al., 2001). Weiterhin hat TNFα auf transkriptioneller Ebene einen Einfluss auf die Expression der eNOS, indem es die Aktivität des eNOS-Promotors deutlich reduziert, und damit zu einer geringeren Proteinmenge in gealterten Endothelzellen beiträgt. (Neumann et al., 2004). Ebenso wird die Stabilität der eNOS mRNA, die unter basalen Bedingungen eine Halbwertzeit von 48h aufweist, durch TNFα auf eine Halbwertzeit von nur noch 3h begrenzt (Yoshizumi et al., 1993). Eine erhöhte Menge an TNFα beeinflusst nicht nur die Menge des NO-produzierenden Enzyms eNOS, sondern hat ebenso eine erhebliche Auswirkung auf dessen Substrat L-Arginin welches zur NO-Synthese essentiell ist. So wirkt TNFα in Endothelzellen inhibierend auf die Expression der Argininosuccinat-Synthase, ein Enzym, welches die Reaktion von Aspartat mit Zitrullin zu Argininosuccinat katalysiert (Goodwin et al., 2007). Argininosuccinat stellt eine direkte Vorstufe von L-Arginin dar und limitiert somit dessen Syntheserate. Ein anderes Enzym des Harnstoffzyklus, die Arginase, welches die Hydrolyse von L-Arginin zu Ornithin und Harnstoff katalysiert und somit in direkter Konkurrenz um L-Arginin mit der eNOS steht, wird durch TNFα in Endothelzellen transkriptionell aufreguliert (Gao et al., 2007). Zusammenfassend tragen die erhöhte Menge an TNFα, denen alternde Endothelzellen ausgesetzt sind sowie deren erhöhte Sensitivität gegenüber TNFα mit zunehmendem Alter erheblich zur gesteigerten Apoptose der Endothelzellen bei. Hierbei wird in einem großen Maße die Menge des anti-apoptotisch wirkenden NOs auf mehreren Ebenen reduziert, was die endotheliale Dysfunktion begünstigt.

Reaktive Sauerstoffspezies

Ein weiterer wichtiger Aspekt, der zu einer endothelialen Dysfunktion und Apoptose beiträgt, sind die in alternden Zellen vermehrt vorkommenden reaktiven Sauerstoffspezies (reactive oxygen species, ROS). Bereits 1956 postulierte Harman seine Theorie zur Alterung. Diese besagt, dass es mit zunehmender Alterung in Zellen zu einer Erhöhung an ROS kommt. ROS, welche in fehlgeleiteten Stoffwechselprozessen entstehen, schädigen dann die Zelle und tragen so zu der Alterung selbst bei (Harman, 1956). Ein Anstieg an ROS wurde ebenso in gealterten Endothelzellen festgestellt (Haendeler et al., 2004). ROS bilden eine Gruppe von reaktiven und deswegen oft schädlichen Formen des Sauerstoffes. Hierzu zählen unter anderem das Superoxid-Anion (O_2^-), das hochreaktive Hydroxyl-Radikal (OH^-) und das Wasserstoffperoxid (H_2O_2). Bedingt durch ihre hohe Reaktivität können ROS diverse zelluläre Makromoleküle schädigen. So werden Proteine und Lipide oxidiert und DNA geschädigt, aber auch niedermolekulare Substanzen abgefangen und somit deren Wirkungsweise auf die Zellen verändert (Sies and Cadenas, 1985). Zusätzlich kann das Superoxid-Anion mit NO reagieren, wodurch das membrangängige Radikal Peroxynitrit ($ONOO^-$) entsteht (Ferrer-Sueta and Radi, 2009). Auf der anderen Seite haben ROS auch essentielle Funktionen in der Zelle, meist aufgrund ihrer hohen und schnellen Reaktivität in verschiedenen Signaltransduktionsprozessen (Bae et al., 2011). So können z.B. Phosphatasen und Kinasen aktiviert und deaktiviert werden, Transkriptionsfaktoren reguliert und demzufolge Genexpressionsmuster verändert werden (Lukosz,.., **Zschauer** et al., 2010). Physiologisch vorgesehen bzw. als fehlgeleitete Reaktion entstehen ROS endogen durch diverse Quellen. Hierbei sind als wichtige prooxidative Systeme in Endothelzellen die NADPH Oxidasen (Nox), Xanthinoxidase (XO), entkoppelte eNOS und die Mitochondrien zu nennen. Nox sind Membran-assoziierte Proteinkomplexe bestehend aus mehreren Untereinheiten (Sirker et al., 2011). In Endothelzellen wurden vier verschiedene Mitglieder der Nox-Proteinfamilie nachgewiesen: Nox1, Nox2, Nox4 und Nox5 (Ago et al., 2011). Nox1 ist unter physiologischen Bedingungen nur sehr schwach exprimiert, die membranassoziierte Nox2 ist nachweisbar und die physiologische Rolle der Nox5 im Endothel ist unklar. Am höchsten exprimiert von allen NADPH Oxidasen im Endothel ist Nox4 (Ray and Shah, 2005). Vorläufige Ergebnisse unserer Arbeitsgruppe zeigen zudem einen Anstieg der Nox4 in alternden Endothelzellen (persönliche Kommunikation P. Czypiorski). Im Gegensatz zu anderen Enzymen, bei denen ROS als unerwünschte Nebenprodukte entstehen können, ist die Hauptaufgabe der Noxs die Produktion von Superoxid-Anionen bzw. H_2O_2 (Bedard and Krause, 2007; Takac et al., 2012). Dies geschieht unter physiologischen Bedingungen im Endothel hauptsächlich durch die Nox4 (Bedard and Krause, 2007). Eine höhere Konzentration an TNFα, die, wie bereits erwähnt, während des Alterungsprozesses auftritt,

steigert die Aktivität der Nox4 in Endothelzellen (Yoshida and Tsunawaki, 2008). Ebenso steht das durch die Nox4 vermehrt generierte O_2^- im Verdacht an der Aktivierung der XO sowie an der Entkopplung der eNOS beteiligt zu sein. XO katalysiert die Oxidation von Hypoxanthin zu Xanthin und danach zur Harnsäure unter Generierung von H_2O_2. Ein vermehrtes Auftreten von ROS kann jedoch die XO zu einem O_2^- -produzierenden Enzym verändern. So wurde die XO in diversen kardiovaskulären Erkrankungen, wie z.B. der Arteriosklerose, als wichtige pathologische, endotheliale Quelle des O_2^- charakterisiert (Spiekermann et al., 2003). Unter der Entkopplung der eNOS versteht man einen ähnlichen Enzym-verändernden Prozess der maßgeblich zu einem Anstieg an endothelialem O_2^- beiträgt. Hierbei wird der streng regulierte Elektronenfluss der enzymatischen Reduktion des molekularen Sauerstoffes im Enzym durch eine Oxidation des essentiellen Kofaktors Tetrahydrobiopterin (BH_4) zu Dihydrobiopterin (BH_2) von der katalytischen Reaktion mit L-Arginin entkoppelt. Dadurch zerfällt der stabilisierende Komplex des Sauerstoffes mit dem Häm-Kofaktor im katalytischen Zentrum und O_2^- entsteht. Bei einer Knappheit des Substrates L-Arginin kommt es ebenfalls zu einem Zerfall des Häm-Sauerstoff-Komplexes, da der reduzierte Sauerstoff nicht mit dem Kohlenstoffatom der Guanidin-Gruppe des Substrat L-Arginin reagieren kann und die eNOS zu einem O_2^- anstatt NO produzierendem Enzym wird (Forstermann and Munzel, 2006). Es wird vor allem das übermäßig produzierte O_2^- der Nox4 und 2 und der XO als Oxidans des BH_4 diskutiert, aber auch das $ONOO^-$ aus der Reaktion des O_2^- mit NO stellt eine mögliche Quelle dar (Kuzkaya et al., 2003; Moens et al., 2008). Zusammenfassend stellt eine übermäßige endotheliale Superoxid-Produktion durch die vorgestellten Enzymsysteme eine große Herausforderung für das Überleben der Endothelzellen dar. Gerade in gealterten Endothelzellen, die einer fehlregulierten Zytokinproduktion sowie erhöhten ROS-Mengen ausgesetzt sind, stellt dies einen Teufelskreis dar, da durch die erhöhte Menge an ROS die Verfügbarkeit des BH_4 verringert wird wodurch die eNOS immer stärker entkoppelt wird. Dies führt zu einer endothelialen Dysfunktion durch Limitierung von NO und einer erhöhten Apoptoserate. Mitochondrien sind Zellorganellen, die ebenfalls eine relevante Quelle für intrazelluläre ROS darstellen. Neben ihrer Funktion als Energielieferant der Zellen spielen Mitochondrien auch eine zentrale Rolle in der Regulation der Apoptose (Martinou and Youle, 2011). Die Energiebereitstellung in Form von ATP erfolgt über die mitochondrielle Atmungskette. Hierbei werden in einer mehrstufigen als oxidative Phosphorylierung bekannten Reaktion, die durch vier Enzymkomplexe katalysiert wird, Elektronen von NADH oder FADH auf den finalen Elektronenakzeptor O_2 übertragen, welcher dadurch zu H_2O reduziert wird. Im Verlauf der oxidativen Phosphorylierung wird ein Protonengradient über der inneren Mitochondrienmembran aufgebaut, der genutzt wird um über den Rückfluss der Protonen

vom Intermembranraum durch die ATP-Synthase in die mitochondrielle Matrix ADP zu ATP zu phosphorylieren. Bereits unter basalen physiologischen Bedingungen werden ca. 4% des O_2 nicht vollständig reduziert, sondern O_2^- vornehmlich durch Komplex I und III gebildet (Puddu et al., 2005). Mitochondrien reagieren sehr sensitiv in Bezug auf eine Erhöhung der dort produzierten ROS. So ist bekannt, dass es während des Alterungsprozesses zu einer mitochondriellen Dysfunktion kommt, die charakterisiert ist durch eine erhöhte ROS-Produktion gepaart mit metabolischer Ineffizienz (Seo et al., 2010). Hier scheint die Schädigung der mitochondriellen DNA (mtDNA) von besonderer Bedeutung zu sein, da diese den dort gebildeten ROS der Atmungskette durch die räumliche Nähe in einem besonderen Maße ausgesetzt ist. Dies macht die mtDNA im Gegensatz zu der nukleären DNA wesentlich anfälliger gegenüber oxidativen Schädigungen, auch da sie nicht um Histone, als verpackender Bestandteil des Chromatins, gewunden vorliegt, die dadurch einen zusätzlichen Schutz bilden. Auf der menschlichen mtDNA sind 13 Untereinheiten der Atmungskette kodiert, daher kann eine Schädigung der mtDNA zu einer fehlerhaften Expression führen. Dies äußert sich wiederum in einer erhöhten Ineffizienz der oxidativen Phosphorylierung, da die Komplexe der Atmungskette aus vielen verschiedenen Untereinheiten aufgebaut sind, die durch eine definierte stöchiometrischen Zusammensetzung charakterisiert sind (Mandavilli et al., 2002). Eine fehlerhafte Atmungskettenkomplex-Stöchiometrie führt zu Fehlfunktionen in der Atmungskette und somit zu einer weiteren Produktion mitochondrieller ROS, was wiederum zu Schädigungen der mtDNA führt. So entsteht ein Teufelskreis der sich in einer Mitochondrienfehlfunktion im Alterungsprozess manifestiert. Zusammenfassend hat eine unregulierte ROS-Produktion erhebliche Auswirkungen auf die Funktionalität der Zelle, die sich im Alterungsprozess akkumulieren, diesen beschleunigen können und die Zelle schlussendlich diese Schädigungen nicht mehr kompensieren kann.

Telomerase Reverse Transkriptase

Ein Protein, das in dem Alterungsprozess eine wesentliche Rolle spielt, ist die Telomerase. Sie ist ein Enzymkomplex bestehend aus zwei Hauptkomponenten, der katalytischen Untereinheit Telomerase Reverse Transkriptase (TERT) und einer RNA Komponente (TERC). Die Telomerase wirkt der Verkürzung der Telomere, den Enden der Chromosomen, entgegen. Mit jedem vollendeten Zellzyklus verkürzen sich die Telomere, da die DNA-Polymerase das terminale Ende der Chromosomen nicht replizieren kann. Dies wird als Endreplikationsproblem bezeichnet. Dabei stellt TERC die RNA-Komponente dar, die TERT als Matrize dient (Greider and Blackburn, 1989). Während des Alterungsprozesses

verkürzen sich somit die Telomere proliferierender Zellen, welches ab einer kritischen Telomerlänge zu einem Zellzyklusarrest führt. Dies wird auch als replikative Seneszenz bezeichnet (Hayflick and Moorhead, 1961). Eine Erhöhung der intrazellulären ROS und eine geringere NO-Bioverfügbarkeit während der Endothelzellalterung, führen zu einer Reduktion der nukleären TERT Proteinmenge. Dadurch kommt es auch in nicht proliferierenden Endothelzellen zu einem seneszenten Phänotyp, der sich in einer Erhöhung der dysfunktionalen Endothelzellen ausprägt (Erusalimsky and Skene, 2009). Die katalytische Untereinheit TERT übt außerdem noch weitere telomerunabhängige Funktionen aus. So fungiert TERT als ein Apoptoseinhibitor in einer Reihe von Zellen (Gorbunova et al., 2002; Oh et al., 2001), darunter auch Endothelzellen (Haendeler et al., 2003b). Erhöhte ROS Produktion führt kurzzeitig zu einer Apoptoseinduktion und langfristig zur Seneszenz in Endothelzellen (Haendeler et al., 2003a; Haendeler et al., 2004). In beiden Fällen kommt es zu einem Kernexport von TERT, der durch eine Src-Kinase-Familien abhängige Phosphorylierung an Tyr_{707} vermittelt wird (Haendeler et al., 2004), der in unserer Arbeitsgruppe auf die Kinasen Src und Yes eingegrenzt werden konnte. Als negativer Regulator des Kernexportes wurde in diesem Zusammenhang die Phosphatase Shp-2 identifiziert (Jakob et al., 2008). Vor wenigen Jahren konnte TERT auch in den Mitochondrien von Zelllinien nachgewiesen werden (Santos et al., 2006). In unserer Arbeitsgruppe wurde zum ersten Mal gezeigt, dass TERT in primären Endothelzellen ebenfalls in Mitochondrien lokalisiert ist. Dort bindet TERT an die mtDNA, übernimmt somit eine schützende Funktion der mtDNA gegenüber exogenen Noxen und unterstützt die Aufrechterhaltung der Mitochondrienfunktion (Haendeler et al., 2009). ROS reduzieren die TERT Proteinmenge im Mitochondrium, hierbei kommt es jedoch nicht zu einem Export des Proteins aus dem Mitochondrium, da anders als beim Nukleus keine Proteine aus den Mitochondrien exportiert werden können (Gerdes et al., 2012). Wir konnten in Mitochondrien von primären humanen Endothelzellen ebenfalls die bekannten TERT-regulierenden Proteine identifizieren und zudem beobachten, dass es unter Bedingungen von erhöhten ROS, wie während des Alterungsprozess, zu einer Src-Kinasen-abhängigen Reduktion der mitochondriellen TERT-Menge kommt (Buchner, **Zschauer** et al., 2010). Da mitochondrielles TERT eine wichtige Schutzfunktion für das Mitochondrium und somit für die Zelle darstellt, ist eine weitere Aufklärung der zugrundeliegenden Regulationsmechanismen von großer Bedeutung um auch in der alternden Endothelzelle eine ausreichend hohe mitochondrielle TERT-Proteinmenge gewährleisten zu können. Dabei könnte die Proteinkinase B (Akt) ein vielversprechendes Ziel darstellen, denn TERT wird in Endothelzellen durch einen Komplex mit AKT und dem Hitzeschockprotein 90 durch Phosphorylierung an Ser_{823} aktiviert (Haendeler et al., 2003b). Dennoch müsste diese Aktivierung kontrolliert erfolgen, da TERT

durch AKT in Krebszellen ebenfalls aktiviert wird (Kang et al., 1999) und aktives TERT eine Rolle in der Krebsentstehung spielt (Kyo et al., 2008).

Antioxidative Systeme

Um ROS entgegen zu wirken, hat die Zelle eine Reihe antioxidativer Systeme, welche eine Akkumulation von ROS verhindern können und diese somit auf ein physiologisches Level eindämmen. Es herrscht also ein Gleichgewicht zwischen prooxidativen und antioxidativen Systemen, man bezeichnet dies als kontrolliertes Redoxgleichgewicht. Eine Störung dieses essentiellen Gleichgewichts, sei es durch eine übermäßige ROS-Produktion, wie sie während des Alterungsprozesses auftreten kann, oder durch eine reduzierte antioxidative Kapazität, bezeichnet man als oxidativen Stress (Sies and Cadenas, 1985). Antioxidative Systeme stellen Enzymsysteme oder Substanzen dar, die in der Lage sind ROS zu reduzieren oder zu binden. Hierzu gehören z.B. Katalase, Superoxiddismutase (SOD), Glutaredoxin (Grx), Peroxiredoxin (Prx), Glutathionperoxidase (Gpx), Glutathion und Thioredoxin (Trx-1). Die Aufgabe der SOD ist es O_2^- zu H_2O_2 zu reduzieren, welches dann von der Katalase zu Wasser und molekularem Sauerstoff umgesetzt werden kann. Gpx können diese Funktion ebenfalls übernehmen, indem sie unter Oxidation von Glutathion organische Peroxide und H_2O_2 reduzieren. Glutathion (GSH) ist ein Pseudotripeptid bestehend aus den Aminosäuren Glutaminsäure, Cystein und Glycin und ist in fast allen Zellen in hohen Konzentrationen enthalten. Es bildet einen wichtigen reduktiven und damit antioxidativen Puffer innerhalb der Zelle und liegt zu ca. 90% reduziert als GSH und zu 10% in der oxidierten dimeren Form Glutathiondisulfid (GSSG) vor. Regeneriert wird GSH durch die NADPH-abhängige Reduktion von GSSG durch die Glutathionreduktase. Zudem stellt Glutathion eine zelluläre Reserve der wichtigen Aminosäure Cystein dar, die in vielen Proteinen im katalytischen Zentrum redoxabhängig zur Signaltransduktion beiträgt (Lukosz,..,**Zschauer** et al., 2010). Das schwefelhaltige Cystein ist sehr sensitiv gegenüber oxidativen Prozessen und wird, gerade unter Bedingungen des oxidativen Stresses, zur Cysteinsulfinsäure und –sulfonsäure oxidiert (Wagner et al., 2002). Mit einem benachbarten Cysteinrest kann eine Oxidation zu einer kovalenten Disulfidbrückenbindung führen. Sowohl die antioxidativen Glutaredoxine als auch Peroxiredoxine enthalten mindestens ein Cystein in ihrem katalytischen Zentrum. Glutaredoxine reduzieren Proteine unter Bildung der Disulfidbrückenbindung im eigenen aktiven Zentrum, welche durch Glutathion wieder reduziert und somit das Enzym regeneriert wird (Lillig et al., 2008). Peroxiredoxine hingegen reduzieren eine Reihe von Hydroperoxiden unter Oxidation der Cysteine in ihrem katalytischen Zentrum, werden jedoch zum großen Teil enzymatisch durch das Thioredoxin-

System regeneriert (Chae et al., 1999). Neben der gemeinsamen Detoxifizierung von Hydroperoxiden zusammen mit dem Prx-System, übt Trx-1 eine Vielzahl zusätzlicher wichtiger Funktionen im Zusammenspiel mit anderen Proteinen in der Zelle aus, auf die ich in dem kommenden Abschnitt näher eingehen möchte.

Thioredoxin

Thioredoxin-1 (Trx-1) ist ein 12kDa großes Protein, welches zuerst 1964 von der Arbeitsgruppe um Peter Reichard in *Escherichia coli* entdeckt worden ist. Dabei wurde Trx-1 als Wasserstoffdonor für die Ribonukleotid-Reduktase, einem wichtigen Enzym in der DNA-Synthese des Bakteriums, identifiziert (Laurent et al., 1964). Kurze Zeit später konnte die Aminosäuresequenz mit 104 Aminosäuren entschlüsselt werden. Im Zuge dessen wurde ebenfalls die konservierte redoxregulatorische Domäne mit der Aminosäurenabfolge Cys_{32}-Gly-Pro-Cys_{35} beschrieben, von der wenige Jahre später die 3D-Struktur aufgeklärt werden konnte und die Thioredoxin-Domäne darstellt, welche namensgebend für die Thioredoxin-Proteinfamilie ist (Holmgren, 1968; Holmgren et al., 1975). Trx-1 ist ein wichtiges antioxidatives und redoxregulierendes Protein, das ubiquitär in eukaryotischen Zellen exprimiert wird und eine Vielzahl an unterschiedlichen Aufgaben übernimmt. Dass Trx-1 essentiell ist, wird belegt dadurch, dass Trx-1-defiziente Mäuse bereits während der Embryonalentwicklung sterben (Matsui et al., 1996). Es sind neben Trx-1, welches cytosolisch und unter bestimmten Umständen nukleär lokalisiert ist, zwei weitere Thioredoxine bekannt, nämlich Trx-2, welches in den Mitochondrien zu finden ist und ebenfalls ubiquitär vorkommt (Spyrou et al., 1997) sowie ein Testis-spezifisches Trx (spTrx), welches in Spermatozoen exprimiert wird (Miranda-Vizuete et al., 2001). Eine wichtige Aufgabe von Trx-1 ist es, durch seine redoxaktive Thioredoxin-Domäne Disulfidbrücken in anderen Proteinen zu reduzieren und somit diese zu regenerieren. Dabei kommt es zu einem nukleophilen Angriff des Cys_{32} auf ein Cystein der Disulfidbrückenbindung des zu reduzierenden Proteins unter Bildung eines intermediären, gemischten Disulfids. Die dreidimensionale Umgebung der Thioredoxin-Domäne ermöglicht diesen Initialschritt, da der Schwefel im Cys_{32} aufgrund der stabilisierenden Wirkung von Wasserstoffbrückenbindungen hauptsächlich als Thiolat vorliegt. Das benachbarte Cys_{35} hingegen liegt in reduziertem Trx-1 meist als Thiol vor. Ist ein gemischtes Disulfid gebildet, werden stabilisierenden Wasserstoffbrückenbindungen zu Cys_{35} ausgebildet, die eine Thiolat-Bildung begünstigen. Im weiteren Reaktionsverlauf kommt es zu einem nukleophilen Angriff des Cys_{35} auf das Cys_{32} des intermediären, gemischten Proteindisulfids und zur Freisetzung des reduzierten Zielproteins unter Bildung eines oxidierten Trx-1 mit einer Disulfidbrückenbindung zwischen Cys_{32} und Cys_{35} (Collet and Messens, 2010). Oxidiertes Trx-1 wird durch die Thioredoxin-

Reduktase (TrxR), welche Bestandteil des Thioredoxin-Systems ist, reduziert und somit regeneriert. Dabei werden Elektronen von NADPH durch das Selenocysteinhaltige Flavoprotein TrxR auf das oxidierte Trx-1 übertragen und dadurch die Thiol-Disulfid-Redoxreaktion mit der Elektronenübertragung auf NADPH gekoppelt (siehe Abbildung 1) (Nordberg and Arner, 2001). Somit kann die Anzahl der intrazellulären Disulfidbrückenbindungen, gerade unter oxidativen Stressbedingungen, verringert werden. Neben diesen beschriebenen Aufgaben als Oxidoreduktase übt Trx-1 viele weitere Funktionen in der Zelle aus, die gerade in Endothelzellen von Bedeutung für das Überleben der Zelle selbst sind. Hierbei besonders hervorzuheben sind Interaktionen mit einer Reihe an weiteren Proteinen. Schwerpunkt meiner Dissertation war es neue Interaktionspartner von Trx-1 zu identifizieren und die funktionellen Konsequenzen der Interaktion zu charakterisieren. Daher möchte ich im nächsten Kapitel zunächst einige bekannte Interaktionen und abschließend neue Interaktionspartner und deren physiologische Relevanz in Verbindung mit Trx-1 vorstellen.

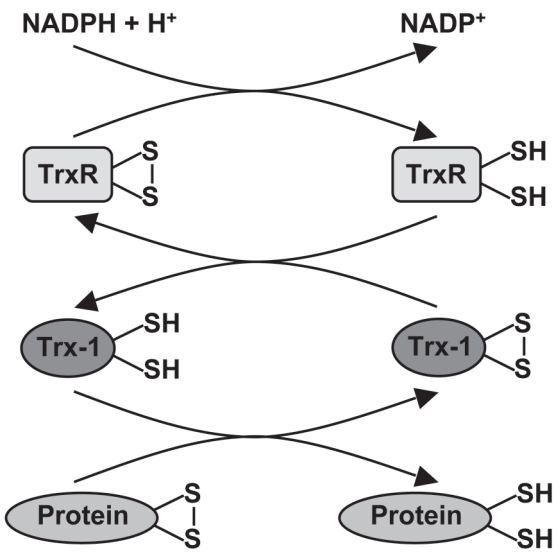

Abbildung 1: Thioredoxin-System. Trx-1 reduziert andere Proteine und wird dabei oxidiert. TrxR reduziert und regeneriert oxidiertes Trx-1 unter $NADPH+H^+$ Verbrauch.

Interaktion von Trx-1 und Proteinen in Endothelzellen

Trx-1, als multifunktionelles Protein, interagiert mit einer Reihe an Proteinen, wobei man als Gemeinsamkeit für die bisher identifizierten Interaktionen eine schützende Funktion für die Zelle nennen kann. Für kardiovaskuläre Zellen von großer Bedeutung ist die Bindung von Trx-1 an die Apoptosis signal-regulating kinase 1 (ASK1), einem Bestandteil des MAP (mitogen activated protein)-Kinase-Signalweges. Es sind drei gut untersuchte MAP-Kinase-Signalwege bekannt, die alle verschiedenste extrazelluläre bzw. intrazelluläre Signale erkennen. Diese Signale werden dann in Signaltransduktionsprozesse eingeleitet und in zelluläre Veränderungen als Reaktionen auf diese umgesetzt. Dabei handelt es sich zum einen um die ERK (extracellular signal-regulated kinase)-Signalkaskade, die hauptsächlich durch Zytokine und Wachstumsfaktoren aktiviert wird und eine Rolle in der Zelldifferenzierung und dem Zellwachstum spielt. Gleichermaßen existieren noch der c-Jun

N-terminal Kinase (JNK) und der p38 MAP-Kinase-Signalweg, die beide durch verschiedene chemische und physikalische Stressfaktoren sowie Zytokine, wie z.B. TNFα, aktiviert werden können. Diese beiden Signalkaskaden regulieren hauptsächlich Stressadaption und Apoptose bzw. Überlebenssignale. Die MAP-Kinasen sind Kinasen, die andere Proteine durch Phosphorylierung aktivieren und somit die extrazellulären bzw. intrazellulären Signale umsetzten. Anfänglich wird eine MAP Kinase Kinase Kinase (MAPKKK) durch einen adäquaten Stimulus, wie z.B. ROS aktiviert. diese phosphoryliert und aktiviert dann eine entsprechende MAP Kinase Kinase (MAPKK), welche wiederum die MAP Kinase (MAPK) durch Phosphorylierung aktiviert. ASK1 ist ein Mitglied der MAPKKK-Proteinfamilie und durch Phosphorylierung und Aktivierung von MAPKK3/MAPKK6 und MAPKK4/MAPKK7 in der Lage, den p38-Signalweg und den JNK-Signalweg zu aktivieren (Ichijo et al., 1997). Damit ASK1 als MAPKKK aktiviert werden kann, muss zunächst ein Homo-Dimer gebildet werden, wodurch eine Autophosphorylierung erst ermöglicht wird und somit ein aktives ASK1-Dimer entsteht. Aktivierte ASK1 induziert Mitochondrien-abhängige Apoptose durch Cytochrom c Freisetzung und aktiviert zudem die Effektorcaspasen 3 und 9 (Hatai et al., 2000). Trx-1 bindet direkt an der N-terminalen Region der ASK1 und verhindert dadurch deren Dimerisierung. Damit stellt Trx-1 einen wichtigen negativen Regulator der ASK1 dar und wirkt durch die Assoziation mit ASK1 anti-apoptotisch. Eine essentielle Voraussetzung für diese Interaktion ist die reduzierte Thioredoxin-Domäne, da oxidiertes Trx-1 nicht in der Lage ist, ASK-1 zu binden. Dies ist einer der Gründe, weshalb oxidativer Stress als starker Aktivator der ASK1 wirkt. ROS, deren Bildung z.B. durch TNFα induziert werden kann, bewirken eine Unterbrechung der Bindung durch Oxidation von Trx-1 und führen somit zur Freisetzung von ASK1, die dann aktiv werden kann und Apoptose induziert (Saitoh et al., 1998). In Bezug auf den Alterungsprozess in Endothelzellen, der charakterisiert ist durch eine Erhöhung der ROS-Spiegel und eine erhöhte Sensitivität gegenüber TNFα, könnte die negative Regulation von ASK1 durch Trx-1, die von entscheidender Bedeutung für das Überleben der Endothelzellen ist, gestört sein. Ebenso ist die Trennung der ASK1-Trx-1 Bindung in einer Reihe von kardiovaskulären Erkrankungen, wie z.B. Arteriosklerose und einer krankhaften Hypertrophie des Herzens, ein Aspekt, der zu einer progressiven Krankheitsentwicklung beiträgt (**Zschauer** et al., 2012). Ein weiteres Protein, das mit Trx-1 direkt interagiert und dessen Interaktion ebenfalls einen erheblichen Einfluss auf die Zelle ausübt, ist das Thioredoxin-interacting protein (Txnip). Txnip ist ein 44 kDa großes Protein und gehört zur Arrestin-Proteinfamilie. Txnip wurde ursprünglich in leukämischen Zellen entdeckt, als ein Protein dessen Expression durch 1,25-Dihydroxycholecalciferol (Vitamin D_3) massiv gesteigert wird (Chen and DeLuca, 1994). Wenig später wurde Txnip mittels eines Hefe-Zweihybrid-Systems als ein Bindungspartner von Trx-1 identifiziert. Daher findet man

Txnip in der Literatur auch unter den Synonymen Vitamin-D up-regulated protein 1 (VDUP1) oder Thioredoxin-binding protein 2 (TBP2) (Chen and DeLuca, 1995; Nishiyama et al., 1999). Die Expression von Txnip ist in Endothelzellen gering, wird jedoch durch eine Reihe pathologischer Stimuli stark induziert. So kommt es zu einer vermehrten Transkription und erhöhter Proteinmenge unter hyperglykämischen Bedingungen, wie sie bei vielen älteren an Diabetes erkrankten Menschen vorkommen. Unter physiologischen Bedingungen stellt der normale Blutfluss mit seinen Scherkräften einen inhibierenden Einfluss auf die Genexpression von Txnip dar. Wird der Blutfluss jedoch gestört, wie z.B. durch arteriosklerotische Gefäßveränderungen, wird Txnip im Gegenzug stark erhöht exprimiert (Yamawaki et al., 2005). Eine vermehrte Expression von Txnip führt zu einer verstärkten Interaktion von Txnip und Trx-1. Dabei bilden beide Proteine eine Disulfidbrückenbindung zwischen Trx-Cys_{32} und Txnip-Cys_{247} aus. Zum Ersten ist wiederum das Vorliegen der reduzierten Thioredoxin-Domäne und zum Zweiten eine bereits bestehende Disulfidbrückenbindung zwischen Cys_{63} und Cys_{247} von Txnip Voraussetzung für die Assoziation beider Proteine. Dabei kommt es zu einem nukleophilen Angriff des Cys_{32} von Trx-1 auf das Cys_{247} von Txnip unter Bildung eines gemischten Disulfids. Neben der direkten Inhibition von Trx-1 kommt es auch zu einer Verdrängung von Trx-1 aus dem ASK1-Trx-1–Komplex. ASK1 ist nun in der Lage, wie oben beschrieben, durch Homodimerbildung den p38-Signalweg und den JNK-Signalweg zu aktivieren und somit inflammatorische und apoptotische Signale innerhalb der Endothelzellen zu stimulieren. Lange wurde diese Interaktion mit Txnip als reine Trx-1 Inhibition betrachtet, neuere Forschungsarbeiten zeigen jedoch auch protektive Effekte dieses Komplexes. Physiologische Konzentrationen von H_2O_2 und TNFα führen zu einem Export von Txnip aus dem Nukleus in das Cytoplasma. Dort kommt es zu einer Bindung von Txnip an Trx-1 und zu einer kurzzeitigen Relokalisation des Komplexes an die Plasmamembran, wodurch es zu einer Phosphorylierung und Aktivierung des vaskulären endothelialen Wachstumsfaktorrezeptors 2 kommt. Dieser bildet den Ausgangspunkt für eine Signalkaskade, die in Endothelzellen Überlebenssignale über den Proteinkinase B (Akt) Signalweg vermittelt. Daher ist der Txnip-Trx-1 Komplex unter physiologischen ROS-Bedingungen eine Strategie der Endothelzellen Überlebenssignale zu aktivieren (World et al., 2011). Des Weiteren wirkt Trx-1 nicht nur durch eine direkte Interaktion mit Zielproteinen, sondern ist auch an der protektiven Wirkung von Multiproteinkomplexen beteiligt. Ein Beispiel hierfür ist die kürzlich aufgeklärte nukleäre Relokalisation der Histondeacetylase 4 (HDAC4) in Kardiomyozyten. HDACs deacetylieren Histone an Lysinresten, wodurch das Lysin wieder eine positive Ladung bekommt und besser an das negativ geladene Phosphat-Rückgrat der DNA binden kann. Dadurch wird die Chromatinstruktur kompakter und die Transkription inhibiert. HDACs sind somit an der

Regulation der Genexpression beteiligt. Durch oxidativen Stress kommt es zu einer

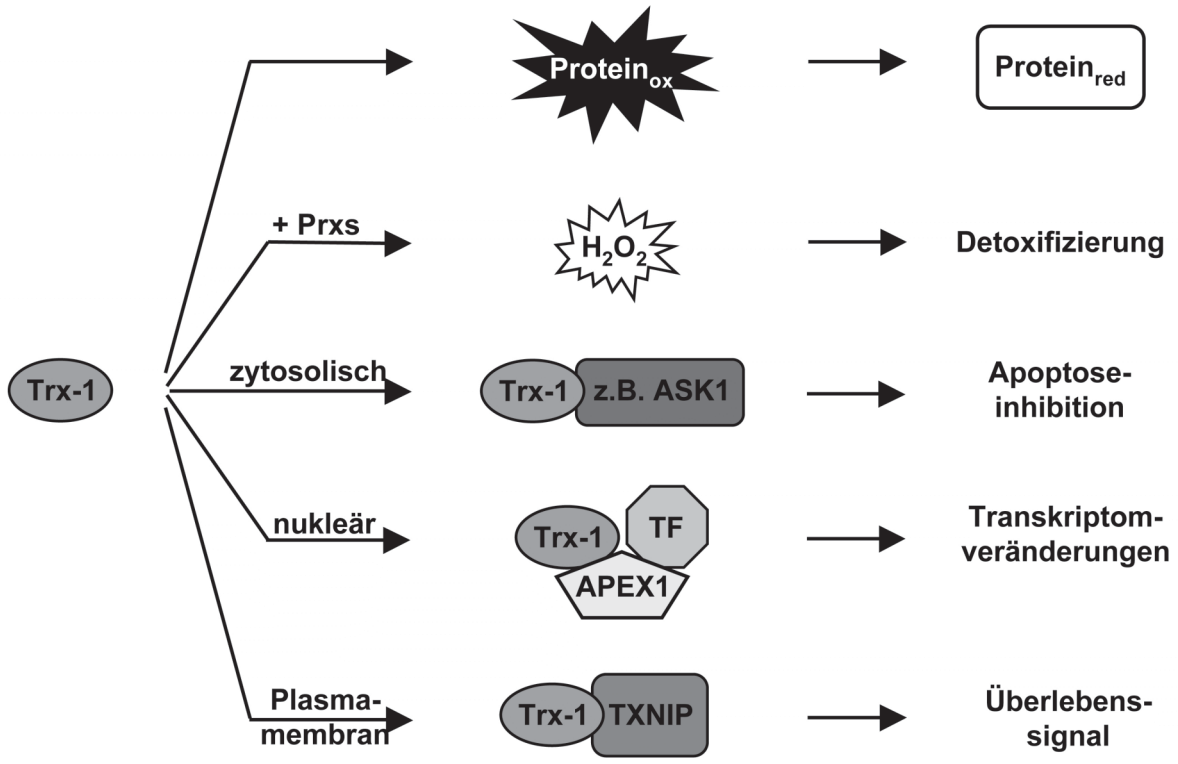

Abbildung 2: Verschiedene Interaktionspartner von Trx-1 und deren physiologische Auswirkung. Trx-1 kann Proteine reduzieren und dadurch wieder aktivieren. Zusammen mit dem Prx-System ist Trx-1 an der Detoxifizierung von Hydroperoxiden beteiligt. Im Zytosol wirkt die Bindung an ASK1 anti-apoptotisch, nukleär werden gemeinsam mit APEX1 Transkriptionsfaktoren reduziert und somit in ihrer Aktivität verändert, Membran-assoziiert wird durch Bindung an Txnip ein Überlebenssignal während oxidativem Stress vermittelt (modifiziert nach (**Zschauer** et al., 2012)). Abkürzungen: Transkriptionsfaktor (TF), reduziert (red) und oxidiert (ox)

Oxidation von HDAC4 unter Bildung einer intramolekularen Disulfidbrückenbindung zwischen Cys_{667} und Cys_{669}. Dieser Oxidation folgt ein nukleärer Export von HDAC4, wodurch die Genexpression hin zu einer krankhaften Hypertrophie der Kardiomyozyten verändert werden kann. Bedingt durch den Kernexport kann HDAC4 nicht mehr an Transkriptionsfaktoren binden und die Transkription ihrer Zielgene inhibieren. Ein Beispiel ist der Myozyten Enhancer Faktor 2 (myocyte enhancer factor 2, MEF2), der nun an Histonacetyltransferasen binden kann, wodurch eine Transkription Hypertrophie-assoziierter Gene, so z.B. atrial natriuretic factor (ANF) and β-myocin heavy chain (β-MHC), ermöglicht wird (Backs and Olson, 2006). Trx-1 steuert diesem Kernexport entgegen, indem es innerhalb eines Multiproteinkomplexes mit dem Hitzeschockprotein DnaJb5 und Txnip die HDAC4 wieder reduziert. Interessanterweise reduziert Trx-1 zunächst eine Disulfidbrückenbindung des Hitzeschockproteins DnaJb5, welches HDAC4 erlaubt an den Komplex zu binden. Nach erfolgreicher Regeneration von Trx-1 reduziert dieses dann im Anschluss HDAC4. Reduziertes HDAC4 kann dann in den Kern re-importiert werden und durch die Interaktion mit MEF2 das ursprüngliche Genexpressionsmuster wiederherstellen (Ago et al., 2008). Neben

diesen erläuterten Interaktionspartnern von Trx-1 sind eine Reihe weiterer Proteine bekannt mit denen Trx-1 ebenfalls in Wechselwirkung tritt und die hier unerwähnt geblieben sind. Aufgrund des großen Einflusses der verschiedenen Trx-1 Interaktionen, war die Aufgabe meiner Dissertation, neue Interaktionspartner von Trx-1 in Endothelzellen zu finden und diese zu charakterisieren. Ebenso sollten bekannte Interaktionen und deren Auswirkungen in Endothelzellen untersucht werden. Die zugehörigen Ergebnisse möchte ich nun in den nächsten Abschnitten vorstellen.

Thioredoxin und Aktin

In Voruntersuchungen unserer Arbeitsgruppe konnte mit Hilfe einer Immunpräzipitation und anschließender Massenspektroskopie gezeigt werden, dass Aktin einen potentiellen Interaktionspartner für Trx-1 darstellt. Bereits vor einigen Jahren konnte in Endothelzellen ein Zusammenhang zwischen oxidativem Stress und dadurch bedingter Aktinfilamentveränderungen und Apoptose hergestellt werden (Huot et al., 1998). Deswegen habe ich die Auswirkungen dieser Interaktion auf die Apoptose der Endothelzellen, Aktin sowie Trx-1 selbst näher untersucht. Aktin ist ein 42 kDa großes Protein und als Bestandteil des Zytoskeletts in allen eukaryotischen Zellen vorhanden. Dabei gehört Aktin mit zu den am häufigsten vorkommenden Proteinen in eukaryotischen Zellen und fungiert hauptsächlich als strukturgebendes, stabilisierendes Protein. Als Eigenschaft von entscheidender Bedeutung ist, dass Aktin sowohl als globuläres Aktin (g-Aktin) als auch als filamentöses Aktin (f-Aktin) in der Zelle vorkommt. F-Aktin fungiert als stabilisierender Teil des Zytoskelettes und besteht aus polymerisiertem g-Aktin. Dabei binden die Aktinmoleküle gerichtet aneinander und geben dem f-Aktin eine Polarität, man unterscheidet so zwischen dem (+) und dem (-) Ende. Diese Polarität hat eine große Auswirkung auf den Aufbau bzw. Abbau der Aktinfilamente. g-Aktin wird am (+) Ende ungefähr zehnmal schneller eingebaut als am (-) Ende, somit entsteht ein gerichtetes Wachstum des f-Aktins. Für die Zelle ist es von ebenso großer Bedeutung, f-Aktin wieder abbauen zu können. Hierfür ist g-Aktin in der Lage ATP zu binden, welches jedoch nicht für die Polymerisation gebraucht wird. Im Aktinfilament wird dann ATP zu ADP+P_i hydrolysiert und g-Aktin kann von dem (-) Ende abdissoziieren, da die Bindungsaffinität zum Aktinfilament für ADP-gebundendes g-Aktin geringer ist als für ATP-gebundendes g-Aktin. Somit existiert ein zelluläres Gleichgewicht zwischen f-Aktin und g-Aktin, welches durch diverse Einflüsse, wie z.B. Aktin-bindende Proteine und Substanzen verschoben werden kann. Um die Interaktion von Trx-1 mit Aktin zu bestätigen, führte ich eine Reihe an Interaktionsstudien mittels Immunpräzipitation aus Proteinlysaten humaner Endothelzellen durch. Hierbei konnte ich in Ko-Immunpräzipitationsexperimenten zeigen,

dass eine Interaktion zwischen den beiden Proteinen existiert. Da Trx-1, Aktin und ASK1 eine wichtige Rolle in der Apoptose der Endothelzellen spielen (Huot et al., 1998; Saitoh et al., 1998), habe ich die Möglichkeit der Ausbildung eines ternären Komplex zwischen Trx-1, Aktin und ASK1 untersucht und weitere Immunpräzipitationsstudien durchgeführt. Mit den Ergebnissen dieser Untersuchungen konnte ich einen Proteinkomplex, in dem alle drei Proteine gleichzeitig vorhanden sind, ausschließen. Aus diesem Grund scheint es in humanen Endothelzellen mindestens drei verschiedene Trx-1 Speicher zu geben: freies Trx-1, Aktin gebundenes Trx-1 und mit ASK1 komplexiertes Trx-1. In weiteren Studien mittels Immunofluoreszenzfärbungen in humanen Endothelzellen konnte eine Ko-Lokalisation von Aktin und Trx-1 beobachten. Interessanterweise erscheint diese in Bereichen der Zelle mit einem hohen Durchsatz an Aufbau und Abbau von Aktinfilamenten, wie z.B. dem Lamellipodium, deutlich intensiver. Diese Beobachtung lässt die Vermutung zu, dass Trx-1 primär mit nicht-polymerisiertem Aktin interagiert. Um diese Theorie zu untersuchen, habe ich humane Endothelzellen mit Cytochalasin D behandelt und anschließend mit den Proteinlysaten Interaktionsstudien durchgeführt. Cytochalasin D ist ein zellpermeabler Pilzmetabolit, der an das (+) Ende eines Aktinfilamentes bindet und somit die Polymerisation des Aktinfilaments verhindert. Durch den weiterhin fortschreitenden Abbau am (-) Ende kommt es zu einer Erhöhung der g-Aktin Menge in der Zelle. Ich konnte mittels Immunopräzipitationen zeigen, dass es zu einer vermehrten Interaktion von Trx-1 mit Aktin in den Cytochalasin D behandelten Endothelzellen kommt. Diese Ergebnisse belegen deutlich, dass Trx-1 mit Aktin in Endothelzellen interagiert und diese Wechselwirkung primär zwischen Trx-1 und g-Aktin vorliegt. Um die physiologischen Hintergründe dieser Interaktion zu charakterisieren, habe ich zunächst die Auswirkung von oxidativem Stress auf das Aktinzytoskelett in Abhängigkeit von Trx-1 untersucht. Es ist bekannt, dass ROS die Bildung von dicken, parallel angeordneten Aktinfilamenten, sogenannten Stressfasern, induzieren (Huot et al., 1998). Bereits eine kurzzeitige H_2O_2 Behandlung führte in humanen Endothelzellen zu deutlich ausgeprägten Aktin-Stressfasern. Im Gegensatz hierzu zeigten Endothelzellen, die vor der H_2O_2-Behandlung mit einem für humanes Trx-1 codierenden Expressionsplasmid transient transfiziert worden waren, diese induzierten Veränderungen im Aktinzytoskelett nicht. Somit inhibiert Trx-1 die H_2O_2-induzierte Stressfaserbildung in humanen Endothelzellen. Damit Aktinfilamente als Antwort auf extrazelluläre Stimuli, wie z.B. Integrin-vermittelte Signale, entstehen können, muss die Focal Adhesion Kinase (FAK) durch Phosphorylierung an Position Tyr_{397} aktiviert werden (Mitra et al., 2005). Durch oxidativen Stress kommt es zu einer Hyperphosphorylierung der FAK und somit zur Ausbildung von Stressfasern (Vepa et al., 1999). Dies kann durch Gabe eines FAK Inhibitors (FAKi) verhindert werden (Slack-Davis et al., 2007).

In weiteren Experimenten wollte ich klären, inwiefern die Bildung der Aktinstressfasern die Apoptose der Endothelzellen und Trx-1 selbst beeinflusst. Hierfür habe ich einen Versuchsaufbau entwickelt, in dem ich unter Verwendung des FAKi die Ausbildung von Aktinstressfasern beeinflussen kann (siehe Abbildung 3). In der als Präinkubation

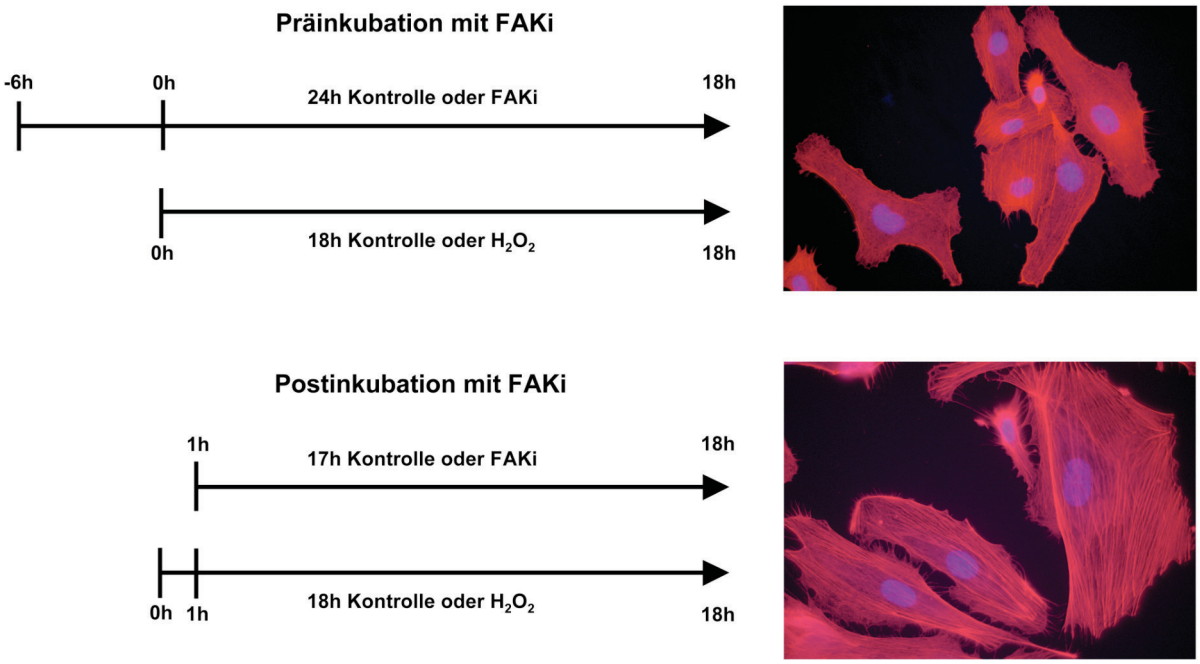

Abbildung 3: Schematischer Versuchsaufbau zur Prä- bzw. Postinkubation der Endothelzellen mit FAKi. Die Präinkubation der Endothelzellen mit dem FAKi vor der H_2O_2-Belastung führte zu keiner Ausbildung der ROS-induzierten Aktinstressfasern, während die Postinkubation der Endothelzellen mit der H_2O_2-Belastung vor der FAKi-Zugabe hingegen eine Ausbildung der Aktinstressfasern zeigte.

bezeichneten Behandlung der Endothelzellen werden diese vor der Inkubation mit H_2O_2 mit dem FAKi vorbehandelt, so dass die Hyperphosphorylierung der FAK unter oxidativem Stress inhibiert werden kann. Mittels durchflusszytometrischer Analysen konnte ich mit H_2O_2 einen signifikanten Anstieg der Apoptoserate feststellen. Ebenso kommt es zur Bildung von ausgeprägten Stressfasern und zu einer Verringerung der Trx-1-Proteinspiegel. Inkubation mit FAKi allein ohne Stimulation der Zellen mit H_2O_2 führte weder zur Apoptoseinduktion und Ausbildung von Stressfasern noch zu einer Abnahme der Trx-1 Proteinspiegel. Interessanterweise konnte ich feststellen, dass es bei H_2O_2-Behandlung nach Präinkubation mit FAKi nicht zu Aktinstressfaserbildung kommt, nicht die Endothelzellapoptose erhöht wird und die Trx-1-Proteinmenge nicht abnimmt. In früheren Arbeiten der Arbeitsgruppe Haendeler wurde eine H_2O_2-induzierte Degradation von Trx-1 durch die Protease Cathepsin D nachgewiesen (Haendeler et al., 2005). Hier konnte ich somit zeigen, dass eine H_2O_2-induzierte Stressfaserbildung eine Vorraussetzung für die Degradierung von Trx-1 und der Apoptoseinduktion der Endothelzellen ist. Bei der Postinkubation hingegen wird FAKi erst nach der Inkubation mit H_2O_2 hinzugegeben, so dass sich Aktinstressfasern ausbilden

können. Im Gegensatz zur Präinkubation kam es bei der Postinkubation mit FAKi zu einer Aktinstressfaserbildung, ebenso wurde weder die Apoptose noch die Degradation von Trx-1 verhindert. Dadurch wird ersichtlich, dass die ROS-bedingte Aktinstressfaserbildung einen unumkehrbaren Punkt in der H_2O_2-induzierten Endothelzellapoptose darstellt. Somit konnte ich zeigen, dass die Ausbildung von Stressfasern irreversibel zur Degradation von Trx-1 und Apoptose der Endothelzellen führt. Die Interaktion von Trx-1 mit Aktin hat eine schützende Funktion für beide Proteine und somit die Endothelzelle, Trx-1 wird nicht abgebaut und Aktin bildet keine Stressfasern aus. Oxidativer Stress führt zu einer Aktinstressfaserbildung, wodurch die Interaktion zwischen Trx-1 und Aktin aufgehoben wird. Dadurch kann das ungebundene Trx-1 durch Cathepsin D degradiert werden, welches wiederum zur Apoptoseinduktion der Endothelzelle führt (siehe Abbildung 4). Eine direkte Bindung von Trx-1 an g-Aktin könnte die Polymerisation des Aktins unter physiologischen Bedingungen verhindern. Ebenso könnte die Bindung der beiden Proteine einen Transport von Trx-1 in die Lysosomen, gefolgt von einem Abbau durch Cathepsin D, inhibieren (Haendeler et al., 2005). Ein weiterer möglicher Mechanismus, wie die Aktinpolymerisation durch Trx-1 blockiert werden könnte ist eine S-nitrosierung des Aktins durch Übertragung der S-Nitroso-Gruppe von Trx-1. Beide Proteine sind als S-nitrosiert beschrieben und interessanterweise führt eine solche Modifikation des Aktins zu einer verringerten Polymerisation und verkürzten

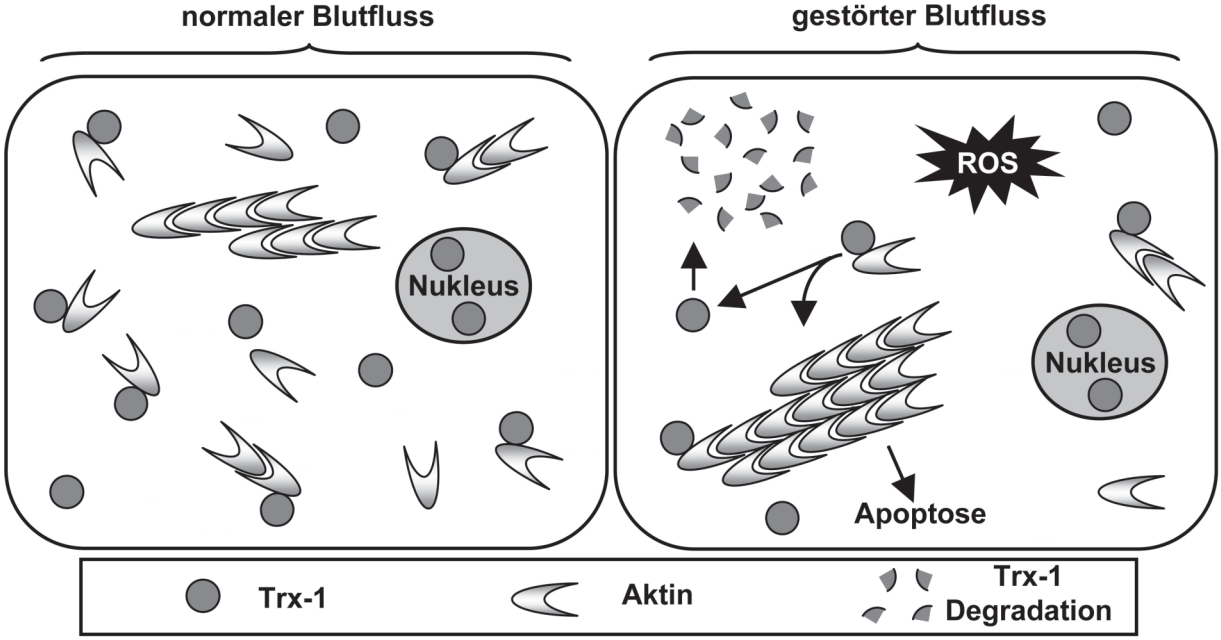

Abbildung 4: Interaktion von Trx-1 und Aktin in Endothelzellen. Unter normalen physiologischen Bedingungen bindet Trx-1 an globuläres Aktin. Diese Bindung schützt Trx-1 vor Degradation, Aktin vor einer Stressfaserbildung und somit die Endothelzelle. Bei oxidativem Stress kommt es zu einer Stressfaserbildung, weswegen die Bindung zwischen Trx-1 und Aktin aufgelöst wird. Freies Trx-1 kann nun durch die Protease Cathepsin D degradiert werden und die Endothelzelle wird apoptotisch (modifiziert nach (**Zschauer** et al., 2012)).

Aktinfilamenten (Dalle-Donne et al., 2000; Haendeler et al., 2002). Zusammenfassend ist daher die Aufrechterhaltung der untersuchten Aktin-Trx-1 Interaktion für das Überleben der Endothelzelle unter oxidativem Stress von entscheidender Bedeutung (**Zschauer** et al., 2011). Denn im Gegensatz zum normalen Blutfluss, unter dem physiologische ROS-Mengen entstehen, kommt es bei gestörtem bzw. turbulentem Blutfluss zu einer drastischen Erhöhung an ROS. Dadurch könnte das alternde Endothel durch die ausgebildeten Stressfasern starrer werden, was ein möglicher Grund für die bekannte Gefäßversteifung im Alter sein kann.

Thioredoxin und Apurinic-apyrimidinic Endonuklease 1

Eine weitere Interaktion, die ich während meiner Dissertation untersucht habe, ist die Interaktion von Trx-1 mit der Apurinic-apyrimidinic Endonuklease 1 (APEX1), einem multifunktionalen Protein. APEX1 übernimmt eine Schlüsselrolle in dem base excision repair (BER)-Signalweg, einem Mechanismus, um abasische DNA-Schäden zu reparieren. Nichtreparierte abasische Positionen führen zu einer genetischen Instabilität und verhindern die DNA-Replikation (Wilson and Barsky, 2001). APEX1 trägt zur Initialisierung der DNA-Reparatur bei, indem es in 5‘-Richtung von der abasischen Position schneidet und dadurch eine normale 3‘-Hydroxylgruppe und ein abasisches Desoxribose-5-phosphat hinterlässt. Zudem rekrutiert APEX1 die DNA Polymerase ß und DNA Ligase 1, welche die DNA-Reparatur durch Einfügen der komplementären Base und Ligation komplettieren (Parsons et al., 2004). Daneben fungiert APEX1 als redoxaktives Enzym und hat sieben konservierte Cysteine (65, 93, 99, 138, 208, 296, 310) in seiner Aminosäuresequenz, von denen die Cysteine 65 und 93 als redoxaktiv beschrieben sind (Walker et al., 1993). APEX1 reduziert vor allem eine Reihe Transkriptionsfaktoren, wodurch deren DNA-Bindungsaffinität erhöht wird (siehe Abbildung 5). Hierzu gehört z.B. der Transkriptionsfaktorkomplex activator protein-1 (AP-1), ein Heterodimer, der sich aus verschiedenen Untereinheiten der c-jun und c-Fos Proteinfamilien zusammensetzt (Vesely et al., 2009). APEX1 reduziert das konservierte Cys_{272} in der DNA-Bindedomäne der c-jun Untereinheit und ermöglicht somit eine stärkere Bindung von AP-1 an die DNA (Xanthoudakis and Curran, 1992). Sobald APEX1 hingegen oxidiert ist, ist die DNA-Bindungsaffinität von AP-1 deutlich verringert. In diesem Zusammenhang konnte interessanterweise gezeigt werden, dass Trx-1 die Fähigkeit besitzt, die reduktive Fähigkeit von APEX1 in HeLa und Cos7 Zellen wiederherzustellen (Hirota et al., 1997). Weitere Studien offenbarten, dass eine nukleäre Lokalisation von Trx-1 und Interaktion mit APEX1 eine Voraussetzung für die reduktive Aktivierung von AP-1 als Antwort auf ionisierende Strahlung in HeLa und Jurkat Zellen darstellt (Wei et al., 2000).

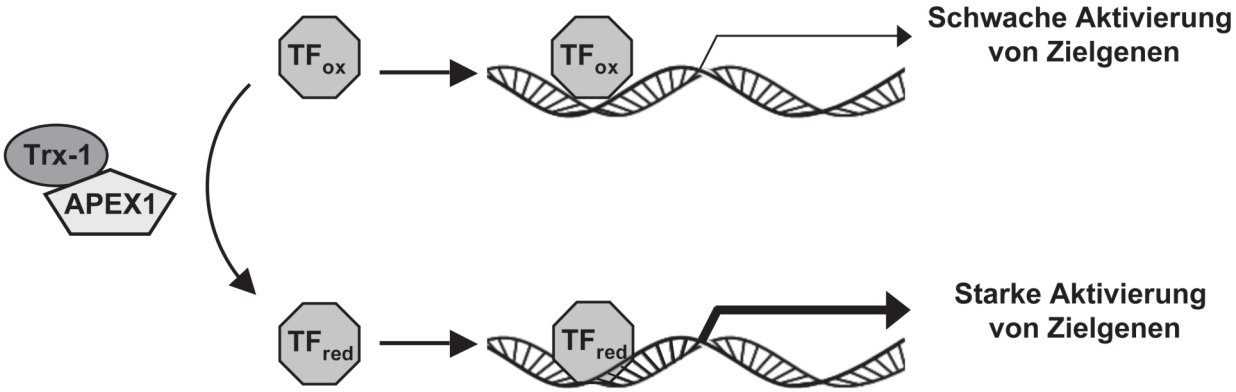

Abbildung 5: Indirekte Regulation von Transkriptionsfaktoren durch APEX1 und Trx-1. Durch die Interaktion von APEX1 mit Trx-1 können oxidierte Transkriptionsfaktoren (TF) reduziert werden. Die Reduktion erhöht die DNA-Bindeeigenschaft des TF und führt zu einer starken Aktivierung von Zielgenen (modifiziert nach (Lukosz,.., **Zschauer** et al., 2010)). Abkürzungen: Transkriptionsfaktor (TF), reduziert (red) und oxidiert (ox)

Ebenso ist eine ähnliche Trx-1/APEX1 Interaktion bekannt, in der Trx-1 gemeinsam mit APEX1 die DNA-Bindung von p53 erhöht und so zu einer erhöhten Expression von p21 in Tumorzellen beiträgt (Ueno et al., 1999). Weitere Studien konnten *in vitro* zeigen, dass sowohl APEX1 als auch Trx-1 allein, die p50-Untereinheit des Transkriptionsfaktors NFκB durch Reduktion aktivieren können. Dies benötigt jedoch hohe Konzentrationen von APEX1 bzw. Trx-1. Interessanterweise erfolgt die reduktive Aktivierung von p50 in Gegenwart der Kombination aus Trx-1 und APEX1 in Konzentrationsbereichen, die alleine keinen Effekt ausüben können (Ando et al., 2008). Diese vorgestellten Studien lassen den Rückschluss zu, dass APEX1 und Trx-1 eine Interaktion mit redoxregulatorischer Eigenschaft eingehen, die durch Aktivierung von Transkriptionsfaktoren zu Transkriptomveränderungen führt (siehe Abbildung 5). Da Untersuchungen in unserer Arbeitsgruppe eine deutliche Verringerung von Trx-1 in seneszenten humanen Endothelzellen zeigen (Altschmied and Haendeler, 2009) und APEX1 ebenso mit zellulärer Seneszenz assoziiert ist (Heo et al., 2009), habe ich die Interaktion der beiden Proteine in humanen Endothelzellen untersucht. Ebenso ist bisher nicht bekannt wie Trx-1 an APEX1 bindet. Da die meisten Studien, welche eine Interaktion von Trx-1 mit APEX1 untersucht haben, *in vitro* Studien sind, habe ich zuerst die Interaktion beider Proteine mittels Immunofluoreszenzaufnahmen analysiert. Ich konnte zwar eine Kolokalisation beider endogenen Proteine in der gesamten Zelle nachweisen, diese erscheint jedoch im Nukleus besonders prominent (Lukosz,.., **Zschauer** et al., 2010). Weiterführend habe ich mittels Immunopräzipitationsstudien eine Interaktion von Trx-1 und APEX1 in humanen Endothelzellen zeigen können. Um eine mögliche Beteiligung von Cysteinen in APEX1 an der Interaktion nachweisen zu können, habe ich Endothelzellen mit einem für humanes APEX1 codierenden Expressionsplasmid mit drei mutierten Cysteinen (C65/93/310S) transfiziert. In diesem sind Cysteine, die an der Bindung von APEX1 an andere Proteine beteiligt sind (Tell et al., 2009), durch Serine ersetzt. Anschließend habe ich

Immunopräzipitationen mit gereinigten cytosolischen sowie nukleären Zellfraktionen durchgeführt. Ich konnte zeigen, dass Trx-1 unabhängig von den Cysteinen 65, 93 und 310 in APEX1 bindet und dass diese Bindung sowohl im Cytosol als auch im Nukleus stattfindet. Im nächsten Schritt habe ich ein Trx-1 Expressionsplasmid, Trx-1 (C32/35S), verwendet, in dem die redoxaktiven Cysteine durch Serine ersetzt sind. Hier konnte ich ebenfalls eine Bindung des mutierten Trx-1 mit APEX1 sowohl im Cytosol als auch im Nukleus nachweisen. Mit diesen Interaktionsstudien konnte ich zeigen, dass die Bindung von Trx-1 und APEX1, sowohl im Cytosol als auch Im Zellkern, unabhängig von Disulfidbrückenbindungen zwischen den Cysteinen C_{32} und C_{35} in Trx-1 und C_{65}, C_{93} und C_{310} in APEX1, ist. Im weiteren Verlauf der Bindungscharakterisierung habe ich Expressionsvektoren für Deletionsmutanten von APEX1 hergestellt. Dabei habe ich zwei Deletionsmutanten generiert, denen die ersten 20 bzw. 60 N-terminalen Aminosäuren mit den in der Literatur diskutierten nukleären Lokalisationssignalen fehlen. Eine weitere Deletionsmutante besteht nur aus den ersten 127 Aminosäuren, die als Redoxdomäne von APEX1 beschrieben ist (Tell et al., 2009). Vorversuche zur Auswirkung dieser APEX1-Deletionsmutanten haben eine antiapoptotische Wirkung der Proteinvariante mit intaktem N-Terminus angedeutet, wohingegen dieser schützende Effekt nicht bei den Mutanten auftritt, deren N-Terminus teilweise oder ganz fehlt. Interessanterweise scheint die anti-apoptotisch wirkenden, C-terminal verkürzte Mutante von APEX1 in Immunofluoreszenzfärbungen immer noch nukleär lokalisiert zu sein.

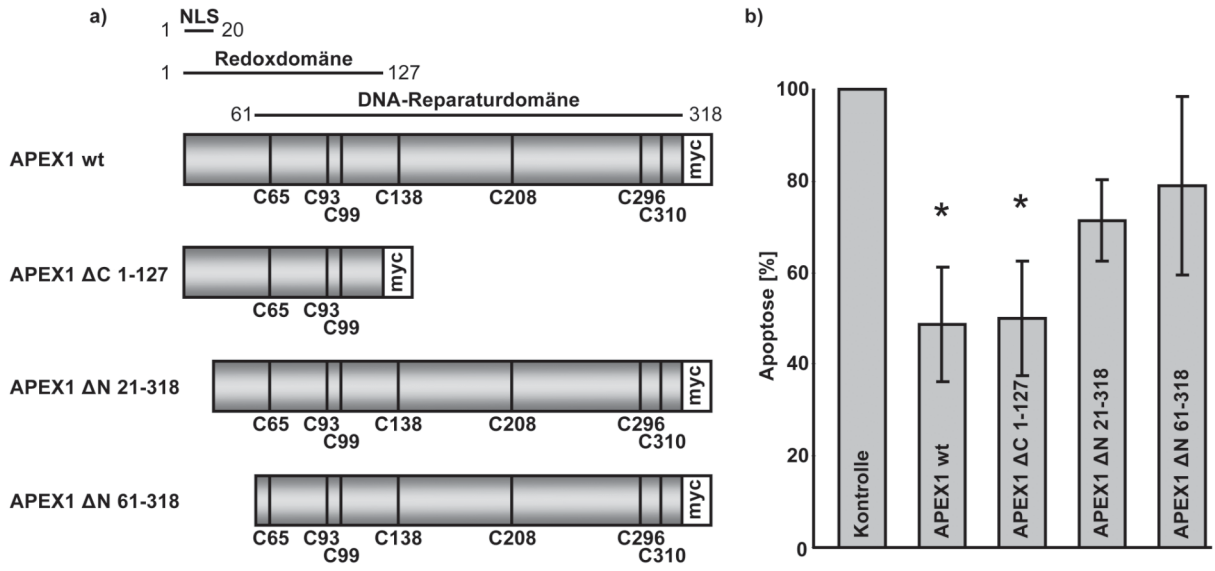

Abbildung 6: Schematische Darstellung der APEX-Deletionsmutanten. a) Übersicht über die generierten APEX1-Deletionskonstrukte, APEX1 wt zeigt die myc-getaggte, nicht deletierte Variante mit dem nukleären Lokalisationssignal (NLS, Aminosäure 1-20), der intakten Redoxdomäne (Aminosäuren 1-127) und der DNA-Reparaturdomäne. APEX1 ΔC 1-127 besteht ausschließlich aus der beschriebenen Redoxdomäne mit intakter NLS. APEX1 ΔN 21-318 bzw. APEX1 ΔN 61-318 fehlen die angegeben Bereiche der N-terminalen Region inkl. der NLS. b) Durchflusszytometrische Apoptosemessung der transient transfizierten Endothelzellen (*p<0,05 vs Kontrolle).

Im Gegensatz hierzu zeigen die Konstrukte ohne N-terminales Ende eine wesentlich verringerte bis keine nukleäre Lokalisation. So könnte eine nukleäre Lokalisation von APEX1 eine Vorraussetzung für die antiapoptotische Eigenschaft von APEX1 sein. Im Zellkern könnte APEX1 im Zusammenspiel mit Trx-1 eine Reihe von Transkriptionsfaktoren reduktiv aktivieren und so zu einem protektiven Genexpressionsmuster beitragen. Im Einklang mit dieser Spekulation ist das Ergebnis, dass die anti-apoptotisch wirkende Deletionsmutante nur aus der Redoxdomäne von APEX1 besteht. Eine Beteiligung der DNA-Reparaturdomäne an der anti-apoptotischen Wirkung wäre somit auszuschließen. Inwiefern die APEX-1 Deletionsmutanten mit Trx-1 in Endothelzellen interagieren können, ist Gegenstand momentaner Untersuchungen.

Ausblick

Neben der Identifizierung von Aktin als einen neuen Interaktionspartner von Trx-1 in Endothelzellen konnte ich zudem eine physiologische Konsequenz dieser Interaktion aufklären. Hierbei kommt es zu einem gegenseitigen Schutz der beiden Proteine vor ROS-bedingten Veränderungen und letztendlich der Endothelzelle vor Apoptose. So wird Trx-1 vor der lysosomalen Degradation durch die Protease Cathepsin D geschützt und Aktin vor einer oxidativ bedingten Ausbildung von Stressfasern (siehe Abbildung 4). Ebenfalls konnte ich die Interaktion von Trx-1 mit APEX1 in humanen Endothelzellen nachweisen. Im weiteren Verlauf der Charakerisierung konnte ich zeigen, dass die Bindung von Trx-1 und APEX1, sowohl im Cytosol als auch Im Zellkern, unabhängig von Disulfidbrückenbindungen zwischen den Cysteinen C_{32} und C_{35} in Trx-1 und C_{65}, C_{93} und C_{310} in APEX1, ist. Als Frage bleibt offen, ob die verbleibenden Cysteine C_{62}, C_{69} und C_{73} von Trx-1 und/oder C_{99}, C_{138}, C_{208} und C_{296} von APEX1 an einer gegenseitigen Disulfidbrückenbindung beteiligt sind. Um im weiteren Verlauf die Interaktionsdomänen, über die eine Bindung erfolgen könnte, zu identifizieren, müssen mit den in Abbildung 6 bereits vorgestellten APEX-Deletionsmutanten Immunpräzipitationsstudien in Endothelzellen durchgeführt und überprüft werden, welche Deletionsmutanten in der Lage sind Trx-1 zu binden. Vielversprechend ist hier die in Vorversuchen gezeigte antiapoptotische Wirkung des APEX1 wt und APEX1 ΔC 1-127, die beide über ein intaktes nukleäres Lokalisationssignal verfügen und deren redoxaktive Domäne ebenfalls vollständig vorhanden ist. So könnte man eine Interaktion von APEX1 mit Trx-1 innerhalb der redoxaktiven Proteindomäne (Aminosäuren 1-127) von APEX1 vermuten, da diese anscheinend die Apoptose von Endothelzellen inhibieren kann. Zudem zeigten immunohistochemische Untersuchungen, dass sowohl APEX1 wt als auch APEX1 ΔC 1-127 im Gegensatz zu APEX1 ΔN 21-318 und APEX1 ΔN 61-318 im Nukleus lokalisiert

sind (nicht veröffentlichte Daten). Da eine reduktive Aktivierung von Transkriptionsfaktoren wahrscheinlich eine der Hauptaufgabe des nukleären APEX1/Trx-1 Komplexes darstellt (siehe Abbildung 5), ist die Eigenschaft von beiden Proteinen vom Cytoplasma in den Nukleus zu translozieren wahrscheinlich eine Voraussetzung dafür und somit eventuell auch für die antiapoptotische Wirkung die von APEX1 wt und APEX1 ΔC 1-127 ausgeübt wird. Falls sich bei einer der Deletionsmutanten zeigt, dass diese nicht mehr an Trx-1 binden kann, wäre die Konsequenz der Überexpression für Endothelzellen unter mechanistischen Gesichtspunkten interessant. Die exakte Bindungsdomäne der beiden Proteine APEX1 und Trx-1 aufzuklären würde vielleicht in der Zukunft zu einer Entwicklung von kleinen spezifischen Peptiden führen, die eine mimetische Funktion ausüben. Dadurch könnte man eventuell die während des Alterungsprozesses verursachten Transkriptomveränderungen durch eine verringerte Interaktion von Trx-1 mit APEX1 aufhalten bzw. umkehren. Eine weitere interessante Forschungsarbeit wäre es weitere neue Interaktionspartner von Trx-1 zu identifizieren und dann die Eigenschaften dieser Interaktion in Endothelzellen aufzuklären.

Zusammenfassung

Physiologische Alterungsprozesse im kardiovaskulären System sind begleitet von einer endothelialen Dysfunktion, die ebenfalls in vielen kardiovaskulären Erkrankungen eine große Rolle spielt. Das Endothel, die innerste Auskleidung der Blutgefäße, zeigt weitreichende Veränderungen im Verlauf des Alterns, die gekennzeichnet sind durch eine verringerte Bioverfügbarkeit von Stickstoffmonoxid und somit einer erhöhten Apoptose. Reaktive Sauerstoffspezies (ROS) tragen wesentlich zur endothelialen Dysfunktion bei. Ein redoxregulierendes Enzym, welches den ROS-Effekten entgegenwirken kann, ist das ubiquitär exprimierte Thioredoxin-1 (Trx-1). Ein wichtiges Strukturelement in Trx-1 ist die Thioredoxin-Domäne mit zwei konservierten Cysteinen (Cys_{32} und Cys_{35}) über die es antioxidativ und anti-apoptotisch wirkt. Trx-1 übt viele seiner schützenden Funktionen durch Interaktionen mit anderen Proteinen aus. So detoxifiziert es zusammen mit Peroxiredoxinen Hydroperoxide, die Bindung von Trx-1 an die Apoptosis signal-regulating kinase 1 (ASK1) verhindert die Induktion der Apoptose durch die ASK1. Zudem reduziert es eine Vielzahl an oxidierten Proteinen und kann im Zusammenspiel mit der Apurinic-apyrimidinic Endonuklease 1 (APEX1) im Nukleus Transkriptionsfaktoren durch deren Reduktion aktivieren. Aufgrund dieser protektiven Einflüsse von Trx-1 in Endothelzellen, war es das Ziel dieser Arbeit neue Interaktionspartner von Trx-1 zu finden und deren physiologische Relevanz zu untersuchen. Ebenso sollten die funktionellen Konsequenzen bekannter Interaktionen charakterisiert werden. Als einen neuen Bindungspartner von Trx-1 konnte ich in humanen Endothelzellen das Zytoskelettprotein Aktin identifizieren. Ich konnte diese Bindung auf eine Interaktion von Trx-1 mit monomerem Aktin eingrenzen. Unter oxidativem Stress kommt es zu Veränderungen des Zytoskeletts in Form einer starken Bündelung von Aktinfilamenten, den sogenannten Stressfasern. In dieser Arbeit konnte ich zeigen, dass die ROS-induzierte Ausbildung dieser Stressfasern eine Voraussetzung für den Abbau von Trx-1 und die Apoptose der Endothelzellen durch ROS ist. So ist anzunehmen, dass die Trx-1/Aktin Bindung beide Proteine schützt, zum Einen Trx-1 durch Schutz vor Degradation und zum Anderen Aktin vor Stressfaserbildung, was in einem Schutz vor Apoptose resultiert. Zusätzlich konnte ich die bekannte Interaktion von Trx-1 mit APEX1 näher charakterisieren. So konnte ich zeigen, dass dieser Komplex ebenfalls in Endothelzellen, sowohl im Cytoplasma als auch im Nukleus existiert und unabängig von den Cysteinen der Thioredoxin-Domäne und den Cysteinen 65, 93, 310 von APEX1 ist. Zudem ist der N-Terminus von APEX1 wichtig für seine anti-apoptotische Wirkung in Endothelzellen.

Summary

Physiological aging processes in the cardiovascular system are associated with endothelial dysfunction that also plays a major role in many cardiovascular diseases. Endothelial cells, the innermost layer of blood vessels, display extensive changes during aging that are linked to the loss of nitric oxide and thus enhanced apoptosis. Reactive oxygen species (ROS) contribute considerably to endothelial dysfunction. One intracellular protein that has been shown to exhibit antioxidative potential is the redox-sensitive oxidoreductase Thioredoxin-1 (Trx-1). Trx-1 is ubiquitously expressed and contains an active site termed Trx-domain with two conserved cysteine residues at position 32 and 35 through which it exerts antioxidative and antiapoptotic functions. Many of these functions are carried out in concert with interacting proteins. Together with the peroxiredoxins Trx-1 detoxifies hydroperoxides. By binding to the apoptosis signal-regulating kinase (ASK1) Trx-1 inhibits the induction of apoptosis. Through the Trx-domain Trx-1 is capable of reducing oxidizied proteins. Upon interaction with apurinic-apyrimidinic endonuclease 1 (APEX1) in the nucleus Trx-1 reduces and activates transcription. Based on these protective effects of Trx-1 the goals of my work were to identify new interaction partners of Trx-1 and characterize their physiological relevance. In the same way it was my ambition to elucidate the functional consequences of known interaction partners in endothelial cells. As a new binding partner of Trx-1 I could identify the cytoskeletal protein actin. Further studies revealed that Trx-1 predominantly interacts with non-polymerized actin. Under conditions of oxidative stress thick bundles of actin are formed, so called stress fibers. I was able to show that ROS-induced stress fiber formation is a prerequesite for Trx-1 degradation and endothelial cell apoptosis. Thus, it can be hypothesized that this interaction results in a mutual protection of both proteins. On the one hand Trx-1 is protected from oxidative stress induced degradation and on the other hand actin stress fiber formation is attenuated resulting in protection of endothelial cells against apoptosis. In addition, I characterized the known interaction of Trx-1 and APEX1. I could demonstrate that this complex also exists in endothelial cells in the cytoplasm as well as in the nucleus. Moreover, binding is independent of disulfide bridge formation between the cysteines of the Trx-domain and the cysteines 65, 93 and 310 of APEX1. Besides that preliminary studies indicated an antiapoptotic function of the APEX1 N-terminus in endothelial cells.

Literaturverzeichnis

Ago, T., Kuroda, J., Kamouchi, M., Sadoshima, J., and Kitazono, T. (2011). Pathophysiological roles of NADPH oxidase/nox family proteins in the vascular system. - Review and perspective. Circ J *75*, 1791-1800.

Ago, T., Liu, T., Zhai, P., Chen, W., Li, H., Molkentin, J.D., Vatner, S.F., and Sadoshima, J. (2008). A redox-dependent pathway for regulating class II HDACs and cardiac hypertrophy. Cell *133*, 978-993.

Altschmied, J., and Haendeler, J. (2009). Thioredoxin-1 and endothelial cell aging: role in cardiovascular diseases. Antioxid Redox Signal *11*, 1733-1740.

Ando, K., Hirao, S., Kabe, Y., Ogura, Y., Sato, I., Yamaguchi, Y., Wada, T., and Handa, H. (2008). A new APE1/Ref-1-dependent pathway leading to reduction of NF-kappaB and AP-1, and activation of their DNA-binding activity. Nucleic Acids Res *36*, 4327-4336.

Asai, K., Kudej, R.K., Shen, Y.T., Yang, G.P., Takagi, G., Kudej, A.B., Geng, Y.J., Sato, N., Nazareno, J.B., Vatner, D.E., Natividad, F., Bishop, S.P., and Vatner, S.F. (2000). Peripheral vascular endothelial dysfunction and apoptosis in old monkeys. Arterioscler Thromb Vasc Biol *20*, 1493-1499.

Backs, J., and Olson, E.N. (2006). Control of cardiac growth by histone acetylation/deacetylation. Circ Res *98*, 15-24.

Bae, Y.S., Oh, H., Rhee, S.G., and Yoo, Y.D. (2011). Regulation of reactive oxygen species generation in cell signaling. Mol Cells *32*, 491-509.

Bedard, K., and Krause, K.H. (2007). The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev *87*, 245-313.

Bruunsgaard, H., Skinhoj, P., Pedersen, A.N., Schroll, M., and Pedersen, B.K. (2000). Ageing, tumour necrosis factor-alpha (TNF-alpha) and atherosclerosis. Clin Exp Immunol *121*, 255-260.

Buchner, N., **Zschauer, T.C.**, Lukosz, M., Altschmied, J., and Haendeler, J. (2010). Downregulation of mitochondrial telomerase reverse transcriptase induced by H2O2 is Src kinase dependent. Exp Gerontol *45*, 558-562.

Chae, H.Z., Kim, H.J., Kang, S.W., and Rhee, S.G. (1999). Characterization of three isoforms of mammalian peroxiredoxin that reduce peroxides in the presence of thioredoxin. Diabetes Res Clin Pract *45*, 101-112.

Chen, K.S., and DeLuca, H.F. (1994). Isolation and characterization of a novel cDNA from HL-60 cells treated with 1,25-dihydroxyvitamin D-3. Biochim Biophys Acta *1219*, 26-32.

Chen, K.S., and DeLuca, H.F. (1995). Cloning of the human 1 alpha,25-dihydroxyvitamin D-3 24-hydroxylase gene promoter and identification of two vitamin D-responsive elements. Biochim Biophys Acta *1263*, 1-9.

Collet, J.F., and Messens, J. (2010). Structure, function, and mechanism of thioredoxin proteins. Antioxid Redox Signal *13*, 1205-1216.

Dalle-Donne, I., Milzani, A., Giustarini, D., Di Simplicio, P., Colombo, R., and Rossi, R. (2000). S-NO-actin: S-nitrosylation kinetics and the effect on isolated vascular smooth muscle. J Muscle Res Cell Motil *21*, 171-181.

Dimmeler, S., Haendeler, J., Nehls, M., and Zeiher, A.M. (1997). Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. J Exp Med *185*, 601-607.

Erusalimsky, J.D., and Skene, C. (2009). Mechanisms of endothelial senescence. Exp Physiol *94*, 299-304.

Ferrer-Sueta, G., and Radi, R. (2009). Chemical biology of peroxynitrite: kinetics, diffusion, and radicals. ACS Chem Biol *4*, 161-177.

Forstermann, U., and Munzel, T. (2006). Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation *113*, 1708-1714.

Gao, X., Xu, X., Belmadani, S., Park, Y., Tang, Z., Feldman, A.M., Chilian, W.M., and Zhang, C. (2007). TNF-alpha contributes to endothelial dysfunction by upregulating arginase in ischemia/reperfusion injury. Arterioscler Thromb Vasc Biol *27*, 1269-1275.

Gaziano, T., Reddy, K.S., Paccaud, F., Horton, S., and Chaturvedi, V. (2006). Cardiovascular Disease. In Disease Control Priorities in Developing Countries, D.T. Jamison, J.G. Breman, A.R. Measham, G. Alleyne, M. Claeson, D.B. Evans, P. Jha, A. Mills, and P. Musgrove, eds. (Washington (DC)).

Gerdes, F., Tatsuta, T., and Langer, T. (2012). Mitochondrial AAA proteases--towards a molecular understanding of membrane-bound proteolytic machines. Biochim Biophys Acta *1823*, 49-55.

Goodwin, B.L., Pendleton, L.C., Levy, M.M., Solomonson, L.P., and Eichler, D.C. (2007). Tumor necrosis factor-alpha reduces argininosuccinate synthase expression and nitric oxide production in aortic endothelial cells. Am J Physiol Heart Circ Physiol *293*, H1115-1121.

Gorbunova, V., Seluanov, A., and Pereira-Smith, O.M. (2002). Expression of human telomerase (hTERT) does not prevent stress-induced senescence in normal human fibroblasts but protects the cells from stress-induced apoptosis and necrosis. J Biol Chem *277*, 38540-38549.

Greider, C.W., and Blackburn, E.H. (1989). A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature *337*, 331-337.

Haendeler, J., Drose, S., Buchner, N., Jakob, S., Altschmied, J., Goy, C., Spyridopoulos, I., Zeiher, A.M., Brandt, U., and Dimmeler, S. (2009). Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage. Arterioscler Thromb Vasc Biol *29*, 929-935.

Haendeler, J., Hoffmann, J., Brandes, R.P., Zeiher, A.M., and Dimmeler, S. (2003a). Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707. Mol Cell Biol *23*, 4598-4610.

Haendeler, J., Hoffmann, J., Diehl, J.F., Vasa, M., Spyridopoulos, I., Zeiher, A.M., and Dimmeler, S. (2004). Antioxidants inhibit nuclear export of telomerase reverse transcriptase and delay replicative senescence of endothelial cells. Circ Res *94*, 768-775.

Haendeler, J., Hoffmann, J., Rahman, S., Zeiher, A.M., and Dimmeler, S. (2003b). Regulation of telomerase activity and anti-apoptotic function by protein-protein interaction and phosphorylation. FEBS Lett *536*, 180-186.

Haendeler, J., Hoffmann, J., Tischler, V., Berk, B.C., Zeiher, A.M., and Dimmeler, S. (2002). Redox regulatory and anti-apoptotic functions of thioredoxin depend on S-nitrosylation at cysteine 69. Nat Cell Biol *4*, 743-749.

Haendeler, J., Popp, R., Goy, C., Tischler, V., Zeiher, A.M., and Dimmeler, S. (2005). Cathepsin D and H2O2 stimulate degradation of thioredoxin-1: implication for endothelial cell apoptosis. J Biol Chem *280*, 42945-42951.

Haendeler, J., Weiland, U., Zeiher, A.M., and Dimmeler, S. (1997). Effects of redox-related congeners of NO on apoptosis and caspase-3 activity. Nitric Oxide *1*, 282-293.

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. J Gerontol *11*, 298-300.

Hatai, T., Matsuzawa, A., Inoshita, S., Mochida, Y., Kuroda, T., Sakamaki, K., Kuida, K., Yonehara, S., Ichijo, H., and Takeda, K. (2000). Execution of apoptosis signal-regulating kinase 1 (ASK1)-induced apoptosis by the mitochondria-dependent caspase activation. J Biol Chem *275*, 26576-26581.

Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. Exp Cell Res *25*, 585-621.

Heo, J.Y., Jing, K., Song, K.S., Seo, K.S., Park, J.H., Kim, J.S., Jung, Y.J., Hur, G.M., Jo, D.Y., Kweon, G.R., Yoon, W.H., Lim, K., Hwang, B.D., Jeon, B.H., and Park, J.I. (2009). Downregulation of APE1/Ref-1 is involved in the senescence of mesenchymal stem cells. Stem Cells *27*, 1455-1462.

Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., and Yodoi, J. (1997). AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. Proc Natl Acad Sci U S A *94*, 3633-3638.

Hirst, D.G., and Robson, T. (2011). Nitric oxide physiology and pathology. Methods Mol Biol *704*, 1-13.

Hoffmann, J., Haendeler, J., Aicher, A., Rossig, L., Vasa, M., Zeiher, A.M., and Dimmeler, S. (2001). Aging enhances the sensitivity of endothelial cells toward apoptotic stimuli: important role of nitric oxide. Circ Res *89*, 709-715.

Holmgren, A. (1968). Thioredoxin. 6. The amino acid sequence of the protein from escherichia coli B. Eur J Biochem *6*, 475-484.

Holmgren, A., Soderberg, B.O., Eklund, H., and Branden, C.I. (1975). Three-dimensional structure of Escherichia coli thioredoxin-S2 to 2.8 A resolution. Proc Natl Acad Sci U S A *72*, 2305-2309.

Huot, J., Houle, F., Rousseau, S., Deschesnes, R.G., Shah, G.M., and Landry, J. (1998). SAPK2/p38-dependent F-actin reorganization regulates early membrane blebbing during stress-induced apoptosis. J Cell Biol *143*, 1361-1373.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science *275*, 90-94.

Jakob, S., Schroeder, P., Lukosz, M., Buchner, N., Spyridopoulos, I., Altschmied, J., and Haendeler, J. (2008). Nuclear protein tyrosine phosphatase Shp-2 is one important negative regulator of nuclear export of telomerase reverse transcriptase. J Biol Chem *283*, 33155-33161.

Kang, S.S., Kwon, T., Kwon, D.Y., and Do, S.I. (1999). Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. J Biol Chem *274*, 13085-13090.

Kim, F., Gallis, B., and Corson, M.A. (2001). TNF-alpha inhibits flow and insulin signaling leading to NO production in aortic endothelial cells. Am J Physiol Cell Physiol *280*, C1057-1065.

Kroncke, K.D., Fehsel, K., and Kolb-Bachofen, V. (1998). Inducible nitric oxide synthase in human diseases. Clin Exp Immunol *113*, 147-156.

Kuzkaya, N., Weissmann, N., Harrison, D.G., and Dikalov, S. (2003). Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. J Biol Chem *278*, 22546-22554.

Kyo, S., Takakura, M., Fujiwara, T., and Inoue, M. (2008). Understanding and exploiting hTERT promoter regulation for diagnosis and treatment of human cancers. Cancer Sci *99*, 1528-1538.

Laurent, T.C., Moore, E.C., and Reichard, P. (1964). Enzymatic Synthesis of Deoxyribonucleotides. Iv. Isolation and Characterization of Thioredoxin, the Hydrogen Donor from Escherichia Coli B. J Biol Chem *239*, 3436-3444.

Lillig, C.H., Berndt, C., and Holmgren, A. (2008). Glutaredoxin systems. Biochim Biophys Acta *1780*, 1304-1317.

Lukosz, M., Jakob, S., Buchner, N., **Zschauer, T.C.**, Altschmied, J., and Haendeler, J. (2010). Nuclear redox signaling. Antioxid Redox Signal *12*, 713-742.

Mandavilli, B.S., Santos, J.H., and Van Houten, B. (2002). Mitochondrial DNA repair and aging. Mutat Res *509*, 127-151.

Mannick, J.B. (2007). Regulation of apoptosis by protein S-nitrosylation. Amino Acids *32*, 523-526.

Martinou, J.C., and Youle, R.J. (2011). Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. Dev Cell *21*, 92-101.

Matsui, M., Oshima, M., Oshima, H., Takaku, K., Maruyama, T., Yodoi, J., and Taketo, M.M. (1996). Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. Dev Biol *178*, 179-185.

Miranda-Vizuete, A., Ljung, J., Damdimopoulos, A.E., Gustafsson, J.A., Oko, R., Pelto-Huikko, M., and Spyrou, G. (2001). Characterization of Sptrx, a novel member of the thioredoxin family specifically expressed in human spermatozoa. J Biol Chem *276*, 31567-31574.

Mitra, S.K., Hanson, D.A., and Schlaepfer, D.D. (2005). Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol *6*, 56-68.

Moens, A.L., Takimoto, E., Tocchetti, C.G., Chakir, K., Bedja, D., Cormaci, G., Ketner, E.A., Majmudar, M., Gabrielson, K., Halushka, M.K., Mitchell, J.B., Biswal, S., Channon, K.M., Wolin, M.S., Alp, N.J., Paolocci, N., Champion, H.C., and Kass, D.A. (2008). Reversal of cardiac hypertrophy and fibrosis from pressure overload by tetrahydrobiopterin: efficacy of recoupling nitric oxide synthase as a therapeutic strategy. Circulation *117*, 2626-2636.

Muller, G., and Morawietz, H. (2009). Nitric oxide, NAD(P)H oxidase, and atherosclerosis. Antioxid Redox Signal *11*, 1711-1731.

Murray, C.J., and Lopez, A.D. (1997). Mortality by cause for eight regions of the world: Global Burden of Disease Study. Lancet *349*, 1269-1276.

Neumann, P., Gertzberg, N., and Johnson, A. (2004). TNF-alpha induces a decrease in eNOS promoter activity. Am J Physiol Lung Cell Mol Physiol *286*, L452-459.

Nishiyama, A., Matsui, M., Iwata, S., Hirota, K., Masutani, H., Nakamura, H., Takagi, Y., Sono, H., Gon, Y., and Yodoi, J. (1999). Identification of thioredoxin-binding protein-2/vitamin D(3) up-regulated protein 1 as a negative regulator of thioredoxin function and expression. J Biol Chem *274*, 21645-21650.

Nordberg, J., and Arner, E.S. (2001). Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic Biol Med *31*, 1287-1312.

Oh, H., Taffet, G.E., Youker, K.A., Entman, M.L., Overbeek, P.A., Michael, L.H., and Schneider, M.D. (2001). Telomerase reverse transcriptase promotes cardiac muscle cell proliferation, hypertrophy, and survival. Proc Natl Acad Sci U S A *98*, 10308-10313.

Parsons, J.L., Dianova, II, and Dianov, G.L. (2004). APE1 is the major 3'-phosphoglycolate activity in human cell extracts. Nucleic Acids Res *32*, 3531-3536.

Puddu, P., Puddu, G.M., Galletti, L., Cravero, E., and Muscari, A. (2005). Mitochondrial dysfunction as an initiating event in atherogenesis: a plausible hypothesis. Cardiology *103*, 137-141.

Ray, R., and Shah, A.M. (2005). NADPH oxidase and endothelial cell function. Clin Sci (Lond) *109*, 217-226.

Riedl, S.J., and Shi, Y. (2004). Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol *5*, 897-907.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J *17*, 2596-2606.

Santos, J.H., Meyer, J.N., and Van Houten, B. (2006). Mitochondrial localization of telomerase as a determinant for hydrogen peroxide-induced mitochondrial DNA damage and apoptosis. Hum Mol Genet *15*, 1757-1768.

Seo, A.Y., Joseph, A.M., Dutta, D., Hwang, J.C., Aris, J.P., and Leeuwenburgh, C. (2010). New insights into the role of mitochondria in aging: mitochondrial dynamics and more. J Cell Sci *123*, 2533-2542.

Sies, H., and Cadenas, E. (1985). Oxidative stress: damage to intact cells and organs. Philos Trans R Soc Lond B Biol Sci *311*, 617-631.

Sirker, A., Zhang, M., and Shah, A.M. (2011). NADPH oxidases in cardiovascular disease: insights from in vivo models and clinical studies. Basic Res Cardiol *106*, 735-747.

Slack-Davis, J.K., Martin, K.H., Tilghman, R.W., Iwanicki, M., Ung, E.J., Autry, C., Luzzio, M.J., Cooper, B., Kath, J.C., Roberts, W.G., and Parsons, J.T. (2007). Cellular characterization of a novel focal adhesion kinase inhibitor. J Biol Chem *282*, 14845-14852.

Spiekermann, S., Landmesser, U., Dikalov, S., Bredt, M., Gamez, G., Tatge, H., Reepschlager, N., Hornig, B., Drexler, H., and Harrison, D.G. (2003). Electron spin resonance characterization of vascular xanthine and NAD(P)H oxidase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. Circulation *107*, 1383-1389.

Spyrou, G., Enmark, E., Miranda-Vizuete, A., and Gustafsson, J. (1997). Cloning and expression of a novel mammalian thioredoxin. J Biol Chem *272*, 2936-2941.

Takac, I., Schroder, K., and Brandes, R.P. (2012). The Nox family of NADPH oxidases: friend or foe of the vascular system? Curr Hypertens Rep *14*, 70-78.

Tell, G., Quadrifoglio, F., Tiribelli, C., and Kelley, M.R. (2009). The many functions of APE1/Ref-1: not only a DNA repair enzyme. Antioxid Redox Signal *11*, 601-620.

Tsutsui, M., Shimokawa, H., Otsuji, Y., and Yanagihara, N. (2010). Pathophysiological relevance of NO signaling in the cardiovascular system: novel insight from mice lacking all NO synthases. Pharmacol Ther *128*, 499-508.

Ueno, M., Masutani, H., Arai, R.J., Yamauchi, A., Hirota, K., Sakai, T., Inamoto, T., Yamaoka, Y., Yodoi, J., and Nikaido, T. (1999). Thioredoxin-dependent redox regulation of p53-mediated p21 activation. J Biol Chem *274*, 35809-35815.

Vepa, S., Scribner, W.M., Parinandi, N.L., English, D., Garcia, J.G., and Natarajan, V. (1999). Hydrogen peroxide stimulates tyrosine phosphorylation of focal adhesion kinase in vascular endothelial cells. Am J Physiol *277*, L150-158.

Vesely, P.W., Staber, P.B., Hoefler, G., and Kenner, L. (2009). Translational regulation mechanisms of AP-1 proteins. Mutat Res *682*, 7-12.

Vita, J.A. (2011). Endothelial function. Circulation *124*, e906-912.

Wagner, E., Luche, S., Penna, L., Chevallet, M., Van Dorsselaer, A., Leize-Wagner, E., and Rabilloud, T. (2002). A method for detection of overoxidation of cysteines: peroxiredoxins are oxidized in vivo at the active-site cysteine during oxidative stress. Biochem J *366*, 777-785.

Walker, L.J., Robson, C.N., Black, E., Gillespie, D., and Hickson, I.D. (1993). Identification of residues in the human DNA repair enzyme HAP1 (Ref-1) that are essential for redox regulation of Jun DNA binding. Mol Cell Biol *13*, 5370-5376.

Wei, S.J., Botero, A., Hirota, K., Bradbury, C.M., Markovina, S., Laszlo, A., Spitz, D.R., Goswami, P.C., Yodoi, J., and Gius, D. (2000). Thioredoxin nuclear translocation and interaction with redox factor-1 activates the activator protein-1 transcription factor in response to ionizing radiation. Cancer Res *60*, 6688-6695.

Wilson, D.M., 3rd, and Barsky, D. (2001). The major human abasic endonuclease: formation, consequences and repair of abasic lesions in DNA. Mutat Res *485*, 283-307.

World, C., Spindel, O.N., and Berk, B.C. (2011). Thioredoxin-interacting protein mediates TRX1 translocation to the plasma membrane in response to tumor necrosis factor-alpha: a key mechanism for vascular endothelial growth factor receptor-2 transactivation by reactive oxygen species. Arterioscler Thromb Vasc Biol *31*, 1890-1897.

Xanthoudakis, S., and Curran, T. (1992). Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. EMBO J *11*, 653-665.

Yamawaki, H., Pan, S., Lee, R.T., and Berk, B.C. (2005). Fluid shear stress inhibits vascular inflammation by decreasing thioredoxin-interacting protein in endothelial cells. J Clin Invest *115*, 733-738.

Yoshida, L.S., and Tsunawaki, S. (2008). Expression of NADPH oxidases and enhanced H(2)O(2)-generating activity in human coronary artery endothelial cells upon induction with tumor necrosis factor-alpha. Int Immunopharmacol *8*, 1377-1385.

Yoshizumi, M., Perrella, M.A., Burnett, J.C., Jr., and Lee, M.E. (1993). Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. Circ Res *73*, 205-209.

Zschauer, T.C., Kunze, K., Jakob, S., Haendeler, J., and Altschmied, J. (2011). Oxidative stress-induced degradation of thioredoxin-1 and apoptosis is inhibited by thioredoxin-1-actin interaction in endothelial cells. Arterioscler Thromb Vasc Biol *31*, 650-656.

Zschauer, T.C., Matsushima, S., Altschmied, J., Shao, D., Sadoshima, J., and Haendeler, J. (2012). Interacting with Thioredoxin-1 - disease or no disease? Antioxid Redox Signal *im Druck*.

Eigene Veröffentlichungen

Nuclear redox signaling.

Lukosz M, Jakob S, Büchner N, **Zschauer TC**, Altschmied J, Haendeler J.

Antioxid Redox Signal. 2010;12:713-742.

Downregulation of mitochondrial telomerase reverse transcriptase induced by H_2O_2 is Src kinase dependent.

Büchner N, **Zschauer TC**, Lukosz M, Altschmied J, Haendeler J.

Exp Gerontol. 2010; 45:558-562.

Oxidative stress-induced degradation of thioredoxin-1 and apoptosis is inhibited by thioredoxin-1-actin interaction in endothelial cells.

Zschauer TC, Kunze K, Jakob S, Haendeler J, Altschmied J.

Arterioscler Thromb Vasc Biol. 2011; 31:650-656.

Interacting with Thioredoxin-1 – disease or no disease ?

Zschauer TC, Matsushima S, Altschmied J, Shao D, Sadoshima J, Haendeler J.

Antioxid Redox Signal. 2012; im Druck.

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Lukosz M, Jakob S, Büchner N, **Zschauer TC**, Altschmied J, Haendeler J.

Antioxid Redox Signal. 2010; 12:713-742.

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Atschmied J: Senior Autor, schrieb zusammen mit Dr. Judith Haendeler die Einleitung und das Fazit. Zudem korrigierte er den Teil über die redoxregulierten Transkriptionsfaktoren und den Teil zu APEX-1, Glutaredoxin und Peroxiredoxin.

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ANTIOXIDANTS & REDOX SIGNALING
Volume 12, Number 6, 2010

DOI: 10.1089/ars.2009.2609

COMPREHENSIVE INVITED REVIEW

Nuclear Redox Signaling

Margarete Lukosz, Sascha Jakob, Nicole Büchner, Tim-Christian Zschauer, Joachim Altschmied, and Judith Haendeler

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, 713–742.

Reviewing Editors: *Aron Fisher, Pascal Goldschmidt-Clermont, Arne Holmgren, Toren Finkel, Junichi Sadoshima, Albert van der Vliet, and Junji Yodoi*

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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 that 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 biological 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 biological 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 underestimated. 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 biological 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 that they have a nuclear localization and 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/GSSG. It is reflected in the balance of several sets of

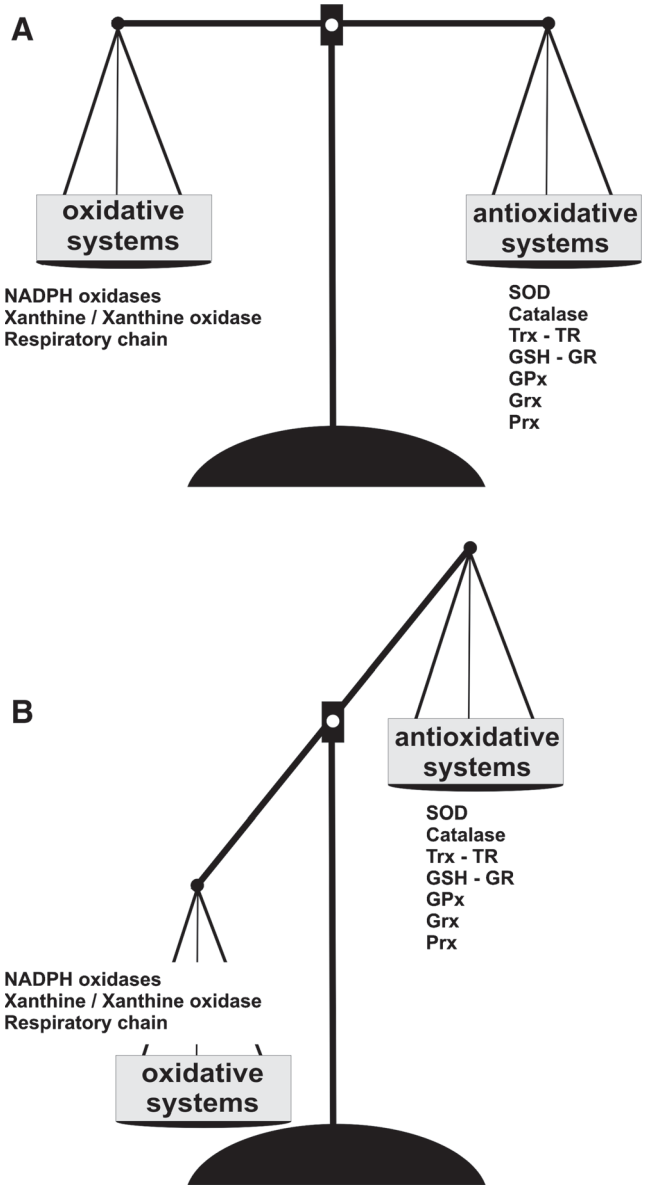

FIG. 1. Balance between oxidative and antioxidative systems. (**A**) The generation of ROS by oxidative systems (*e.g.*, NADPH oxidase, xanthine/xanthine oxidase, and the respiratory chain) is controlled by the activity of antioxidant systems, like superoxide dismutase (SOD), catalase, thioredoxin/thioredoxin 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.

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 >1 (234, 236), whereas the NADH/NAD^+ ratio is <1 (221, 261). Generally, the nucleus provides a reductive environment.

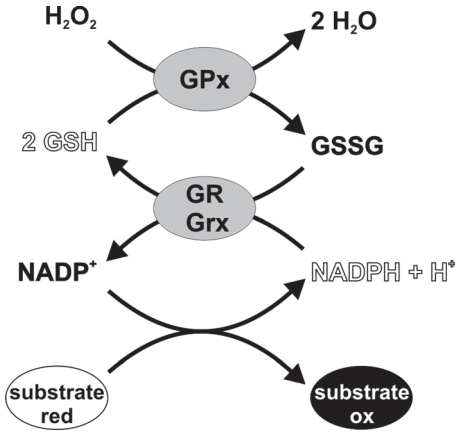

FIG. 2. The GSH/GR/GPx system: a general model of the GSH/GR/GPx system. Glutathione peroxidase (GPx) reduces H_2O_2 to H_2O and thereby oxidizes GSH to GSSG. GSSG itself is reduced by glutathione reductase (GR) to regenerate GSH. The reductant in this reaction is NADPH + H^+, created by metabolic substrate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

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. Since 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 ~4 (210); therefore, the concentration of free NADPH in the nucleus must be ~500 n*M*.

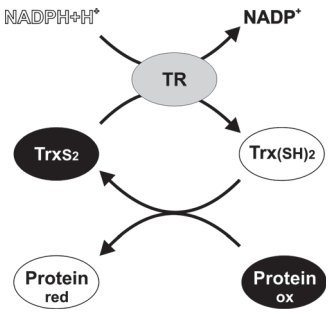

FIG. 3. The Trx/TR system: simplified model of the Trx/TR system and Trx function. The Trx/TR system consists of two oxidoreductase enzymes, thioredoxin (Trx) and thioredoxin reductase (TR). Reduced Trx ($Trx_{(SH)2}$) directly interacts with oxidized proteins by forming disulfide bridges. This involves 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^+.

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. Several nuclear proteins will now be introduced whose activity and functions depend on the redox balance and on nuclear redox signaling.

C. Thioredoxin-1 (Trx-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 (Grxs) and peroxiredoxins (Prxs)

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 ref. 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, see refs. 102 and 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) 1 (APEX1)

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).

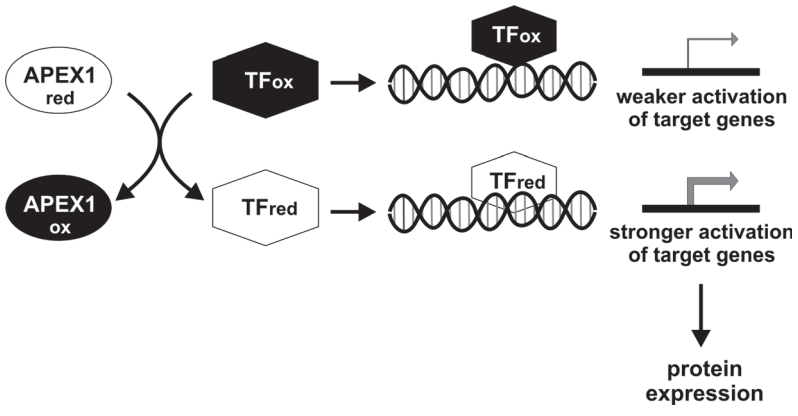

FIG. 4. APEX1. General model of APEX nuclease (multifunctional DNA-repair enzyme) 1 (APEX1) redox regulator function. APEX1 converts a transcription factor (TF) from an oxidized, less DNA binding competent state to its reduced state that can bind more avidly to the regulatory regions of a variety of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

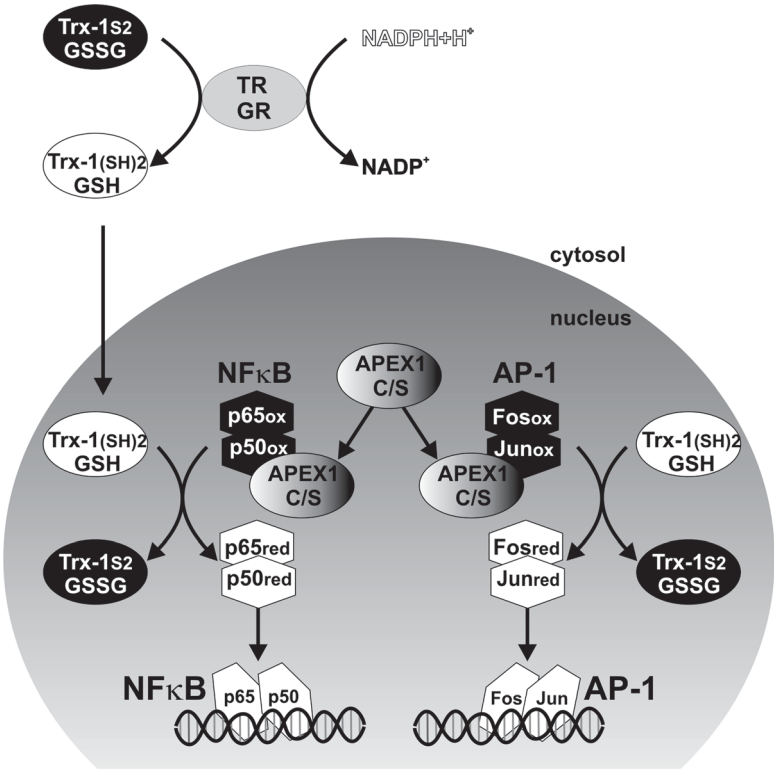

FIG. 5. APEX1 as a redox chaperone: model for the redox chaperone function of APEX1. A mutant of APEX nuclease (multifunctional DNA-repair enzyme) 1, in which all seven cysteine residues are replaced with serine (APEX C/S), can bind to the p50 and Jun subunits of the transcription factor complexes nuclear 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 >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 also 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

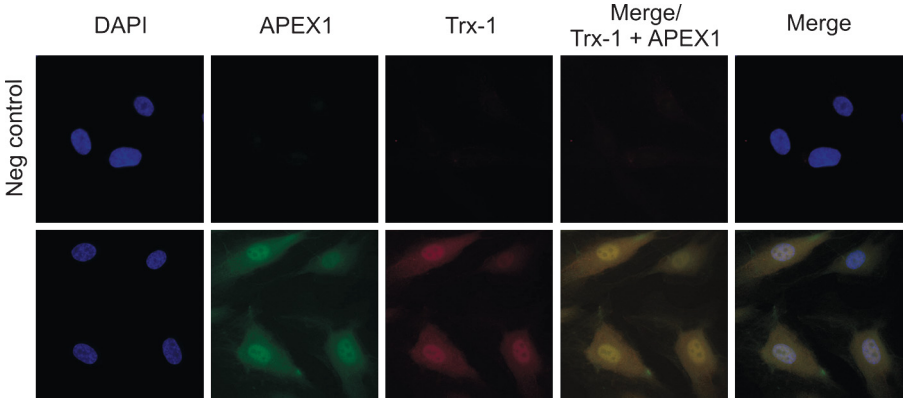

FIG. 6. Interaction of endogenous APEX1 and Trx-1 in human endothelial cells. Nuclear association of endogenous thioredoxin-1 (Trx-1) and APEX nuclease (multifunctional DNA-repair enzyme) 1 (APEX1). Representative immunostainings of human umbilical vein endothelial cells are shown. Nuclear staining with 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.

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.

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.

A. Activator protein-1 (AP-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 canonical 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 ref. 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.

B. Cyclic AMP response element–binding protein (CREB)

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 refs. 145, 175, and 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 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 (Sp1)

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 refs. 100 and 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 upon 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 ref. 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. After 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)

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 upon 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 ref. 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 ref. 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 ref. 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 by 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 refs. 28, 84, 217, and 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)

NFI/CTF was originally described as being required for the replication of adenovirus DNA (for review, see ref. 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 ref. 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.

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.

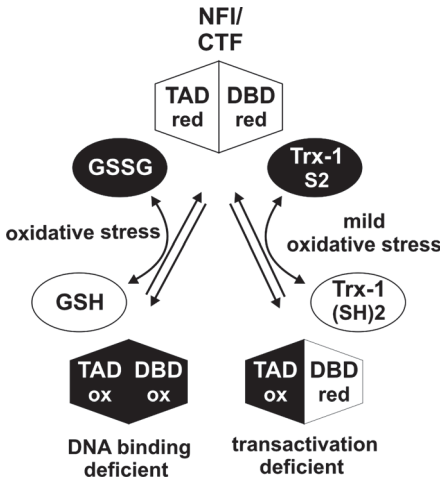

FIG. 7. Redox regulation of NFI/CTF: dual regulation of NFI/CTF transcriptional activity. Nuclear factor I/CAAT transcription factor (NFI/CTF) must be reduced to activate its target genes. Oxidative stress leads to oxidation of its transactivation domain (TAD) and DNA binding domain (DBD), which prevents 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)

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 (<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 refs. 205, 206, 246, and 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 a 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 (Nrf-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 *Keap*1- 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). After 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 refs. 107, 158, and 159).

Besides the cytosolic retention of Nrf-2 by Keap1 and its release upon 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

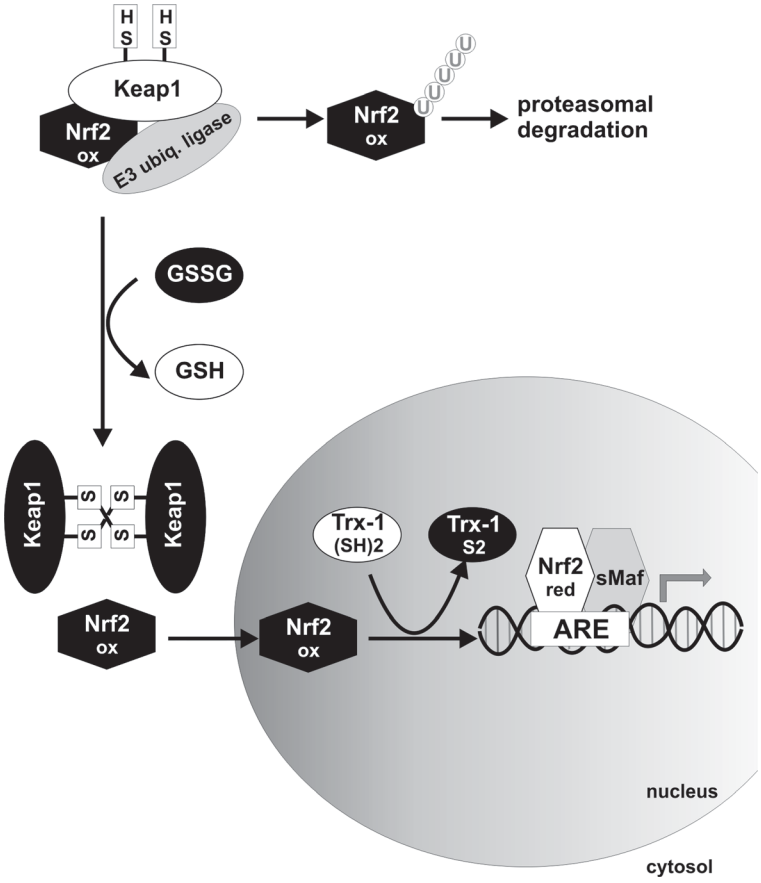

FIG. 8. Activation of Nrf-2: general model of gene induction by the Keap1/Nrf-2 pathway. Nuclear factor erythroid 2–related factor 2/NF-E2–related factor 2 (Nrf-2) is sequestered in the cytosol by Kelch-like ECH associated protein 1 (Keap1). In addition to binding Nrf-2, Keap1 functions as an adaptor 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 depend on nuclear Trx-1 (76) (Fig. 8).

J. Homeobox B5 (HoxB5)

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 (HDAC2)

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 after 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 genes, and dendritic

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		

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 refs. 98 and 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 paragraphs discuss the kinases mentioned earlier in more detail; a brief overview is given in Table 2.

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 ref. 259). This family consists of three groups: (a) the conventional PKCs (cPKCs: α, βI, βII γ); (b) the novel PKCs (nPKCs: $\delta, \varepsilon, \theta, \mu$); 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). However, other 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).

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

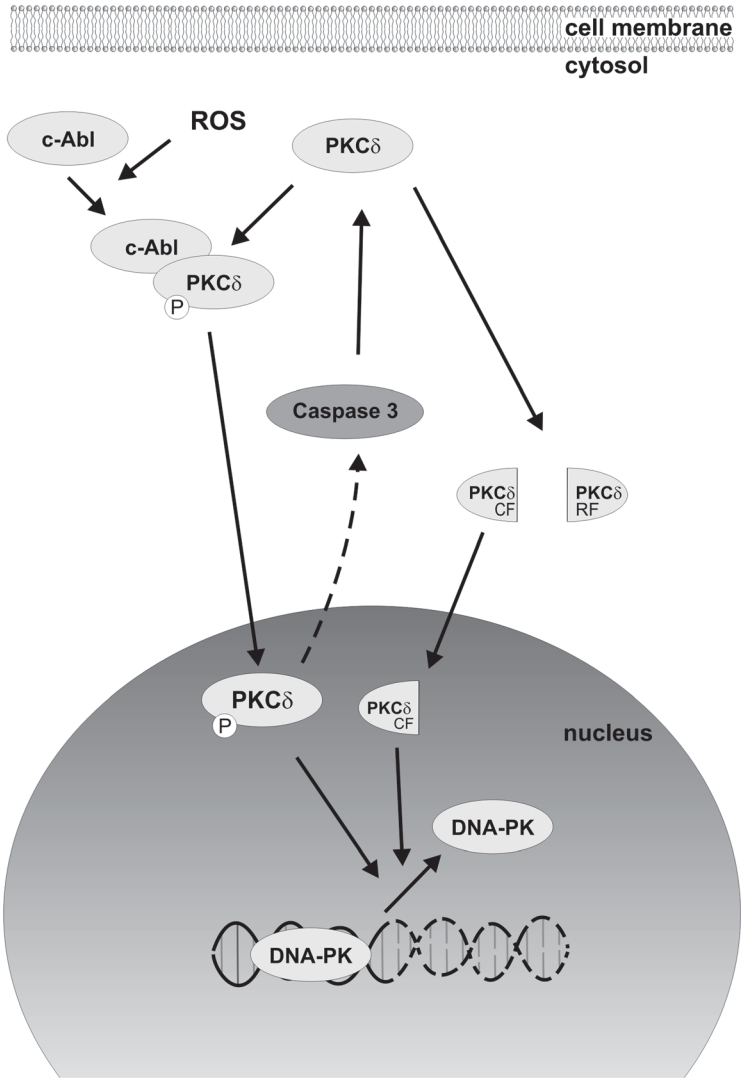

FIG. 9. Role of nuclear PKC*δ*. ROS induce association of protein kinase C *δ* (PKC*δ*) and c-Abl. This leads to phosphorylation, activation, and nuclear import of PKC*δ*. In a direct or indirect way, phosphorylated PKC*δ* activates caspase 3, which in turn leads to cleavage of PKC*δ* into a catalytically 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 (PKA). 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). 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 ref. 127). JNKs translocate into the nucleus and phosphorylate c-Jun and activating transcription factor 2 (ATF2) (for review, see ref. 41), leading to the formation of an AP-1 complex and to the transcription of genes coding for proapoptotic proteins [*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

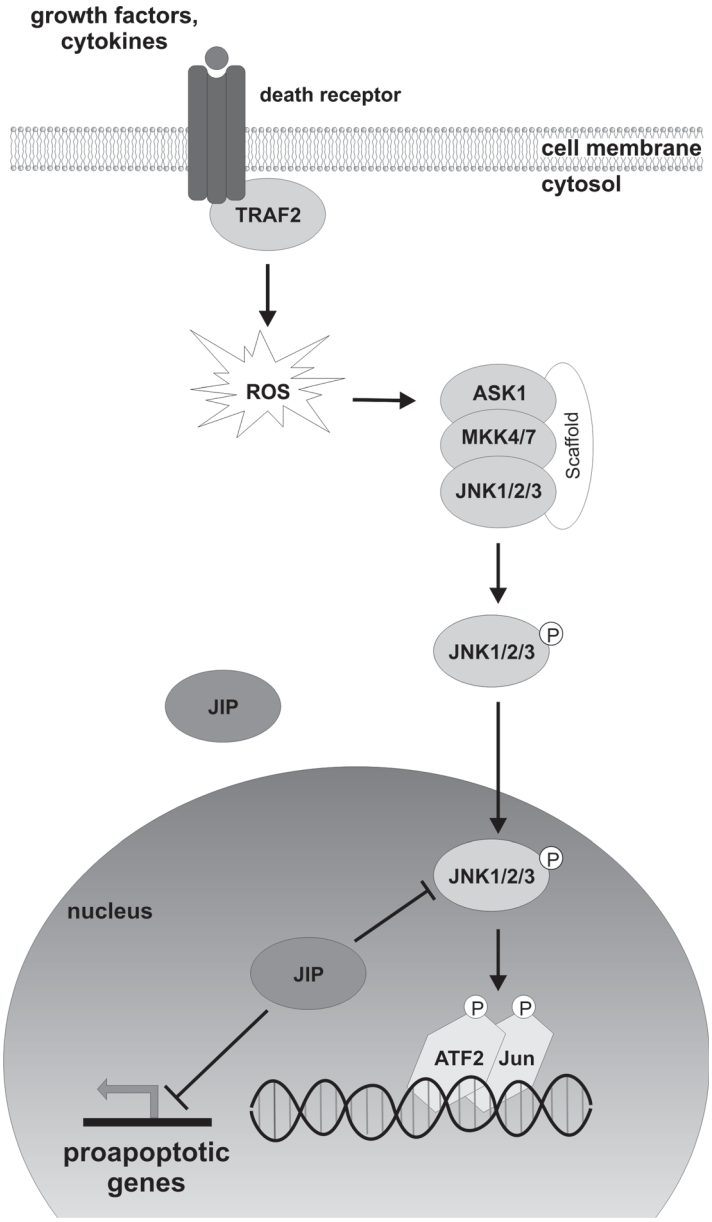

FIG. 10. Nuclear targeting and signaling of JNKs in the regulation of apoptosis. Growth factors and cytokines elevate ROS levels mediated by death receptor–coupled Traf2. This induces the activation of c-Jun NH_2-terminal kinases (JNKs) through upstream kinases apoptosis-signaling kinase 1 (ASK1) and 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.

of individual 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 were 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 (Akt). 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. Upon 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

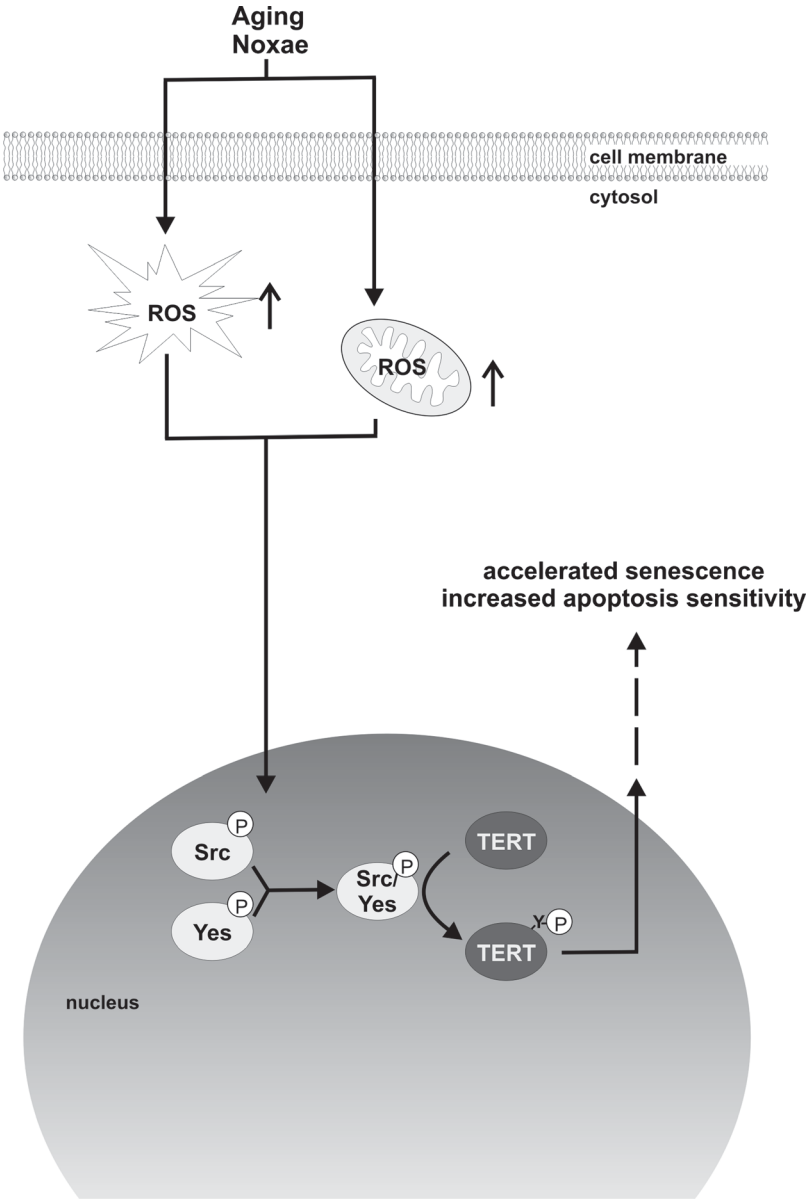

FIG. 11. Nuclear Src and Yes induce TERT export under conditions of oxidative stress. Cytosolic and mitochondrial ROS induce increased activity of nuclear Src and Yes. This leads to tyrosine phosphorylation of telomerase reverse transcriptase (TERT) and its nuclear export. The functional consequences 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, upon 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 (ERK1/2). 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 a 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

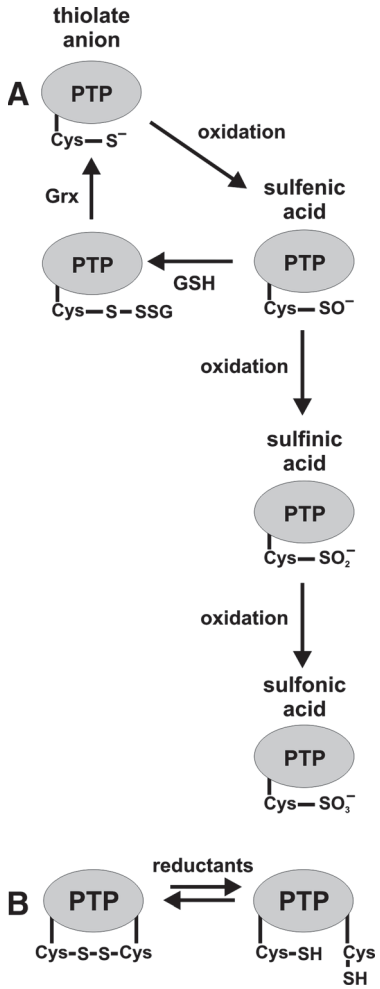

FIG. 12. Model for the regulation of PTP activity. (**A**) Under physiologic conditions, the catalytic cysteine of active protein tyrosine phosphatases (PTPs) is in the thiolate anion form. Oxidation leads to reversible sulfenic acid formation. Further oxidation is irreversible and leads to sulfinic 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 (TC-PTP). 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 change in

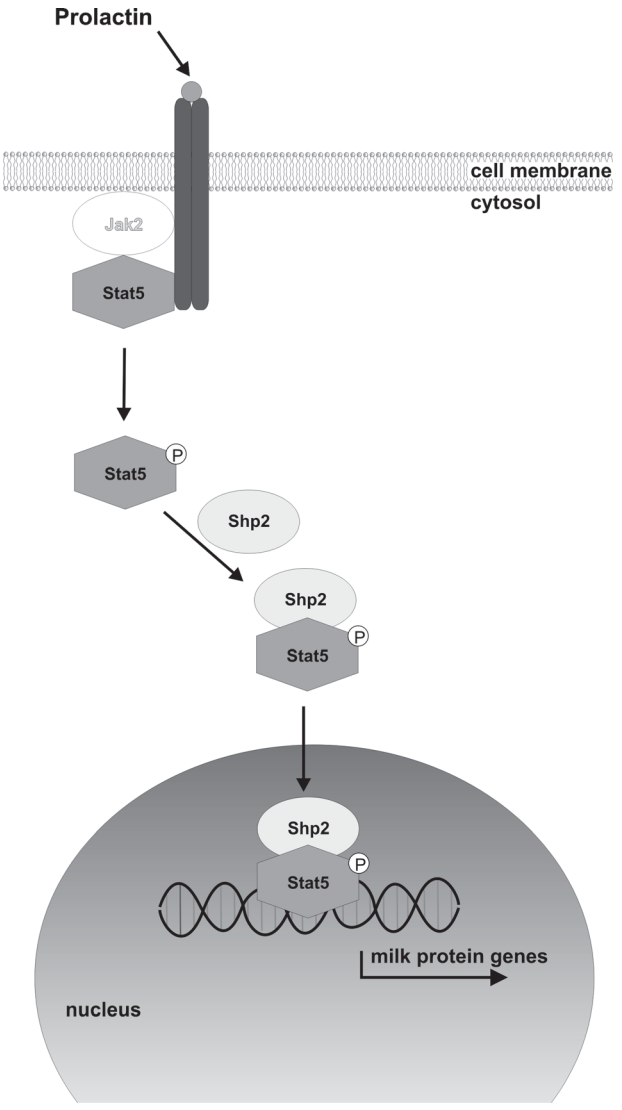

FIG. 13. Nuclear translocation of Shp-2 induces STAT5-dependent gene transcription. Prolactin induces tyrosine phosphorylation of STAT5 at tyrosine 694 by the Janus kinase 2 (JAK2), which leads to complex formation of Shp-2 and STAT5. This complex translocates into the nucleus, binds to DNA *via* STAT5,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

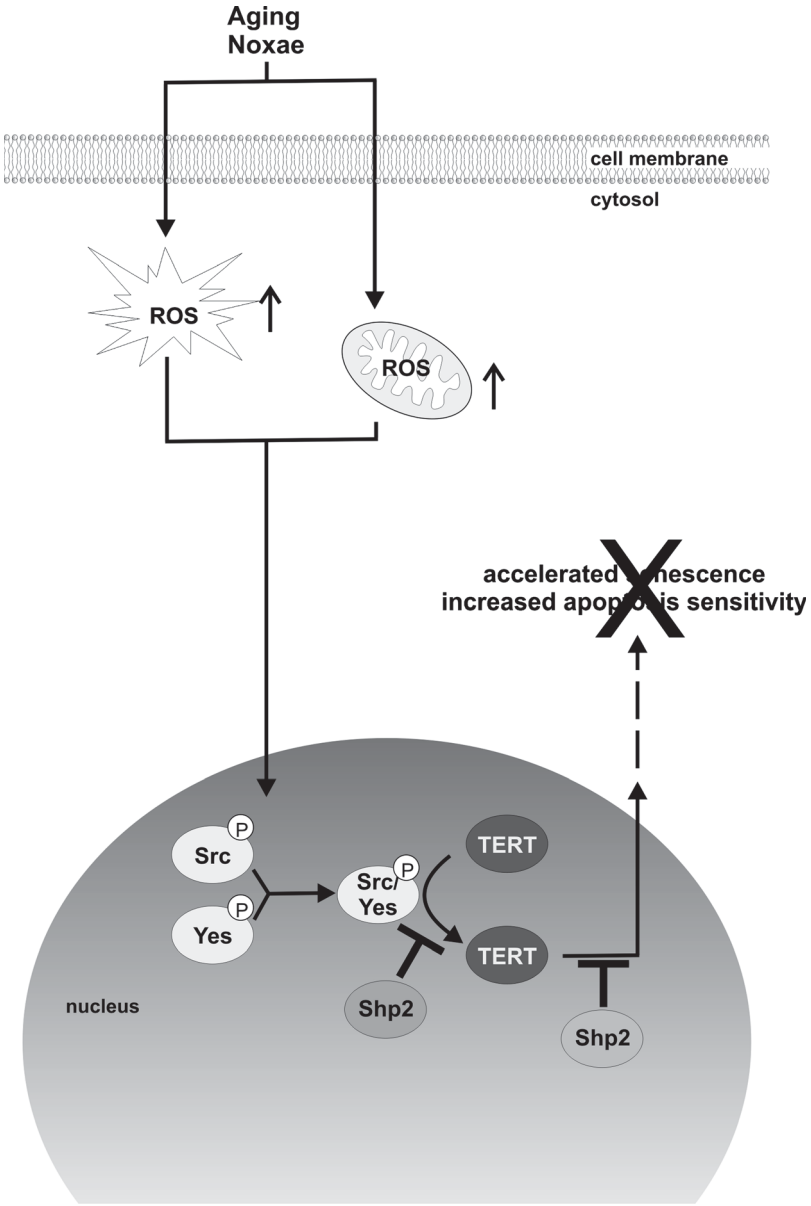

FIG. 14. Shp-2 inhibits TERT nuclear export induced by oxidative stress. ROS induce increased activity of nuclear Src and Yes. This leads to tyrosine phosphorylation of telomerase reverse transcriptase (TERT) and to its nuclear export. Nuclear Shp-2 prevents this export.

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. Upon 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

The first two authors contributed equally to this work. We apologize for the failure to cite many of the important and relevant articles in this field due to space limitations. This study was in part supported by the Deutsche Forschungsgemeinschaft (HA-2868/3-2, SFB 612 TP B15, and SFB 728 TP B5 to J.H.). M.L. received a scholarship from the Research Training Group 1089.

References

1. Abate C, Patel L, Rauscher FJ 3rd, and Curran T. Redox regulation of fos and jun DNA-binding activity in vitro. *Science* 249: 1157–1161, 1990.
2. Adachi M, Sekiya M, Miyachi T, Matsuno K, Hinoda Y, Imai K, and Yachi A. Molecular cloning of a novel protein-tyrosine phosphatase SH-PTP3 with sequence similarity to the src-homology region 2. *FEBS Lett* 314: 335–339, 1992.
3. Adachi T, Pimentel DR, Heibeck T, Hou X, Lee YJ, Jiang B, Ido Y, and Cohen RA. *S*-Glutathiolation of Ras mediates redox-sensitive signaling by angiotensin II in vascular smooth muscle cells. *J Biol Chem* 279: 29857–29862, 2004.
4. Ahmad S, Banville D, Zhao Z, Fischer EH, and Shen SH. A widely expressed human protein-tyrosine phosphatase containing src homology 2 domains. *Proc Natl Acad Sci U S A* 90: 2197–2201, 1993.
5. Akamatsu Y, Ohno T, Hirota K, Kagoshima H, Yodoi J, and Shigesada K. Redox regulation of the DNA binding activity in transcription factor PEBP2: the roles of two conserved cysteine residues. *J Biol Chem* 272: 14497–14500, 1997.
6. Ammendola R, Mesuraca M, Russo T, and Cimino F. Sp1 DNA binding efficiency is highly reduced in nuclear extracts from aged rat tissues. *J Biol Chem* 267: 17944–17948, 1992.
7. Ammendola R, Mesuraca M, Russo T, and Cimino F. The DNA-binding efficiency of Sp1 is affected by redox changes. *Eur J Biochem* 225: 483–489, 1994.
8. Ando K, Hirao S, Kabe Y, Ogura Y, Sato I, Yamaguchi Y, Wada T, and Handa H. A new APE1/Ref-1-dependent pathway leading to reduction of NF-kappaB and AP-1, and activation of their DNA-binding activity. *Nucleic Acids Res* 36: 4327–4336, 2008.
9. Andricopulo AD, Akoachere MB, Krogh R, Nickel C, McLeish MJ, Kenyon GL, Arscott LD, Williams CH Jr,

Davioud-Charvet E, and Becker K. Specific inhibitors of *Plasmodium falciparum* thioredoxin reductase as potential antimalarial agents. *Bioorg Med Chem Lett* 16: 2283–2292, 2006.
10. Aoki N and Matsuda T. A nuclear protein tyrosine phosphatase TC-PTP is a potential negative regulator of the PRL-mediated signaling pathway: dephosphorylation and deactivation of signal transducer and activator of transcription 5a and 5b by TC-PTP in nucleus. *Mol Endocrinol* 16: 58–69, 2002.
11. Arnone MI, Zannini M, and Di Lauro R. The DNA binding activity and the dimerization ability of the thyroid transcription factor I are redox regulated. *J Biol Chem* 270: 12048–12055, 1995.
12. Ashley RH. Challenging accepted ion channel biology: p64 and the CLIC family of putative intracellular anion channel proteins [Review]. *Mol Membr Biol* 20: 1–11, 2003.
13. Bandyopadhyay S and Gronostajski RM. Identification of a conserved oxidation-sensitive cysteine residue in the NFI family of DNA-binding proteins. *J Biol Chem* 269: 29949–29955, 1994.
14. Bandyopadhyay S, Starke DW, Mieyal JJ, and Gronostajski RM. Thioltransferase (glutaredoxin) reactivates the DNA-binding activity of oxidation-inactivated nuclear factor I. *J Biol Chem* 273: 392–397, 1998.
15. Banmeyer I, Marchand C, Verhaeghe C, Vucic B, Rees JF, and Knoops B. Overexpression of human peroxiredoxin 5 in subcellular compartments of Chinese hamster ovary cells: effects on cytotoxicity and DNA damage caused by peroxides. *Free Radic Biol Med* 36: 65–77, 2004.
16. Bannister AJ, Cook A, and Kouzarides T. In vitro DNA binding activity of Fos/Jun and BZLF1 but not C/EBP is affected by redox changes. *Oncogene* 6: 1243–1250, 1991.
17. Barlow CA, Kitiphongspattana K, Siddiqui N, Roe MW, Mossman BT, and Lounsbury KM. Protein kinase A-mediated CREB phosphorylation is an oxidant-induced survival pathway in alveolar type II cells. *Apoptosis* 13: 681–692, 2008.
18. Barlow CA, Shukla A, Mossman BT, and Lounsbury KM. Oxidant-mediated cAMP response element binding protein activation: calcium regulation and role in apoptosis of lung epithelial cells. *Am J Respir Cell Mol Biol* 34: 7–14, 2006.
19. Barrett WC, DeGnore JP, Konig S, Fales HM, Keng YF, Zhang ZY, Yim MB, and Chock PB. Regulation of PTP1B via glutathionylation of the active site cysteine 215. *Biochemistry* 38: 6699–6705, 1999.
20. Belich MP, Salmeron A, Johnston LH, and Ley SC. TPL-2 kinase regulates the proteolysis of the NF-kappaB-inhibitory protein NF-kappaB1 p105. *Nature* 397: 363–368, 1999.
21. Benbrook DM and Jones NC. Heterodimer formation between CREB and JUN proteins. *Oncogene* 5: 295–302, 1990.
22. Bharti A, Kraeft SK, Gounder M, Pandey P, Jin S, Yuan ZM, Lees-Miller SP, Weichselbaum R, Weaver D, Chen LB, Kufe D, and Kharbanda S. Inactivation of DNA-dependent protein kinase by protein kinase Cdelta: implications for apoptosis. *Mol Cell Biol* 18: 6719–6728, 1998.
23. Björkblom B, Östman N, Hongisto V, Komarovski V, Filen JJ, Nyman TA, Kallunki T, Courtney MJ, and Coffey ET. Constitutively active cytoplasmic c-Jun N-terminal kinase 1 is a dominant regulator of dendritic architecture: role of microtubule-associated protein 2 as an effector. *J Neurosci* 25: 6350–6361, 2005.
24. Björkblom B, Vainio JC, Hongisto V, Herdegen T, Courtney MJ, and Coffey ET. All JNKs can kill, but nuclear localization is critical for neuronal death. *J Biol Chem* 283: 19704–19713, 2008.
25. Blass M, Kronfeld I, Kazimirsky G, Blumberg PM, and Brodie C. Tyrosine phosphorylation of protein kinase Cdelta is essential for its apoptotic effect in response to etoposide. *Mol Cell Biol* 22: 182–195, 2002.
26. Bloom D, Dhakshinamoorthy S, and Jaiswal AK. Site-directed mutagenesis of cysteine to serine in the DNA binding region of Nrf2 decreases its capacity to upregulate antioxidant response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene. *Oncogene* 21: 2191–2200, 2002.
27. Bloomfield KL, Osborne SA, Kennedy DD, Clarke FM, and Tonissen KF. Thioredoxin-mediated redox control of the transcription factor Sp1 and regulation of the thioredoxin gene promoter. *Gene* 319: 107–116, 2003.
28. Bourdon JC. p53 and its isoforms in cancer. *Br J Cancer* 97: 277–282, 2007.
29. Breitschopf K, Zeiher AM, and Dimmeler S. Pro-atherogenic factors induce telomerase inactivation in endothelial cells through an Akt-dependent mechanism. *FEBS Lett* 493: 21–25, 2001.
30. Camper-Kirby D, Welch S, Walker A, Shiraishi I, Setchell KD, Schaefer E, Kajstura J, Anversa P, and Sussman MA. Myocardial Akt activation and gender: increased nuclear activity in females versus males. *Circ Res* 88: 1020–1027, 2001.
31. Carpenter G. Employment of the epidermal growth factor receptor in growth factor-independent signaling pathways. *J Cell Biol* 146: 697–702, 1999.
32. Chandrasekaran V and Taylor EW. Molecular modeling of the oxidized form of nuclear factor-kappa B suggests a mechanism for redox regulation of DNA binding and transcriptional activation. *J Mol Graph Model* 26: 861–867, 2008.
33. Chen CY, Willard D, and Rudolph J. Redox regulation of SH2-domain-containing protein tyrosine phosphatases by two backdoor cysteines. *Biochemistry* 48: 1399–1409, 2009.
34. Chen H, Li X, and Epstein PN. MnSOD and catalase transgenes demonstrate that protection of islets from oxidative stress does not alter cytokine toxicity. *Diabetes* 54: 1437–1446, 2005.
35. Chinenov Y and Kerppola TK. Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene* 20: 2438–2452, 2001.
36. Chou WC, Chen HY, Yu SL, Cheng L, Yang PC, and Dang CV. Arsenic suppresses gene expression in promyelocytic leukemia cells partly through Sp1 oxidation. *Blood* 106: 304–310, 2005.
37. Chughtai N, Schimchowitsch S, Lebrun JJ, and Ali S. Prolactin induces SHP-2 association with Stat5, nuclear translocation, and binding to the beta-casein gene promoter in mammary cells. *J Biol Chem* 277: 31107–31114, 2002.
38. Claiborne A, Yeh JI, Mallett TC, Luba J, Crane EJ 3rd, Charrier V, and Parsonage D. Protein-sulfenic acids: diverse roles for an unlikely player in enzyme catalysis and redox regulation. *Biochemistry* 38: 15407–15416, 1999.
39. Conour JE, Graham WV, and Gaskins HR. A combined in vitro/bioinformatic investigation of redox regulatory mechanisms governing cell cycle progression. *Physiol Genom* 18: 196–205, 2004.
40. Darnell JE Jr, Kerr IM, and Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and

other extracellular signaling proteins. *Science* 264: 1415–1421, 1994.
41. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell* 103: 239–252, 2000.
42. de Jong RN and van der Vliet PC. Mechanism of DNA replication in eukaryotic cells: cellular host factors stimulating adenovirus DNA replication. *Gene* 236: 1–12, 1999.
43. Del Maestro R and McDonald W. Subcellular localization of superoxide dismutases, glutathione peroxidase and catalase in developing rat cerebral cortex. *Mech Ageing Dev* 48: 15–31, 1989.
44. Delphin C, Cahen P, Lawrence JJ, and Baudier J. Characterization of baculovirus recombinant wild-type p53: dimerization of p53 is required for high-affinity DNA binding and cysteine oxidation inhibits p53 DNA binding. *Eur J Biochem* 223: 683–692, 1994.
45. Denu JM and Tanner KG. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* 37: 5633–5642, 1998.
46. DeVries TA, Neville MC, and Reyland ME. Nuclear import of PKCdelta is required for apoptosis: identification of a novel nuclear import sequence. *EMBO J* 21: 6050–6060, 2002.
47. Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M, and Talalay P. Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci U S A* 99: 11908–11913, 2002.
48. Dunphy WG and Kumagai A. The cdc25 protein contains an intrinsic phosphatase activity. *Cell* 67: 189–196, 1991.
49. Egloff MP, Johnson DF, Moorhead G, Cohen PT, Cohen P, and Barford D. Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J* 16: 1876–1887, 1997.
50. Eklund H, Cambillau C, Sjoberg BM, Holmgren A, Jornvall H, Hoog JO, and Branden CI. Conformational and functional similarities between glutaredoxin and thioredoxins. *EMBO J* 3: 1443–1449, 1984.
51. Ema M, Hirota K, Mimura J, Abe H, Yodoi J, Sogawa K, Poellinger L, and Fujii-Kuriyama Y. Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. *EMBO J* 18: 1905–1914, 1999.
52. Esposito F, Cuccovillo F, Morra F, Russo T, and Cimino F. DNA binding activity of the glucocorticoid receptor is sensitive to redox changes in intact cells. *Biochim Biophys Acta* 1260: 308–314, 1995.
53. Fan M and Chambers TC. Role of mitogen-activated protein kinases in the response of tumor cells to chemotherapy. *Drug Resist Update* 4: 253–267, 2001.
54. Feng GS, Hui CC, and Pawson T. SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases. *Science* 259: 1607–1611, 1993.
55. Freeman RM Jr, Plutzky J, and Neel BG. Identification of a human src homology 2-containing protein-tyrosine-phosphatase: a putative homolog of *Drosophila* corkscrew. *Proc Natl Acad Sci U S A* 89: 11239–11243, 1992.
56. Fu W, Begley JG, Killen MW, and Mattson MP. Anti-apoptotic role of telomerase in pheochromocytoma cells. *J Biol Chem* 274: 7264–7271, 1999.
57. Gaiddon C, Moorthy NC, and Prives C. Ref-1 regulates the transactivation and pro-apoptotic functions of p53 in vivo. *EMBO J* 18: 5609–5621, 1999.
58. Galang CK and Hauser CA. Cooperative DNA binding of the human HoxB5 (Hox-2.1) protein is under redox regulation in vitro. *Mol Cell Biol* 13: 4609–4617, 1993.
59. Georgiadis MM, Luo M, Gaur RK, Delaplane S, Li X, and Kelley MR. Evolution of the redox function in mammalian apurinic/apyrimidinic endonuclease. *Mutat Res* 643: 54–63, 2008.
60. Ghayur T, Hugunin M, Talanian RV, Ratnofsky S, Quinlan C, Emoto Y, Pandey P, Datta R, Huang Y, Kharbanda S, Allen H, Kamen R, Wong W, and Kufe D. Proteolytic activation of protein kinase C delta by an ICE/CED 3-like protease induces characteristics of apoptosis. *J Exp Med* 184: 2399–2404, 1996.
61. Giebler HA, Loring JE, van Orden K, Colgin MA, Garrus JE, Escudero KW, Brauweiler A, and Nyborg JK. Anchoring of CREB binding protein to the human T-cell leukemia virus type 1 promoter: a molecular mechanism of Tax transactivation. *Mol Cell Biol* 17: 5156–5164, 1997.
62. Gladyshev VN, Liu A, Novoselov SV, Krysan K, Sun QA, Kryukov VM, Kryukov GV, and Lou MF. Identification and characterization of a new mammalian glutaredoxin (thioltransferase), Grx2. *J Biol Chem* 276: 30374–30380, 2001.
63. Goldkorn T, Balaban N, Matsukuma K, Chea V, Gould R, Last J, Chan C, and Chavez C. EGF-receptor phosphorylation and signaling are targeted by H_2O_2 redox stress. *Am J Respir Cell Mol Biol* 19: 786–798, 1998.
64. Goren I, Tavor E, Goldblum A, and Honigman A. Two cysteine residues in the DNA-binding domain of CREB control binding to CRE and CREB-mediated gene expression. *J Mol Biol* 313: 695–709, 2001.
65. Gronostajski RM. Roles of the NFI/CTF gene family in transcription and development. *Gene* 249: 31–45, 2000.
66. Guan JS, Haggarty SJ, Giacometti E, Dannenberg JH, Joseph N, Gao J, Nieland TJ, Zhou Y, Wang X, Mazitschek R, Bradner JE, DePinho RA, Jaenisch R, and Tsai LH. HDAC2 negatively regulates memory formation and synaptic plasticity. *Nature* 459: 55–60, 2009.
67. Guehmann S, Vorbrueggen G, Kalkbrenner F, and Moelling K. Reduction of a conserved Cys is essential for Myb DNA-binding. *Nucleic Acids Res* 20: 2279–2286, 1992.
68. Haendeler J, Hoffmann J, Brandes RP, Zeiher AM, and Dimmeler S. Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707. *Mol Cell Biol* 23: 4598–4610, 2003.
69. Haendeler J, Hoffmann J, Diehl JF, Vasa M, Spyridopoulos I, Zeiher AM, and Dimmeler S. Antioxidants inhibit nuclear export of telomerase reverse transcriptase and delay replicative senescence of endothelial cells. *Circ Res* 94: 768–775, 2004.
70. Haendeler J, Hoffmann J, Rahman S, Zeiher AM, and Dimmeler S. Regulation of telomerase activity and anti-apoptotic function by protein-protein interaction and phosphorylation. *FEBS Lett* 536: 180–186, 2003.
71. Hagiwara M, Brindle P, Harootunian A, Armstrong R, Rivier J, Vale W, Tsien R, and Montminy MR. Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A. *Mol Cell Biol* 13: 4852–4859, 1993.
72. Hai T and Curran T. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc Natl Acad Sci U S A* 88: 3720–3724, 1991.

73. Hainaut P and Milner J. Redox modulation of p53 conformation and sequence-specific DNA binding in vitro. *Cancer Res* 53: 4469–4473, 1993.
74. Hansen JM, Gong SG, Philbert M, and Harris C. Misregulation of gene expression in the redox-sensitive NF-kappab-dependent limb outgrowth pathway by thalidomide. *Dev Dyn* 225: 186–194, 2002.
75. Hansen JM, Moriarty-Craige S, and Jones DP. Nuclear and cytoplasmic peroxiredoxin-1 differentially regulate NF-kappaB activities. *Free Radic Biol Med* 43: 282–288, 2007.
76. Hansen JM, Watson WH, and Jones DP. Compartmentation of Nrf-2 redox control: regulation of cytoplasmic activation by glutathione and DNA binding by thioredoxin-1. *Toxicol Sci* 82: 308–317, 2004.
77. Hanson S, Kim E, and Deppert W. Redox factor 1 (Ref-1) enhances specific DNA binding of p53 by promoting p53 tetramerization. *Oncogene* 24: 1641–1647, 2005.
78. Hayashi S, Hajiro-Nakanishi K, Makino Y, Eguchi H, Yodoi J, and Tanaka H. Functional modulation of estrogen receptor by redox state with reference to thioredoxin as a mediator. *Nucleic Acids Res* 25: 4035–4040, 1997.
79. Hirota K, Matsui M, Iwata S, Nishiyama A, Mori K, and Yodoi J. AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proc Natl Acad Sci U S A* 94: 3633–3638, 1997.
80. Hirota K, Matsui M, Murata M, Takashima Y, Cheng FS, Itoh T, Fukuda K, and Yodoi J. Nucleoredoxin, glutaredoxin, and thioredoxin differentially regulate NF-kappaB, AP-1, and CREB activation in HEK293 cells. *Biochem Biophys Res Commun* 274: 177–182, 2000.
81. Hirota K, Murata M, Sachi Y, Nakamura H, Takeuchi J, Mori K, and Yodoi J. Distinct roles of thioredoxin in the cytoplasm and in the nucleus: a two-step mechanism of redox regulation of transcription factor NF-kappaB. *J Biol Chem* 274: 27891–27897, 1999.
82. Hof P, Pluskey S, Dhe-Paganon S, Eck MJ, and Shoelson SE. Crystal structure of the tyrosine phosphatase SHP-2. *Cell* 92: 441–450, 1998.
83. Hoh J, Jin S, Parrado T, Edington J, Levine AJ, and Ott J. The p53MH algorithm and its application in detecting p53-responsive genes. *Proc Natl Acad Sci U S A* 99: 8467–8472, 2002.
84. Hollstein M, Sidransky D, Vogelstein B, and Harris CC. p53 mutations in human cancers. *Science* 253: 49–53, 1991.
85. Holmgren A. Thioredoxin 6: the amino acid sequence of the protein from *Escherichia coli* B. *Eur J Biochem* 6: 475–484, 1968.
86. Holmgren A. Glutathione-dependent synthesis of deoxyribonucleotides: purification and characterization of glutaredoxin from *Escherichia coli*. *J Biol Chem* 254: 3664–3671, 1979.
87. Holmgren A. Thioredoxin. *Annu Rev Biochem* 54: 237–271, 1985.
88. Holmgren A, Soderberg BO, Eklund H, and Branden CI. Three-dimensional structure of *Escherichia coli* thioredoxin-S2 to 2.8 A resolution. *Proc Natl Acad Sci U S A* 72: 2305–2309, 1975.
89. Holt SE, Aisner DL, Baur J, Tesmer VM, Dy M, Ouellette M, Trager JB, Morin GB, Toft DO, Shay JW, Wright WE, and White MA. Functional requirement of p23 and Hsp90 in telomerase complexes. *Genes Dev* 13: 817–826, 1999.
90. Huang LE, Arany Z, Livingston DM, and Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. *J Biol Chem* 271: 32253–32259, 1996.
91. Huang RP and Adamson ED. Characterization of the DNA-binding properties of the early growth response-1 (Egr-1) transcription factor: evidence for modulation by a redox mechanism. *DNA Cell Biol* 12: 265–273, 1993.
92. Hughes MN. Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite. *Biochim Biophys Acta* 1411: 263–272, 1999.
93. Hutchison KA, Matic G, Meshinchi S, Bresnick EH, and Pratt WB. Redox manipulation of DNA binding activity and BuGR epitope reactivity of the glucocorticoid receptor. *J Biol Chem* 266: 10505–10509, 1991.
94. Isakov N, Witte S, and Altman A. PICOT-HD: a highly conserved protein domain that is often associated with thioredoxin and glutaredoxin modules. *Trends Biochem Sci* 25: 537–539, 2000.
95. Ischiropoulos H, Zhu L, Chen J, Tsai M, Martin JC, Smith CD, and Beckman JS. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch Biochem Biophys* 298: 431–437, 1992.
96. Jakob S, Schroeder P, Lukosz M, Buchner N, Spyridopoulos I, Altschmied J, and Haendeler J. Nuclear protein tyrosine phosphatase shp-2 is one important negative regulator of nuclear export of telomerase reverse transcriptase. *J Biol Chem* 283: 33155–33161, 2008.
97. Jayaraman L, Murthy KG, Zhu C, Curran T, Xanthoudakis S, and Prives C. Identification of redox/repair protein Ref-1 as a potent activator of p53. *Genes Dev* 11: 558–570, 1997.
98. Johnson GL and Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298: 1911–1912, 2002.
99. Johnson LN, Noble ME, and Owen DJ. Active and inactive protein kinases: structural basis for regulation. *Cell* 85: 149–158, 1996.
100. Kaczynski J, Cook T, and Urrutia R. Sp1- and Krüppel-like transcription factors. *Genome Biol* 4: 206, 2003.
101. Kagoshima H, Akamatsu Y, Ito Y, and Shigesada K. Functional dissection of the alpha and beta subunits of transcription factor PEBP2 and the redox susceptibility of its DNA binding activity. *J Biol Chem* 271: 33074–33082, 1996.
102. Kalinina EV, Chernov NN, and Saprin AN. Involvement of thio-, peroxi-, and glutaredoxins in cellular redox-dependent processes. *Biochemistry (Mosc)* 73: 1493–1510, 2008.
103. Kambe F, Nomura Y, Okamoto T, and Seo H. Redox regulation of thyroid-transcription factors, Pax-8 and TTF-1, is involved in their increased DNA-binding activities by thyrotropin in rat thyroid FRTL-5 cells. *Mol Endocrinol* 10: 801–812, 1996.
104. Kaneshiro K, Tsutsumi S, Tsuji S, Shirahige K, and Aburatani H. An integrated map of p53-binding sites and histone modification in the human ENCODE regions. *Genomics* 89: 178–188, 2007.
105. Kang SS, Kwon T, Kwon DY, and Do SI. Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J Biol Chem* 274: 13085–13090, 1999.
106. Kemble DJ and Sun G. Direct and specific inactivation of protein tyrosine kinases in the Src and FGFR families by reversible cysteine oxidation. *Proc Natl Acad Sci U S A* 106: 5070–5075, 2009.
107. Kensler TW, Wakabayashi N, and Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* 47: 89–116, 2007.
108. Khokhlatchev AV, Canagarajah B, Wilsbacher J, Robinson M, Atkinson M, Goldsmith E, and Cobb MH. Phosphor-

ylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. *Cell* 93: 605–615, 1998.
109. Kinnula VL, Lehtonen S, Sormunen R, Kaarteenaho-Wiik R, Kang SW, Rhee SG, and Soini Y. Overexpression of peroxiredoxins I, II, III, V, and VI in malignant mesothelioma. *J Pathol* 196: 316–323, 2002.
110. Knoepfel L, Steinkuhler C, Carri MT, and Rotilio G. Role of zinc-coordination and of the glutathione redox couple in the redox susceptibility of human transcription factor Sp1. *Biochem Biophys Res Commun* 201: 871–877, 1994.
111. Kuge S, Toda T, Iizuka N, and Nomoto A. Crm1 (XpoI) dependent nuclear export of the budding yeast transcription factor yAP-1 is sensitive to oxidative stress. *Genes Cells* 3: 521–532, 1998.
112. Kumagai A and Dunphy WG. The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell-free system. *Cell* 64: 903–914, 1991.
113. Kwon J, Lee SR, Yang KS, Ahn Y, Kim YJ, Stadtman ER, and Rhee SG. Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. *Proc Natl Acad Sci U S A* 101: 16419–16424, 2004.
114. Lam MH, Michell BJ, Fodero-Tavoletti MT, Kemp BE, Tonks NK, and Tiganis T. Cellular stress regulates the nucleocytoplasmic distribution of the protein-tyrosine phosphatase TCPTP. *J Biol Chem* 276: 37700–37707, 2001.
115. Lamalice L, Houle F, Jourdan G, and Huot J. Phosphorylation of tyrosine 1214 on VEGFR2 is required for VEGF-induced activation of Cdc42 upstream of SAPK2/p38. *Oncogene* 23: 434–445, 2004.
116. Lando D, Pongratz I, Poellinger L, and Whitelaw ML. A redox mechanism controls differential DNA binding activities of hypoxia-inducible factor (HIF) 1alpha and the HIF-like factor. *J Biol Chem* 275: 4618–4627, 2000.
117. Lane DP. p53, guardian of the genome. *Nature* 358: 15–16, 1992.
118. Laurent TC, Moore EC, and Reichard P. Enzymatic synthesis of deoxyribonucleotides. IV, isolation and characterization of thioredoxin, the hydrogen donor from *Escherichia coli* B. *J Biol Chem* 239: 3436–3444, 1964.
119. Lee W, Haslinger A, Karin M, and Tjian R. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. *Nature* 325: 368–372, 1987.
120. Leslie NR, Bennett D, Lindsay YE, Stewart H, Gray A, and Downes CP. Redox regulation of PI 3-kinase signalling via inactivation of PTEN. *EMBO J* 22: 5501–5510, 2003.
121. Li L, Lorenzo PS, Bogi K, Blumberg PM, and Yuspa SH. Protein kinase Cdelta targets mitochondria, alters mitochondrial membrane potential, and induces apoptosis in normal and neoplastic keratinocytes when overexpressed by an adenoviral vector. *Mol Cell Biol* 19: 8547–8558, 1999.
122. Liang X, Lu B, Scott GK, Chang CH, Baldwin MA, and Benz CC. Oxidant stress impaired DNA-binding of estrogen receptor from human breast cancer. *Mol Cell Endocrinol* 146: 151–161, 1998.
123. Lillig CH, Berndt C, and Holmgren A. Glutaredoxin systems. *Biochim Biophys Acta* 1780: 1304–1317, 2008.
124. Lillig CH and Holmgren A. Thioredoxin and related molecules: from biology to health and disease. *Antioxid Redox Signal* 9: 25–47, 2007.
125. Lin A and Dibling B. The true face of JNK activation in apoptosis. *Aging Cell* 1: 112–116, 2002.
126. Lind C, Gerdes R, Schuppe-Koistinen I, and Cotgreave IA. Studies on the mechanism of oxidative modification of human glyceraldehyde-3-phosphate dehydrogenase by glutathione: catalysis by glutaredoxin. *Biochem Biophys Res Commun* 247: 481–486, 1998.
127. Liu H, Nishitoh H, Ichijo H, and Kyriakis JM. Activation of apoptosis signal-regulating kinase 1 (ASK1) by tumor necrosis factor receptor-associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin. *Mol Cell Biol* 20: 2198–2208, 2000.
128. Liu J and Lin A. Role of JNK activation in apoptosis: a double-edged sword. *Cell Res* 15: 36–42, 2005.
129. Lönn ME, Hudemann C, Berndt C, Cherkasov V, Capani F, Holmgren A, and Lillig CH. Expression pattern of human glutaredoxin 2 isoforms: identification and characterization of two testis/cancer cell-specific isoforms. *Antioxid Redox Signal* 10: 547–557, 2008.
130. Lorenzen JA, Dadabay CY, and Fischer EH. COOH-terminal sequence motifs target the T cell protein tyrosine phosphatase to the ER and nucleus. *J Cell Biol* 131: 631–643, 1995.
131. Lundberg M, Johansson C, Chandra J, Enoksson M, Jacobsson G, Ljung J, Johansson M, and Holmgren A. Cloning and expression of a novel human glutaredoxin (Grx2) with mitochondrial and nuclear isoforms. *J Biol Chem* 276: 26269–26275, 2001.
132. Luo M, Delaplane S, Jiang A, Reed A, He Y, Fishel M, Nyland RL 2nd, Borch RF, Qiao X, Georgiadis MM, and Kelley MR. Role of the multifunctional DNA repair and redox signaling protein Ape1/Ref-1 in cancer and endothelial cells: small-molecule inhibition of the redox function of Ape1. *Antioxid Redox Signal* 10: 1853–1867, 2008.
133. Lysell J, Stjernholm Vladic Y, Ciarlo N, Holmgren A, and Sahlin L. Immunohistochemical determination of thioredoxin and glutaredoxin distribution in the human cervix, and possible relation to cervical ripening. *Gynecol Endocrinol* 17: 303–310, 2003.
134. Makino Y, Okamoto K, Yoshikawa N, Aoshima M, Hirota K, Yodoi J, Umesono K, Makino I, and Tanaka H. Thioredoxin: a redox-regulating cellular cofactor for glucocorticoid hormone action: cross talk between endocrine control of stress response and cellular antioxidant defense system. *J Clin Invest* 98: 2469–2477, 1996.
135. Makino Y, Yoshikawa N, Okamoto K, Hirota K, Yodoi J, Makino I, and Tanaka H. Direct association with thioredoxin allows redox regulation of glucocorticoid receptor function. *J Biol Chem* 274: 3182–3188, 1999.
136. Manevich Y, Feinstein SI, and Fisher AB. Activation of the antioxidant enzyme 1-CYS peroxiredoxin requires glutathionylation mediated by heterodimerization with pi GST. *Proc Natl Acad Sci U S A* 101: 3780–3785, 2004.
137. Markovic J, Borras C, Ortega A, Sastre J, Vina J, and Pallardo FV. Glutathione is recruited into the nucleus in early phases of cell proliferation. *J Biol Chem* 282: 20416–20424, 2007.
138. Markovic J, Mora NJ, Broseta AM, Gimeno A, de-la-Concepcion N, Vina J, and Pallardo FV. The depletion of nuclear glutathione impairs cell proliferation in 3t3 fibroblasts. *PLoS One* 4: e6413, 2009.
139. Martin JL. Thioredoxin: a fold for all reasons. *Structure* 3: 245–250, 1995.
140. Mates JM, Segura JA, Alonso FJ, and Marquez J. Intracellular redox status and oxidative stress: implications for cell proliferation, apoptosis, and carcinogenesis. *Arch Toxicol* 82: 273–299, 2008.
141. Matthews JR, Botting CH, Panico M, Morris HR, and Hay RT. Inhibition of NF-kappaB DNA binding by nitric oxide. *Nucleic Acids Res* 24: 2236–2242, 1996.

142. Matthews JR, Wakasugi N, Virelizier JL, Yodoi J, and Hay RT. Thioredoxin regulates the DNA binding activity of NF-kappa B by reduction of a disulphide bond involving cysteine 62. *Nucleic Acids Res* 20: 3821–3830, 1992.
143. Mattila E, Auvinen K, Salmi M, and Ivaska J. The protein tyrosine phosphatase TCPTP controls VEGFR2 signalling. *J Cell Sci* 121: 3570–3580, 2008.
144. Mattson MP and Klapper W. Emerging roles for telomerase in neuronal development and apoptosis. *J Neurosci Res* 63: 1–9, 2001.
145. Mayr B and Montminy M. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol* 2: 599–609, 2001.
146. McBride AA, Klausner RD, and Howley PM. Conserved cysteine residue in the DNA-binding domain of the bovine papillomavirus type 1 E2 protein confers redox regulation of the DNA-binding activity in vitro. *Proc Natl Acad Sci U S A* 89: 7531–7535, 1992.
147. McEwan IJ. Nuclear receptors: one big family. *Methods Mol Biol* 505: 3–18, 2009.
148. McMahon M, Itoh K, Yamamoto M, Chanas SA, Henderson CJ, McLellan LI, Wolf CR, Cavin C, and Hayes JD. The Cap'n'Collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. *Cancer Res* 61: 3299–3307, 2001.
149. Meister A and Anderson ME. Glutathione. *Annu Rev Biochem* 52: 711–760, 1983.
150. Meng TC, Fukada T, and Tonks NK. Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. *Mol Cell* 9: 387–399, 2002.
151. Mirkovic N, Voehringer DW, Story MD, McConkey DJ, McDonnell TJ, and Meyn RE. Resistance to radiation-induced apoptosis in Bcl-2-expressing cells is reversed by depleting cellular thiols. *Oncogene* 15: 1461–1470, 1997.
152. Monteiro G, Horta BB, Pimenta DC, Augusto O, and Netto LE. Reduction of 1-Cys peroxiredoxins by ascorbate changes the thiol-specific antioxidant paradigm, revealing another function of vitamin C. *Proc Natl Acad Sci U S A* 104: 4886–4891, 2007.
153. Morel Y and Barouki R. The repression of nuclear factor I/CCAAT transcription factor (NFI/CTF) transactivating domain by oxidative stress is mediated by a critical cysteine (Cys-427). *Biochem J* 348: 235–240, 2000.
154. Morel Y, Coumoul X, Nalpas A, and Barouki R. Nuclear factor I/CCAAT box transcription factor trans-activating domain is a negative sensor of cellular stress. *Mol Pharmacol* 58: 1239–1246, 2000.
155. Myrset AH, Bostad A, Jamin N, Lirsac PN, Toma F, and Gabrielsen OS. DNA and redox state induced conformational changes in the DNA-binding domain of the Myb oncoprotein. *EMBO J* 12: 4625–4633, 1993.
156. Nakshatri H, Bhat-Nakshatri P, and Currie RA. Subunit association and DNA binding activity of the heterotrimeric transcription factor NF-Y is regulated by cellular redox. *J Biol Chem* 271: 28784–28791, 1996.
157. Natoli G and De Santa F. Shaping alternative NF-kappaB-dependent gene expression programs: new clues to specificity. *Cell Death Differ* 13: 693–696, 2006.
158. Nguyen T, Pickett CB, and Nioi P. The Nrf2-ARE signaling pathway and its activation by oxidative stress. *J Biol Chem* 284: 13291–13295, 2009.
159. Nguyen T, Yang CS, and Pickett CB. The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. *Free Radic Biol Med* 37: 433–441, 2004.
160. Nigg EA, Baeuerle PA, and Luhrmann R. Nuclear import-export: in search of signals and mechanisms. *Cell* 66: 15–22, 1991.
161. Nolan GP and Baltimore D. The inhibitory ankyrin and activator Rel proteins. *Curr Opin Genet Dev* 2: 211–220, 1992.
162. Nordberg J and Arner ES. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* 31: 1287–1312, 2001.
163. Nott A, Watson PM, Robinson JD, Crepaldi L, and Riccio A. S-Nitrosylation of histone deacetylase 2 induces chromatin remodelling in neurons. *Nature* 455: 411–415, 2008.
164. Novak A, Goyal N, and Gronostajski RM. Four conserved cysteine residues are required for the DNA binding activity of nuclear factor I. *J Biol Chem* 267: 12986–12990, 1992.
165. O'Neill E and Kolch W. Conferring specificity on the ubiquitous Raf/MEK signalling pathway. *Br J Cancer* 90: 283–288, 2004.
166. Oh H, Taffet GE, Youker KA, Entman ML, Overbeek PA, Michael LH, and Schneider MD. Telomerase reverse transcriptase promotes cardiac muscle cell proliferation, hypertrophy, and survival. *Proc Natl Acad Sci U S A* 98: 10308–10313, 2001.
167. Okamoto K, Tanaka H, Ogawa H, Makino Y, Eguchi H, Hayashi S, Yoshikawa N, Poellinger L, Umesono K, and Makino I. Redox-dependent regulation of nuclear import of the glucocorticoid receptor. *J Biol Chem* 274: 10363–10371, 1999.
168. Okuno H, Akahori A, Sato H, Xanthoudakis S, Curran T, and Iba H. Escape from redox regulation enhances the transforming activity of Fos. *Oncogene* 8: 695–701, 1993.
169. Ordway JM, Eberhart D, and Curran T. Cysteine 64 of Ref-1 is not essential for redox regulation of AP-1 DNA binding. *Mol Cell Biol* 23: 4257–4266, 2003.
170. Osawa M, Itoh S, Ohta S, Huang Q, Berk BC, Marmarosh NL, Che W, Ding B, Yan C, and Abe J. ERK1/2 associates with the c-Met-binding domain of growth factor receptor-bound protein 2 (Grb2)-associated binder-1 (Gab1): role in ERK1/2 and early growth response factor-1 (Egr-1) nuclear accumulation. *J Biol Chem* 279: 29691–29699, 2004.
171. Pai HV, Starke DW, Lesnefsky EJ, Hoppel CL, and Mieyal JJ. What is the functional significance of the unique location of glutaredoxin 1 (GRx1) in the intermembrane space of mitochondria? *Antioxid Redox Signal* 9: 2027–2033, 2007.
172. Pallardo FV, Markovic J, Garcia JL, and Vina J. Role of nuclear glutathione as a key regulator of cell proliferation. *Mol Aspects Med* 30: 77–85, 2009.
173. Pan S and Berk BC. Glutathiolation regulates tumor necrosis factor-alpha-induced caspase-3 cleavage and apoptosis: key role for glutaredoxin in the death pathway. *Circ Res* 100: 213–219, 2007.
174. Paolillo M, Feliciello A, Porcellini A, Garbi C, Bifulco M, Schinelli S, Ventra C, Stabile E, Ricciardelli G, Schettini G, and Avvedimento EV. The type and the localization of cAMP-dependent protein kinase regulate transmission of cAMP signals to the nucleus in cortical and cerebellar granule cells. *J Biol Chem* 274: 6546–6552, 1999.
175. Papavassiliou AG. The CREB/ATF family of transcription factors: modulation by reversible phosphorylation. *Anticancer Res* 14: 1801–1805, 1994.
176. Park SW, Huq MD, Hu X, and Wei LN. Tyrosine nitration on p65: a novel mechanism to rapidly inactivate nuclear factor-kappaB. *Mol Cell Proteomics* 4: 300–309, 2005.

177. Parks D, Bolinger R, and Mann K. Redox state regulates binding of p53 to sequence-specific DNA, but not to nonspecific or mismatched DNA. *Nucleic Acids Res* 25: 1289–1295, 1997.
178. Parsons SJ and Parsons JT. Src family kinases, key regulators of signal transduction. *Oncogene* 23: 7906–7909, 2004.
179. Peng CY, Graves PR, Thoma RS, Wu Z, Shaw AS, and Piwnica-Worms H. Mitotic and G_2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science* 277: 1501–1505, 1997.
180. Persson C, Sjoblom T, Groen A, Kappert K, Engstrom U, Hellman U, Heldin CH, den Hertog J, and Ostman A. Preferential oxidation of the second phosphatase domain of receptor-like PTP-alpha revealed by an antibody against oxidized protein tyrosine phosphatases. *Proc Natl Acad Sci U S A* 101: 1886–1891, 2004.
181. Pineda-Molina E, Klatt P, Vazquez J, Marina A, Garcia de Lacoba M, Perez-Sala D, and Lamas S. Glutathionylation of the p50 subunit of NF-kappaB: a mechanism for redox-induced inhibition of DNA binding. *Biochemistry* 40: 14134–14142, 2001.
182. Qanungo S, Starke DW, Pai HV, Mieyal JJ, and Nieminen AL. Glutathione supplementation potentiates hypoxic apoptosis by S-glutathionylation of p65-NFkappaB. *J Biol Chem* 282: 18427–18436, 2007.
183. Quan X, Tsoulos P, Kuritzky A, Zhang R, and Stochaj U. The carrier Msn5p/Kap142p promotes nuclear export of the hsp70 Ssa4p and relocates in response to stress. *Mol Microbiol* 62: 592–609, 2006.
184. Quan X, Yu J, Bussey H, and Stochaj U. The localization of nuclear exporters of the importin-beta family is regulated by Snf1 kinase, nutrient supply and stress. *Biochim Biophys Acta* 1773: 1052–1061, 2007.
185. Rainwater R, Parks D, Anderson ME, Tegtmeyer P, and Mann K. Role of cysteine residues in regulation of p53 function. *Mol Cell Biol* 15: 3892–3903, 1995.
186. Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, and Kensler TW. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proc Natl Acad Sci U S A* 98: 3410–3415, 2001.
187. Rhee SG, Chae HZ, and Kim K. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic Biol Med* 38: 1543–1552, 2005.
188. Rhee SG, Kang SW, Chang TS, Jeong W, and Kim K. Peroxiredoxin, a novel family of peroxidases. *IUBMB Life* 52: 35–41, 2001.
189. Richards JP, Bächinger HP, Goodman RH, and Brennan RG. Analysis of the structural properties of cAMP-responsive element-binding protein (CREB) and phosphorylated CREB. *J Biol Chem* 271: 13716–13723, 1996.
190. Rodriguez-Manzaneque MT, Tamarit J, Belli G, Ros J, and Herrero E. Grx5 is a mitochondrial glutaredoxin required for the activity of iron/sulfur enzymes. *Mol Biol Cell* 13: 1109–1121, 2002.
191. Rogers LK, Gupta S, Welty SE, Hansen TN, and Smith CV. Nuclear and nucleolar glutathione reductase, peroxidase, and transferase activities in livers of male and female Fischer-344 rats. *Toxicol Sci* 69: 279–285, 2002.
192. Rudolph KL, Chang S, Millard M, Schreiber-Agus N, and DePinho RA. Inhibition of experimental liver cirrhosis in mice by telomerase gene delivery. *Science* 287: 1253–1258, 2000.
193. Safe S and Abdelrahim M. Sp transcription factor family and its role in cancer. *Eur J Cancer* 41: 2438–2448, 2005.
194. Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, and Ichijo H. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* 17: 2596–2606, 1998.
195. Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H, and Elledge SJ. Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. *Science* 277: 1497–1501, 1997.
196. Sanchez-Perez I, Murguia JR, and Perona R. Cisplatin induces a persistent activation of JNK that is related to cell death. *Oncogene* 16: 533–540, 1998.
197. Sassone-Corsi P. Transcription factors responsive to cAMP. *Annu Rev Cell Dev Biol* 11: 355–377, 1995.
198. Savitsky PA and Finkel T. Redox regulation of Cdc25C. *J Biol Chem* 277: 20535–20540, 2002.
199. Schäfer D, Hamm-Kunzelmann B, Hermfisse U, and Brand K. Differences in DNA-binding efficiency of Sp1 to aldolase and pyruvate kinase promoter correlate with altered redox states in resting and proliferating rat thymocytes. *FEBS Lett* 391: 35–38, 1996.
200. Schafer FQ and Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 30: 1191–1212, 2001.
201. Scheidereit C. IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. *Oncogene* 25: 6685–6705, 2006.
202. Schröder E and Ponting CP. Evidence that peroxiredoxins are novel members of the thioredoxin fold superfamily. *Protein Sci* 7: 2465–2468, 1998.
203. Schroeder P, Popp R, Wiegand B, Altschmied J, and Haendeler J. Nuclear redox-signaling is essential for apoptosis inhibition in endothelial cells: important role for nuclear thioredoxin-1. *Arterioscler Thromb Vasc Biol* 27: 2325–2331, 2007.
204. Schulze PC, De Keulenaer GW, Yoshioka J, Kassik KA, and Lee RT. Vitamin D_3-upregulated protein-1 (VDUP-1) regulates redox-dependent vascular smooth muscle cell proliferation through interaction with thioredoxin. *Circ Res* 91: 689–695, 2002.
205. Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol* 15: 551–578, 1999.
206. Semenza GL. HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus. *Cell* 107: 1–3, 2001.
207. Sen R and Baltimore D. Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell* 47: 921–928, 1986.
208. Sen R and Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 46: 705–716, 1986.
209. Shenolikar S. Protein serine/threonine phosphatases: new avenues for cell regulation. *Annu Rev Cell Biol* 10: 55–86, 1994.
210. Shigemori K, Ishizaki T, Matsukawa S, Sakai A, Nakai T, and Miyabo S. Adenine nucleotides via activation of ATP-sensitive K + channels modulate hypoxic response in rat pulmonary artery. *Am J Physiol* 270: L803–L809, 1996.
211. Shiraishi I, Melendez J, Ahn Y, Skavdahl M, Murphy E, Welch S, Schaefer E, Walsh K, Rosenzweig A, Torella D,

Nurzynska D, Kajstura J, Leri A, Anversa P, and Sussman MA. Nuclear targeting of Akt enhances kinase activity and survival of cardiomyocytes. *Circ Res* 94: 884–891, 2004.
212. Silva CM and Cidlowski JA. Direct evidence for intra- and intermolecular disulfide bond formation in the human glucocorticoid receptor: inhibition of DNA binding and identification of a new receptor-associated protein. *J Biol Chem* 264: 6638–6647, 1989.
213. Smeenk L, van Heeringen SJ, Koeppel M, van Driel MA, Bartels SJ, Akkers RC, Denissov S, Stunnenberg HG, and Lohrum M. Characterization of genome-wide p53-binding sites upon stress response. *Nucleic Acids Res* 36: 3639–3654, 2008.
214. Smith GC and Jackson SP. The DNA-dependent protein kinase. *Genes Dev* 13: 916–934, 1999.
215. Soboll S, Grundel S, Harris J, Kolb-Bachofen V, Ketterer B, and Sies H. The content of glutathione and glutathione S-transferases and the glutathione peroxidase activity in rat liver nuclei determined by a non-aqueous technique of cell fractionation. *Biochem J* 311: 889–894, 1995.
216. Sohn J and Rudolph J. Catalytic and chemical competence of regulation of cdc25 phosphatase by oxidation/reduction. *Biochemistry* 42: 10060–10070, 2003.
217. Soussi T and Beroud C. Significance of TP53 mutations in human cancer: a critical analysis of mutations at CpG dinucleotides. *Hum Mutat* 21: 192–200, 2003.
218. Starovasnik MA, Blackwell TK, Laue TM, Weintraub H, and Klevit RE. Folding topology of the disulfide-bonded dimeric DNA-binding domain of the myogenic determination factor MyoD. *Biochemistry* 31: 9891–9903, 1992.
219. Stavreus-Evers A, Masironi B, Landgren BM, Holmgren A, Eriksson H, and Sahlin L. Immunohistochemical localization of glutaredoxin and thioredoxin in human endometrium: a possible association with pinopodes. *Mol Hum Reprod* 8: 546–551, 2002.
220. Stella L, Pallottini V, Moreno S, Leoni S, De Maria F, Turella P, Federici G, Fabrini R, Dawood KF, Bello ML, Pedersen JZ, and Ricci G. Electrostatic association of glutathione transferase to the nuclear membrane: evidence of an enzyme defense barrier at the nuclear envelope. *J Biol Chem* 282: 6372–6379, 2007.
221. Stubbs M, Veech RL, and Krebs HA. Control of the redox state of the nicotinamide-adenine dinucleotide couple in rat liver cytoplasm. *Biochem J* 126: 59–65, 1972.
222. Sun X, Wu F, Datta R, Kharbanda S, and Kufe D. Interaction between protein kinase C delta and the c-Abl tyrosine kinase in the cellular response to oxidative stress. *J Biol Chem* 275: 7470–7473, 2000.
223. Tell G, Damante G, Caldwell D, and Kelley MR. The intracellular localization of APE1/Ref-1: more than a passive phenomenon? *Antioxid Redox Signal* 7: 367–384, 2005.
224. Tell G, Pellizzari L, Cimarosti D, Pucillo C, and Damante G. Ref-1 controls pax-8 DNA-binding activity. *Biochem Biophys Res Commun* 252: 178–183, 1998.
225. Tell G, Pines A, Paron I, D'Elia A, Bisca A, Kelley MR, Manzini G, and Damante G. Redox effector factor-1 regulates the activity of thyroid transcription factor 1 by controlling the redox state of the N transcriptional activation domain. *J Biol Chem* 277: 14564–14574, 2002.
226. Tell G, Scaloni A, Pellizzari L, Formisano S, Pucillo C, and Damante G. Redox potential controls the structure and DNA binding activity of the paired domain. *J Biol Chem* 273: 25062–25072, 1998.
227. Tell G, Zecca A, Pellizzari L, Spessotto P, Colombatti A, Kelley MR, Damante G, and Pucillo C. An "environment to nucleus" signaling system operates in B lymphocytes: redox status modulates BSAP/Pax-5 activation through Ref-1 nuclear translocation. *Nucleic Acids Res* 28: 1099–1105, 2000.
228. ten Hoeve J, de Jesus Ibarra-Sanchez M, Fu Y, Zhu W, Tremblay M, David M, and Shuai K. Identification of a nuclear Stat1 protein tyrosine phosphatase. *Mol Cell Biol* 22: 5662–5668, 2002.
229. Tienrungroj W, Meshinchi S, Sanchez ER, Pratt SE, Grippo JF, Holmgren A, and Pratt WB. The role of sulfhydryl groups in permitting transformation and DNA binding of the glucocorticoid receptor. *J Biol Chem* 262: 6992–7000, 1987.
230. Toledano MB and Leonard WJ. Modulation of transcription factor NF-kappa B binding activity by oxidation-reduction in vitro. *Proc Natl Acad Sci U S A* 88: 4328–4332, 1991.
231. Tonks NK. PTP1B: from the sidelines to the front lines! *FEBS Lett* 546: 140–148, 2003.
232. Tsujita Y, Muraski J, Shiraishi I, Kato T, Kajstura J, Anversa P, and Sussman MA. Nuclear targeting of Akt antagonizes aspects of cardiomyocyte hypertrophy. *Proc Natl Acad Sci U S A* 103: 11946–11951, 2006.
233. Valko M, Rhodes CJ, Moncol J, Izakovic M, and Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact* 160: 1–40, 2006.
234. Veech RL, Eggleston LV, and Krebs HA. The redox state of free nicotinamide-adenine dinucleotide phosphate in the cytoplasm of rat liver. *Biochem J* 115: 609–619, 1969.
235. Velichkova M and Hasson T. Keap1 regulates the oxidation-sensitive shuttling of Nrf2 into and out of the nucleus via a Crm1-dependent nuclear export mechanism. *Mol Cell Biol* 25: 4501–4513, 2005.
236. Wahllander A, Soboll S, Sies H, Linke I, and Muller M. Hepatic mitochondrial and cytosolic glutathione content and the subcellular distribution of GSH-S-transferases. *FEBS Lett* 97: 138–140, 1979.
237. Wakabayashi N, Dinkova-Kostova AT, Holtzclaw WD, Kang MI, Kobayashi A, Yamamoto M, Kensler TW, and Talalay P. Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proc Natl Acad Sci U S A* 101: 2040–2045, 2004.
238. Wakasugi N, Tagaya Y, Wakasugi H, Mitsui A, Maeda M, Yodoi J, and Tursz T. Adult T-cell leukemia-derived factor/thioredoxin, produced by both human T-lymphotropic virus type I- and Epstein-Barr virus-transformed lymphocytes, acts as an autocrine growth factor and synergizes with interleukin 1 and interleukin 2. *Proc Natl Acad Sci U S A* 87: 8282–8286, 1990.
239. Walker DR, Bond JP, Tarone RE, Harris CC, Makalowski W, Boguski MS, and Greenblatt MS. Evolutionary conservation and somatic mutation hotspot maps of p53: correlation with p53 protein structural and functional features. *Oncogene* 18: 211–218, 1999.
240. Wang J, Boja ES, Tan W, Tekle E, Fales HM, English S, Mieyal JJ, and Chock PB. Reversible glutathionylation regulates actin polymerization in A431 cells. *J Biol Chem* 276: 47763–47766, 2001.
241. Wang J, Pan S, and Berk BC. Glutaredoxin mediates Akt and eNOS activation by flow in a glutathione reductase-dependent manner. *Arterioscler Thromb Vasc Biol* 27: 1283–1288, 2007.

242. Wang J, Tekle E, Oubrahim H, Mieyal JJ, Stadtman ER, and Chock PB. Stable and controllable RNA interference: investigating the physiological function of glutathionylated actin. *Proc Natl Acad Sci U S A* 100: 5103–5106, 2003.
243. Wang X, Martindale JL, Liu Y, and Holbrook NJ. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. *Biochem J* 333: 291–300, 1998.
244. Wei SJ, Botero A, Hirota K, Bradbury CM, Markovina S, Laszlo A, Spitz DR, Goswami PC, Yodoi J, and Gius D. Thioredoxin nuclear translocation and interaction with redox factor-1 activates the activator protein-1 transcription factor in response to ionizing radiation. *Cancer Res* 60: 6688–6695, 2000.
245. Weinberg RL, Veprintsev DB, and Fersht AR. Cooperative binding of tetrameric p53 to DNA. *J Mol Biol* 341: 1145–1159, 2004.
246. Wenger RH. Cellular adaptation to hypoxia: O_2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O_2-regulated gene expression. *FASEB J* 16: 1151–1162, 2002.
247. Wenger RH, Stiehl DP, and Camenisch G. Integration of oxygen signaling at the consensus HRE. *Sci STKE* 2005: re12, 2005.
248. Wilson DM 3rd and Barsky D. The major human abasic endonuclease: formation, consequences and repair of abasic lesions in DNA. *Mutat Res* 485: 283–307, 2001.
249. Wingert RA, Galloway JL, Barut B, Foott H, Fraenkel P, Axe JL, Weber GJ, Dooley K, Davidson AJ, Schmid B, Paw BH, Shaw GC, Kingsley P, Palis J, Schubert H, Chen O, Kaplan J, and Zon LI. Deficiency of glutaredoxin 5 reveals Fe-S clusters are required for vertebrate haem synthesis. *Nature* 436: 1035–1039, 2005.
250. Witte S, Villalba M, Bi K, Liu Y, Isakov N, and Altman A. Inhibition of the c-Jun N-terminal kinase/AP-1 and NF-kappaB pathways by PICOT, a novel protein kinase C-interacting protein with a thioredoxin homology domain. *J Biol Chem* 275: 1902–1909, 2000.
251. Wood ZA, Schroder E, Robin Harris J, and Poole LB. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci* 28: 32–40, 2003.
252. Wu Y, Moser M, Bautch VL, and Patterson C. HoxB5 is an upstream transcriptional switch for differentiation of the vascular endothelium from precursor cells. *Mol Cell Biol* 23: 5680–5691, 2003.
253. Xanthoudakis S and Curran T. Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. *EMBO J* 11: 653–665, 1992.
254. Xanthoudakis S, Miao G, Wang F, Pan YC, and Curran T. Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme. *EMBO J* 11: 3323–3335, 1992.
255. Xanthoudakis S, Miao GG, and Curran T. The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains. *Proc Natl Acad Sci U S A* 91: 23–27, 1994.
256. Yamaguchi T, Miki Y, and Yoshida K. Protein kinase C delta activates IkappaB-kinase alpha to induce the p53 tumor suppressor in response to oxidative stress. *Cell Signal* 19: 2088–2097, 2007.
257. Yamamoto T, Sekine Y, Kashima K, Kubota A, Sato N, Aoki N, and Matsuda T. The nuclear isoform of protein-tyrosine phosphatase TC-PTP regulates interleukin-6-mediated signaling pathway through STAT3 dephosphorylation. *Biochem Biophys Res Commun* 297: 811–817, 2002.
258. Yamanaka H, Maehira F, Oshiro M, Asato T, Yanagawa Y, Takei H, and Nakashima Y. A possible interaction of thioredoxin with VDUP1 in HeLa cells detected in a yeast two-hybrid system. *Biochem Biophys Res Commun* 271: 796–800, 2000.
259. Yoshida K. PKCdelta signaling: mechanisms of DNA damage response and apoptosis. *Cell Signal* 19: 892–901, 2007.
260. Yuan ZM, Utsugisawa T, Ishiko T, Nakada S, Huang Y, Kharbanda S, Weichselbaum R, and Kufe D. Activation of protein kinase C delta by the c-Abl tyrosine kinase in response to ionizing radiation. *Oncogene* 16: 1643–1648, 1998.
261. Zhang Q, Piston DW, and Goodman RH. Regulation of corepressor function by nuclear NADH. *Science* 295: 1895–1897, 2002.
262. Zhu H, Fu W, and Mattson MP. The catalytic subunit of telomerase protects neurons against amyloid beta-peptide-induced apoptosis. *J Neurochem* 75: 117–124, 2000.
263. Zipper LM and Mulcahy RT. Erk activation is required for Nrf2 nuclear localization during pyrrolidine dithiocarbamate induction of glutamate cysteine ligase modulatory gene expression in HepG2 cells. *Toxicol Sci* 73: 124–134, 2003.

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Date of first submission to ARS Central, March 31, 2009; date of final revised submission September 2, 2009; date of acceptance, September 5, 2009.

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

Downregulation of mitochondrial telomerase reverse transcriptase induced by H_2O_2 is Src kinase dependent.

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Exp Gerontol. 2010; 45:558-562.

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Haendeler J: Senior Autorin, hatte die Idee zur Studie, war an der Versuchsplanung beteiligt und schrieb mit Nicole Büchner das Manuskript.

Experimental Gerontology 45 (2010) 558–562

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Downregulation of mitochondrial telomerase reverse transcriptase induced by H_2O_2 is Src kinase dependent

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ARTICLE INFO

Article history:
Received 31 October 2009
Received in revised form 23 February 2010
Accepted 2 March 2010
Available online 6 March 2010

Keywords:
Mitochondria
Telomerase reverse transcriptase
Src kinase
Akt1
Endothelial cells
H_2O_2

ABSTRACT

Telomerase with its catalytic subunit telomerase reverse transcriptase (TERT) prevents telomere erosion in the nucleus. In addition, telomerase has also telomere-independent functions in protection from apoptosis. Unexpectedly, TERT was found in the mitochondria. However, its regulation in this organelle is completely unknown. Here, we demonstrate that mitochondrial TERT is downregulated by exposure to H_2O_2 in primary human endothelial cells. This depletion is dependent on the Src phosphorylation site within TERT, tyrosine 707. In accordance with this finding, we also detected Src in the mitochondria and demonstrated that Src is activated upon H_2O_2 treatment. This regulation of mitochondrial TERT is reminiscent of the situation in the nucleus from where TERT is exported under conditions of oxidative stress in a Src kinase dependent manner. In addition, Akt1 was also found in the mitochondria and H_2O_2 treatment led to reduced active Akt1 in these organelles, suggesting that similar regulatory mechanisms operate in mitochondria and the nucleus.

1. Introduction

The enzyme telomerase counteracts the shortening of telomeres, the physical ends of the chromosomes. Thereby it prevents the onset of replicative senescence and genetic instability (Blackburn, 2000; Buys, 2000; Collins, 2000; Hemann et al., 2001). Its catalytic subunit is the telomerase reverse transcriptase (TERT) (Collins, 2000). Several studies demonstrated that nuclear TERT increases cell survival and resistance against oxidative stress after short term incubation with different stimuli, which did not affect telomere length (Haendeler et al., 2003a,b; Rahman et al., 2005; Ramirez et al., 2003; Ren et al., 2001). Another unexpected finding was the observation that TERT is also localized in mitochondria (Ahmed et al., 2008; Haendeler et al., 2009; Santos et al., 2004, 2006). Since the circular mitochondrial DNA does not contain any telomeric repeats, mitochondrial TERT has to have also a telomere-independent role within mitochondria. However, the exact functions of TERT in mitochondria remain controversial. Santos et al. demonstrated in fibroblasts overexpressing TERT that it exacerbates oxidative injury (Santos et al., 2004, 2006). In contrast, Ahmed et al. showed in the same cells a protective role for mitochondrial TERT after oxidative stress (Ahmed et al., 2008). Furthermore, we recently revealed that endogenous, mitochondrial TERT protects mitochondrial DNA from damage and importantly contributes to the respiratory chain activity under basal conditions in endothelial cells as well as in human embryonic kidney cells (Haendeler et al., 2009). Besides this controversy, it is completely unknown how TERT is regulated in mitochondria. However, since changes in reactive oxygen species formation regulate nuclear TERT, one could speculate that also mitochondrial TERT is affected by oxidative stress. Moreover, there exists accumulating evidence that mitochondria are one compartment within the cell producing reactive oxygen species that contribute to aging processes, which has been demonstrated by several lines of evidence. Mice overexpressing mitochondrially targeted catalase show a significant increase in life span and a reduction in oxidative damage to DNA and consequently in apoptosis (Schriner et al., 2005). Moreover, overexpression of mitochondrially localized antioxidant enzymes lengthens lifespan of *Drosophila* (Orr and Sohal, 1994; Ruan et al., 2002) and deletion of manganese superoxide dismutase in mice results in the age-related decline of mitochondrial function, culminating in increased apoptosis (Kokoszka et al., 2001). Thus, aging and apoptotic processes are associated with increased reactive oxygen species formation within the mitochondria. Because also TERT is involved in aging and apoptotic processes, we elucidated for the first time the regulation of mitochondrial TERT by oxidative stress and the role of the Src kinase therein.

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doi:10.1016/j.exger.2010.03.003

2. Materials and methods

2.1. Cloning of expression vectors for mitochondrially targeted TERT

The N-terminal mitochondrial targeting sequence was amplified from pCMV/myc/mito (Invitrogen) using the primers mito-link for1 (5′-CAATTGCTAGCCACCATGTCCGTCCTGACGCCG-3′) and mito-link rev1 (5′-GCATGAATTCCAACGAATGGATCTTGGCGCG-3′) in a standard PCR reaction. The resulting amplification product was cut with NheI and EcoRI, inserted into hTERT expression vectors for TERT wild-type and TERT(Y707F), respectively (Haendeler et al., 2003a) and verified by sequencing.

2.2. Cell culture, transfection

Human embryonic kidney (HEK) cells were cultured in DMEM basal medium with 10% heat-inactivated fetal calf serum. Human umbilical vein endothelial cells (HUVEC) were cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/ml), bovine brain extract (12 μg/ml), gentamicin (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. HUVEC were transiently transfected with Superfect (QIAGEN) as described previously (Haendeler et al., 2002). The transfection efficiency in HUVEC was between 20% and 30%.

2.3. Cellular fractionation

Cellular fractionation and isolation of mitochondria was performed as described previously (Yang et al., 1997). To obtain total cell lysates, cells were lysed in RIPA-buffer as described previously (Haendeler et al., 2003a).

2.4. Immunoblotting

Immunoblotting was performed with antibodies directed against TERT (1:500, overnight, 4 °C, Rockland), total Src (1:500, Invitrogen), phospho-SrcY418 (1:500, Invitrogen), phospho-SrcY529 (1:1000, Abcam), phospho-Akt1 (1:1000, Cell Signaling Technol.), Akt1 (1:1000, Cell Signaling Technol.), tubulin (1:5000, Sigma), Thioredoxin-1 (1:500, BD Biosciences), and translocase of inner membrane 23 (TIM23, 1:500, BD Biosciences). Blots were incubated with primary antibodies overnight at 4 °C and with secondary HRP-coupled antibodies 2 h at room temperature. Antibodies were detected by the enhanced chemiluminescence system (GE Healthcare). Semi-quantitative analyses were performed on scanned immunoblots using Scion Image 1.6 (SCION Corporation) or ImageJ (Abramoff et al., 2004).

2.5. Statistics

Statistical analyses were performed with student's *T*-test using winLSTAT 2008.

3. Results

3.1. TERT and the Src kinase are localized in mitochondria

We recently demonstrated that TERT is localized in mitochondria, where it protects mitochondrial DNA from damage and preserves mitochondrial functions (Haendeler et al., 2009). However, the regulation of mitochondrial TERT is completely unknown. From our studies of nuclear TERT in endothelial cells as well as in human embryonic kidney cells, we know that TERT is exported from the nucleus under conditions of oxidative stress in a Src kinase dependent manner (Haendeler et al., 2003a, 2004). Moreover, it has recently been demonstrated that the Src kinase is localized in mitochondria in rat brain and that the complexes of the respiratory chain are substrates for Src, indicating that respiratory chain activity is partially dependent on tyrosine phosphorylation (Arachiche et al., 2008). Therefore, we first investigated whether the Src kinase is present in mitochondrial fractions from human embryonic kidney cells as well as endothelial cells. Indeed, the Src kinase is localized in the mitochondria of these cell types (Figs. 1A and 2).

3.2. Mitochondrial Src kinase is activated by treatment with H_2O_2

Next, we determined whether treatment with H_2O_2 activates the Src kinase in mitochondria. Therefore, human endothelial cells were incubated with 200 μM H_2O_2 and active Src kinase was measured by immunoblot analysis of mitochondrial lysates. For that purpose immunoblots were performed with an antibody against active Src, phosphorylated at tyrosine 418 and an antibody against inactive Src, phosphorylated at tyrosine 529. The ratio between

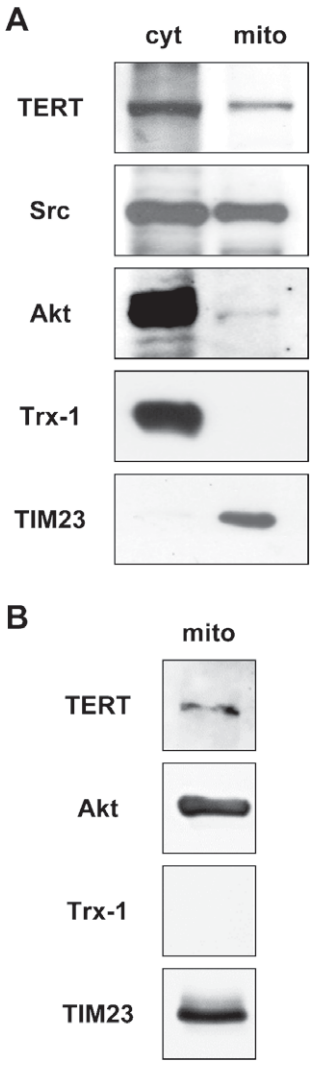

Fig. 1. Localization of TERT, Akt1 and Src kinase in mitochondria. (A) Mitochondrial and cytosolic fractions of HEK 293 cells were isolated as described in Section 2. Lysate proteins were resolved by SDS–PAGE, blotted onto PVDF membranes and the blots probed with antibodies against TERT, Src and Akt1. Antibodies against thioredoxin-1 (Trx-1) and translocase of inner membrane 23 (TIM23) were used to control for purity of the fractions. (B) Immunoblots with mitochondrial lysates from HUVEC were probed with antibodies against TERT, Akt1, Tim23 and Trx-1.

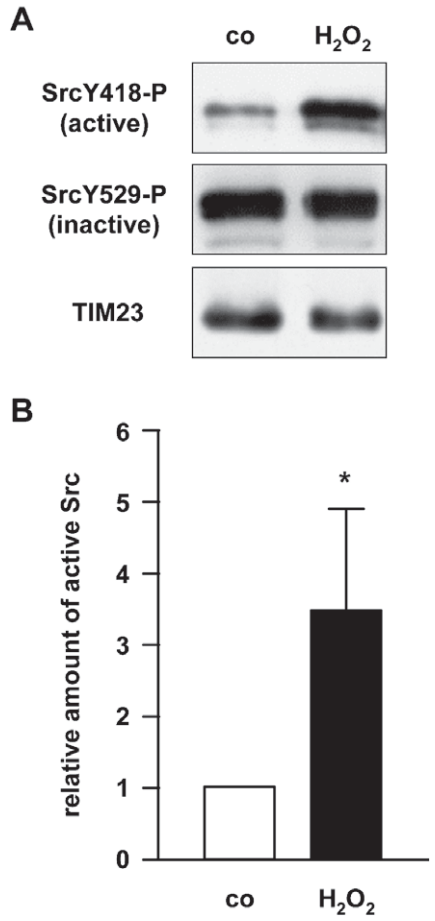

Fig. 2. Src kinase is activated by H_2O_2 in mitochondria. HUVEC were treated with 200 μM H_2O_2 for 3–4 h (H_2O_2) or left untreated (co) and mitochondrial lysates prepared as described in Section 2. The proteins were resolved by SDS–PAGE and blotted onto PVDF membranes. Blots were probed with antibodies against active Src phosphorylated on tyrosine 418 (SrcY418-P), inactive Src phosphorylated on tyrosine 529 (SrcY529-P) and translocase of inner membrane 23 (TIM23). (A) Representative blot. (B) Blots were quantitated densitometrically and the levels of SrcY418-P and SrcY529-P normalized to TIM23 levels. The graph shows the ratio of active to inactive Src. Data are mean +/− SEM of 4 independent experiments. * Significantly different to control ($p < 0.05$).

these two differently phosphorylated forms reflects the activity of Src in the mitochondria. Incubation with H_2O_2 increased the phosphorylation at tyrosine 418 within Src, whereas the phosphorylation at tyrosine 529 remained unaltered (Fig. 2A). Semi-quantitative analysis of the immunoblots revealed that exposure to H_2O_2 significantly increased the activity of the Src kinase in the mitochondria (Fig. 2B).

3.3. Mitochondrial Akt1 is deactivated by treatment with H_2O_2

We previously demonstrated that nuclear TERT is complexed with Akt1 and Heat Shock protein 90 (Haendeler et al., 2003b). Disruption of this complex resulted in inactivation of Akt1, which subsequently led to a reduction of nuclear TERT and to induction of apoptosis (Haendeler et al., 2003b). Therefore, we next determined whether Akt1 is also localized in the mitochondria. Indeed, Akt1 was identified in mitochondria of HEK293 cells as well as endothelial cells (Fig. 1A and B). Since Akt1 is negatively regulated by increased oxidative stress in endothelial cells (Hoffmann et al., 2001), we hypothesized that incubation with H_2O_2 could also reduce active Akt1 in the mitochondria. To determine active Akt1, we measured phosphorylation of Akt1 on serine 473 in mitochondria of endothelial cells. Incubation with H_2O_2 reduced active Akt1 (Fig. 3A and B). These data suggest that mitochondrial TERT and nuclear TERT are regulated similarly.

3.4. Downregulation of mitochondrial TERT by H_2O_2 depends on tyrosine 707 in TERT and thereby on the Src kinase

Having demonstrated that exposure to H_2O_2 increases mitochondrial Src kinase activity and decreases mitochondrial Akt1 activity, we next wanted to investigate whether mitochondrial TERT is regulated by H_2O_2. Taking into account that TERT is exported from the nucleus (Haendeler et al., 2003a) and that after nuclear export, the TERT level is increased in the cytosol and in the mitochondria (Ahmed et al., 2008; Haendeler et al., 2003a), we decided to use TERT exclusively targeted to the mitochondria (mitoTERT) containing a myc-tag to prevent detection of endogenous TERT, which may be newly imported into these organelles. After overexpression of myc-tagged mitoTERT in endothelial cells and treatment with 200 μM H_2O_2, we measured the levels of mitochondrial TERT by immunoblot. Interestingly, H_2O_2 induced a significant reduction of TERT in the mitochondria (Fig. 4A and B). From our previous studies, we knew that tyrosine 707 in TERT is a putative Src kinase phosphorylation site (Haendeler et al., 2003a; Jakob et al., 2008). Mutation of tyrosine 707 to phenylalanine resulted in complete inhibition of nuclear TERT export triggered by the Src kinase. Therefore, we inserted a mitochondrial targeting sequence into the TERT(Y707F) expression construct to

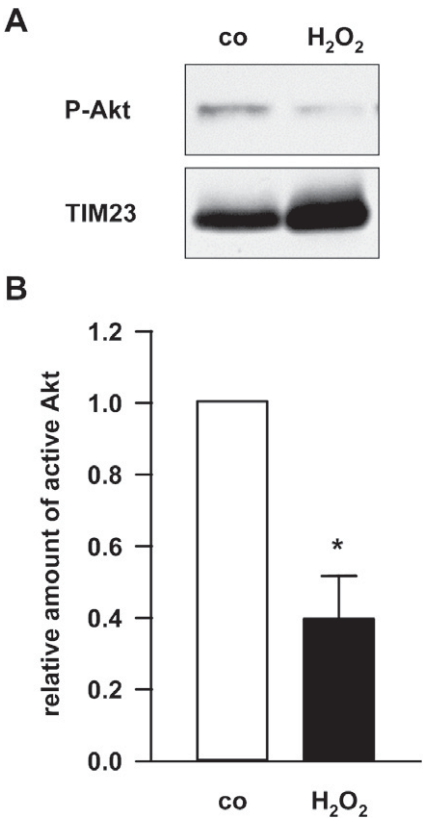

Fig. 3. Akt1 is deactivated by H_2O_2 in mitochondria. HUVEC were treated with 200 μM H_2O_2 for 3–4 h (H_2O_2) or left untreated (co) and mitochondrial lysates prepared as described in Section 2. The proteins were resolved by SDS–PAGE and blotted onto PVDF membranes. Blots were probed with antibodies against active Akt1 phosphorylated on Serine 473 (P-Akt) and translocase of inner membrane 23 (TIM23). (A) Representative blot. (B) Blots were quantitated densitometrically and the levels of active Akt1 (P-Akt) were normalized to TIM23 levels. Data are mean +/− SEM of 4 independent experiments. * Significantly different to control ($p < 0.05$).

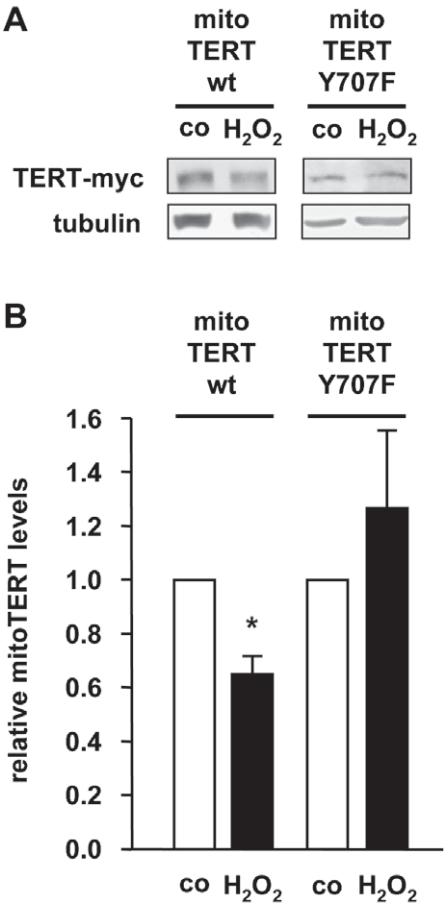

Fig. 4. Mitochondrial TERT wild-type, but not TERT(Y707F) is reduced by H_2O_2. HUVEC were transfected with expression vectors for mitochondrially targeted, myc-tagged human TERT wild-type (mitoTERT wt) or a mutant, in which tyrosine 707 was exchanged to phenylalanine (mitoTERT Y707F), treated with 200 μM H_2O_2 for 3–4 h (H_2O_2) or left untreated (co). Total lysates were prepared as described in Section 2, proteins resolved by SDS–PAGE and blotted onto PVDF membranes. Blots were probed with an anti-myc antibody (TERT-myc) and an antibody against tubulin. (A) Representative blot. (B) Blots were quantitated densitometrically and the levels of TERT normalized to tubulin. The graph shows the relative amount of TERT protein. Data are mean +/− SEM of 3–6 independent experiments. * Significantly different to untreated cells expressing mitochondrial TERT wild-type ($p < 0.05$).

force TERT(Y707F) into the mitochondria, to elucidate whether the downregulation of mitochondrial TERT levels is also dependent on the Src kinase. As shown in Fig. 4, downregulation of mitochondrial TERT by H_2O_2 was completely abrogated by mutation of this single tyrosine residue, demonstrating that the mitochondrial Src kinase is responsible for H_2O_2-induced downregulation of mitochondrial TERT.

4. Discussion

In our present study we investigated for the first time regulatory mechanisms of mitochondrial TERT. Here, we demonstrate that in primary human endothelial cells the Src kinase and Akt1 are localized in the mitochondria. The Src kinase is activated and Akt1 is deactivated upon stimulation with H_2O_2. H_2O_2 treatment results in downregulation of mitochondrial TERT levels, which is dependent on tyrosine 707 within TERT, indicating a phosphorylation-dependent mechanism.

The functions and role of nuclear TERT have extensively been studied and this years Nobel Prize in Physiology or Medicine was awarded for the discovery of how chromosomes are protected by telomeres and the enzyme telomerase, which underscores the importance of this enzyme. Therefore, it is important to understand the regulation of the catalytic subunit of telomerase, TERT. Over the last 10 years, it has become evident that nuclear TERT is not only regulated transcriptionally, but also post-translationally by binding proteins and phosphorylation. In human endothelial cells, we and others demonstrated that nuclear TERT requires protein kinase B (Akt) for its activation under basal conditions (Breitschopf et al., 2001; Haendeler et al., 2003b). In contrast, under conditions of oxidative stress, Src kinases are activated and TERT is exported from the nucleus, which results in the onset of replicative senescence and in increased apoptosis sensitivity (Haendeler et al., 2003a, 2004). Recent studies demonstrated a mitochondrial localization of TERT. However, the regulation of mitochondrial TERT is completely unknown. Several studies were undertaken to investigate, whether Akt1 and Src kinase are localized in mitochondria. Indeed, Akt1 and Src kinase were found to be localized in mitochondria of fibroblasts and rat brain, respectively (Antico Arciuch et al., 2009; Arachiche et al., 2008). Akt1 has been shown to cycle between the nucleus and the mitochondria dependent on the redox status of the cell (Antico Arciuch et al., 2009) and complexes of the respiratory chain have been demonstrated to be substrates of the Src kinase (Arachiche et al., 2008). Taking into account that mitochondrial TERT enhances respiratory chain activity and mitochondrial function (Haendeler et al., 2009) and the known regulation of TERT by the kinases Akt1 and Src in the nucleus as mentioned above, it is tempting to speculate that Akt1 and Src can also regulate mitochondrial TERT. Indeed, we also found the kinases Akt1 and Src in the mitochondria of primary human endothelial cells as well as human embryonic kidney cells (Figs. 1 and 2). Therefore, these kinases could also regulate mitochondrial TERT. Interestingly, H_2O_2 treatment led to a reduction in active Akt1, to an induction in active Src kinase and to a reduction in mitochondrial TERT levels. Thus, one may speculate that mitochondrial TERT is regulated in a similar fashion as nuclear TERT. This speculation is underscored by our finding that oxidative stress-induced downregulation of mitochondrial TERT (Fig. 4) like nuclear TERT (Haendeler et al., 2003a) depends on tyrosine 707. However, it has to be noted that export of nuclear TERT results in an increased amount of mitochondrial TERT (Ahmed et al., 2008). Taken these findings together, one has to suggest that oxidative stress initially leads to a reduction of TERT levels in the mitochondria, which is followed by a nuclear export of TERT and a concomitant mitochondrial import, but this is pure speculation and requires further experimental proof. However, the finding that an increased amount of TERT is found in the mitochondria after oxidative stress-induced nuclear export (Ahmed et al., 2008), suggests also that the mitochondrial Src kinase has to be inactivated at that time. Since we identified the tyrosine phosphatase Shp-2 as the counterplayer for Src dependent nuclear export of TERT (Jakob et al., 2008) and Arachiche et al. found that Shp-2 is also localized in the mitochondria of rat brain (Arachiche et al., 2008), it is tempting to speculate that Shp-2 inactivates the mitochondrial Src kinase.

In conclusion, our study demonstrates for the first time that the Src kinase and Akt1 are present also in the mitochondria of human endothelial cells and human embryonic kidney cells. Mitochondrial Src is activated, Akt1 is deactivated and mitochondrial TERT levels are downregulated by H_2O_2. This downregulation is dependent on a Src kinase dependent phosphorylation site, tyrosine 707, within TERT. Therefore, both mitochondrial and nuclear TERT might be regulated by the same mechanisms at least in primary human endothelial cells.

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to J.H. (HA2868/3-2), by the Sonderfors-

chungsbereich 728 project B5 (to J.H.) and by the Bundesministerium fuer Bildung und Forschung (03NUK003C). We thank Diane Schmiegelt and Kerstin Kunze for expert technical assistance. The authors have nothing to disclose.

References

Abramoff, M.D., Magelhaes, P.J., Ram, S.J., 2004. Image processing with ImageJ. Biophotonics Int. 11, 36–42.

Ahmed, S., Passos, J.F., Birket, M.J., Beckmann, T., Brings, S., Peters, H., Birch-Machin, M.A., von Zglinicki, T., Saretzki, G., 2008. Telomerase does not counteract telomere shortening but protects mitochondrial function under oxidative stress. J. Cell Sci. 121, 1046–1053.

Antico Arciuch, V.G., Galli, S., Franco, M.C., Lam, P.Y., Cadenas, E., Carreras, M.C., Poderoso, J.J., 2009. Akt1 intramitochondrial cycling is a crucial step in the redox modulation of cell cycle progression. PLoS One 4, e7523.

Arachiche, A., Augereau, O., Decossas, M., Pertuiset, C., Gontier, E., Letellier, T., Dachary-Prigent, J., 2008. Localization of PTP-1B, SHP-2, and Src exclusively in rat brain mitochondria and functional consequences. J. Biol. Chem. 283, 24406–24411.

Blackburn, E.H., 2000. Telomere states and cell fates. Nature 408, 53–56.

Breitschopf, K., Zeiher, A.M., Dimmeler, S., 2001. Pro-atherogenic factors induce telomerase inactivation in endothelial cells through an Akt-dependent mechanism. FEBS Lett. 493, 21–25.

Buys, C.H., 2000. Telomeres, telomerase, and cancer. N. Engl. J. Med. 342, 1282–1283.

Collins, K., 2000. Mammalian telomeres and telomerase. Curr. Opin. Cell Biol. 12, 378–383.

Haendeler, J., Hoffmann, J., Tischler, V., Berk, B.C., Zeiher, A.M., Dimmeler, S., 2002. Redox regulatory and anti-apoptotic functions of thioredoxin depend on S-nitrosylation at cysteine 69. Nat. Cell Biol. 4, 743–749.

Haendeler, J., Hoffmann, J., Brandes, R.P., Zeiher, A.M., Dimmeler, S., 2003a. Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707. Mol. Cell Biol. 23, 4598–4610.

Haendeler, J., Hoffmann, J., Rahman, S., Zeiher, A.M., Dimmeler, S., 2003b. Regulation of telomerase activity and anti-apoptotic function by protein–protein interaction and phosphorylation. FEBS Lett. 536, 180–186.

Haendeler, J., Hoffmann, J., Diehl, J.F., Vasa, M., Spyridopoulos, I., Zeiher, A.M., Dimmeler, S., 2004. Antioxidants inhibit nuclear export of telomerase reverse transcriptase and delay replicative senescence of endothelial cells. Circ. Res. 94, 768–775.

Haendeler, J., Drose, S., Buchner, N., Jakob, S., Altschmied, J., Goy, C., Spyridopoulos, I., Zeiher, A.M., Brandt, U., Dimmeler, S., 2009. Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage. Arterioscler. Thromb. Vasc. Biol. 29, 929–935.

Hemann, M.T., Strong, M.A., Hao, L.Y., Greider, C.W., 2001. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. Cell 107, 67–77.

Hoffmann, J., Haendeler, J., Aicher, A., Rossig, L., Vasa, M., Zeiher, A.M., Dimmeler, S., 2001. Aging enhances the sensitivity of endothelial cells toward apoptotic stimuli: important role of nitric oxide. Circ. Res. 89, 709–715.

Jakob, S., Schroeder, P., Lukosz, M., Buchner, N., Spyridopoulos, I., Altschmied, J., Haendeler, J., 2008. Nuclear protein tyrosine phosphatase Shp-2 is one important negative regulator of nuclear export of telomerase reverse transcriptase. J. Biol. Chem. 283, 33155–33161.

Kokoszka, J.E., Coskun, P., Esposito, L.A., Wallace, D.C., 2001. Increased mitochondrial oxidative stress in the Sod2 (+/−) mouse results in the age-related decline of mitochondrial function culminating in increased apoptosis. Proc. Natl. Acad. Sci. USA 98, 2278–2283.

Orr, W.C., Sohal, R.S., 1994. Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster*. Science 263, 1128–1130.

Rahman, R., Latonen, L., Wiman, K.G., 2005. HTERT antagonizes p53-induced apoptosis independently of telomerase activity. Oncogene 24, 1320–1327.

Ramirez, R.D., Herbert, B.S., Vaughan, M.B., Zou, Y., Gandia, K., Morales, C.P., Wright, W.E., Shay, J.W., 2003. Bypass of telomere-dependent replicative senescence (M1) upon overexpression of Cdk4 in normal human epithelial cells. Oncogene 22, 433–444.

Ren, J.G., Xia, H.L., Tian, Y.M., Just, T., Cai, G.P., Dai, Y.R., 2001. Expression of telomerase inhibits hydroxyl radical-induced apoptosis in normal telomerase negative human lung fibroblasts. FEBS Lett. 488, 133–138.

Ruan, H., Tang, X.D., Chen, M.L., Joiner, M.L., Sun, G., Brot, N., Weissbach, H., Heinemann, S.H., Iverson, L., Wu, C.F., Hoshi, T., 2002. High-quality life extension by the enzyme peptide methionine sulfoxide reductase. Proc. Natl. Acad. Sci. USA 99, 2748–2753.

Santos, J.H., Meyer, J.N., Skorvaga, M., Annab, L.A., Van Houten, B., 2004. Mitochondrial hTERT exacerbates free-radical-mediated mtDNA damage. Aging Cell 3, 399–411.

Santos, J.H., Meyer, J.N., Van Houten, B., 2006. Mitochondrial localization of telomerase as a determinant for hydrogen peroxide-induced mitochondrial DNA damage and apoptosis. Hum. Mol. Genet. 15, 1757–1768.

Schriner, S.E., Linford, N.J., Martin, G.M., Treuting, P., Ogburn, C.E., Emond, M., Coskun, P.E., Ladiges, W., Wolf, N., Van Remmen, H., Wallace, D.C., Rabinovitch, P.S., 2005. Extension of murine life span by overexpression of catalase targeted to mitochondria. Science 308, 1909–1911.

Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.-I., Jones, D.P., Wang, X., 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275, 1129–1132.

Oxidative stress-induced degradation of thioredoxin-1 and apoptosis is inhibited by thioredoxin-1-actin interaction in endothelial cells.

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Arterioscler Thromb Vasc Biol. 2011; 31:650-656.

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Oxidative Stress–Induced Degradation of Thioredoxin-1 and Apoptosis Is Inhibited by Thioredoxin-1–Actin Interaction in Endothelial Cells

Tim-Christian Zschauer, Kerstin Kunze, Sascha Jakob, Judith Haendeler, Joachim Altschmied

Objective—Thioredoxin-1 (Trx-1), one important antioxidative enzyme in endothelial cells, is required for apoptosis inhibition. Apoptosis induction is dependent on cytoskeletal changes, which depend on actin rearrangements. Therefore, we wanted to elucidate whether a physical interaction exists between Trx-1 and actin and what the functional consequences are.

Methods and Results—Combined immunoprecipitation/mass spectrometry identified actin as a new binding partner for Trx-1. A separate pool of Trx-1 forms a complex with apoptosis signaling kinase 1. Actin is required for stress fiber formation; thus, the interaction of actin with Trx-1 might interfere with this process. Stress fiber formation, which is directly linked to the phosphorylation of focal adhesion kinase (FAK), occurs as early as 1 hour after H_2O_2 treatment. It is inhibited by Trx-1 overexpression, treatment with exogenous Trx-1, or inhibition of FAK. Prolonged incubation with H_2O_2 induced stress fiber formation, reduced Trx-1 protein levels, and increased apoptosis. All these processes were inhibited by preincubation with the FAK inhibitor PF573228. On the contrary, incubation with PF573228 1 hour after H_2O_2 treatment did not block stress fiber formation, degradation of Trx-1, or apoptosis.

Conclusion—These data demonstrate that the actin–Trx-1 complex protects Trx-1 from degradation and, thus, endothelial cells from apoptosis. Reciprocally, Trx-1 prevents stress fiber formation. **(*Arterioscler Thromb Vasc Biol.* 2011;31:650-656.)**

Key Words: apoptosis ■ thioredoxin-1 ■ actin ■ oxidative stress ■ stress fibers

The thioredoxin system consists of 2 antioxidant oxidoreductase enzymes, thioredoxin-1 (Trx-1) and the thioredoxin reductase 1. Trx-1 is a small, 12-kDa, ubiquitous protein with 2 redox-active cysteine residues in an exposed active center, having the exact same amino acid sequence as *Escherichia coli* Trx, -Cys-Gly-Pro-Cys- (Cys32 and Cys35 within human Trx-1), which is essential for its redox regulatory function.[1] Therefore, this site is conserved among species from bacteria to humans.[1–3] Genetic targeting of Trx-1 leads to a lethal phenotype in mice.[4] The physiological functions of Trx-1 in different types of organisms have evolved from a common fundamental reaction to a large number of different specialized functions. Trx-1 regulates apoptosis, cell growth, differentiation, migration, angiogenesis, tumorigenesis, and development.[5,6] Besides its enzymatic activity as an oxidoreductase, Trx-1 directly interacts with other proteins.[7] The interaction partners of Trx-1 can be subdivided in different classes of proteins depending on the cellular localization of Trx-1 itself. In the nucleus, Trx-1 binds directly to different transcription factors and thereby modulates their DNA-binding activity, eg, p53, nuclear factor-κB, and activator protein 1.[8–13] With respect to apoptosis inhibition, only the apoptosis signaling kinase 1 (ASK-1) and the thioredoxin-interacting protein (TXNIP) have been described.[14–21] By binding to ASK-1, Trx-1 protects cells from apoptosis. Oxidation of Trx-1 results in loss of ASK-1 binding.[17] Moreover, interaction of Trx-1 with TXNIP inhibits the antiproliferative function of the latter in vascular smooth muscle cells, suggesting a proapoptotic function for TXNIP.[21]

The aim of the present study was to identify new Trx-1 interaction partners, which could provide further insights into the protection of Trx-1 from degradation and, thus, into its antiapoptotic functions in endothelial cells. Here, we discovered actin as a new binding partner for Trx-1. The Trx-1–actin complex did not contain ASK-1. Thus, at least 2 different pools of Trx-1 exist, one binding to actin and the other one interacting with ASK-1. Both of them are required to protect endothelial cells from apoptosis. Moreover, interaction with Trx-1 inhibited H_2O_2-induced rigid bundle (so-called stress fiber) formation of actin, Trx-1 degradation, and thereby apoptosis induction in endothelial cells. Interestingly, once stress fibers have been formed, Trx-1 degradation and apoptosis induction cannot be blocked anymore. Therefore, the Trx-1–actin interaction seems to result in a mutual protection of the 2 proteins.

Received on: August 16, 2010; final version accepted on: December 21, 2010.

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Arterioscler Thromb Vasc Biol **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.110.218982**

Materials and Methods

Cell Culture

Human primary endothelial cells (ECs) were cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/mL), bovine brain extract (12 μg/mL), gentamicin (50 μg/mL), amphotericin B (50 ng/mL), epidermal growth factor (10 ng/mL), and 10% fetal calf serum (Lonza, Cologne, Germany). After detachment with trypsin, cells were grown for at least 18 hours as described previously.[22,23]

Plasmids and Transfection

Human Trx-1 was cloned out of endothelial cell–derived cDNA as described previously and inserted in pcDNA 4 (Invitrogen, Karlsruhe, Germany) or in the FLAG vector (Sigma-Aldrich, Munich, Germany).[24] ECs were transiently transfected with Superfect (Qiagen, Hilden, Germany) as described previously.[24]

Immunoprecipitation and Immunoblotting

Lysates (500 μg) were immunoprecipitated with 5 μg of the respective antibody overnight at 4°C. After incubation with protein A and protein G–Sepharose (GE Healthcare, Munich, Germany) for 2 hours at 4°C, the resulting beads were washed and boiled in SDS-PAGE sample buffer, and proteins were resolved by SDS-PAGE. Immunoblotting was performed with antibodies directed against Trx-1 (1:500, overnight, 4°C, BD Biosciences, Karlsruhe, Germany), γ-actin (1:500), GAPDH (1:8000), ASK-1 (1:250) (overnight 4°C, Santa Cruz Biotechnology, Heidelberg, Germany), and phospho-focal adhesion kinase (phospho-FAK) (Tyr397) (1:250), FAK (1:1000) (both overnight 4°C, New England Biolabs, Frankfurt, Germany). Semiquantitative analyses were performed on scanned immunoblots using ImageJ 1.42q.[25] Mass Spectrometry analysis was performed on gel isolated proteins by the Proteome Factory (Berlin, Germany).

Immunostaining

Cells were fixed in 4% paraformaldehyde and permeabilized using 0.3% Triton X-100 and 3% bovine serum albumin in PBS. For immunostaining, cells were incubated with an antibody against Trx-1 and Xpress (both 1:50, Invitrogen, Karlsruhe, Germany) overnight at 4°C and stained with anti-rabbit or anti-mouse Alexa Fluor 488–coupled antibodies (Invitrogen). Actin was stained with Alexa Fluor 568 phalloidin (1:500, Invitrogen) at room temperature for 20 minutes. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (1:2000, Invitrogen). Cells were visualized using an Axiovert 40 microscope from Zeiss (magnification, ×40, oil, Jena, Germany).

Detection of Cell Death by Fluorescence-Activated Cell Sorting

Detection of cell death was performed by fluorescence-activated cell sorting (FACS) analysis using annexin V–APC binding and 7 amino-actinomycin D (7-AAD) staining (BD Pharmingen, Heidelberg, Germany). In brief, cells were trypsinized of the dish and pelleted. After washing twice with annexin binding buffer cell pellets were resuspended in annexin binding buffer and incubated with 2.5 ng/mL annexin V–APC and 2.5 ng/mL 7-AAD for 20 minutes and analyzed using FACS.

Statistics

Statistical analyses were performed with the Student *t* test or Wilcoxon test using WinSTAT 2008.

Results

Actin Is a New Interaction Partner of Trx-1

We described previously that Trx-1 acts antioxidatively and is required to protect ECs from apoptosis.[22,24,26] Oxidative stress–induced stress fiber formation has been demonstrated to induce apoptosis.[27] Therefore, we hypothesized that a potential connection between Trx-1 and actin exists. Using Trx-1 immunoprecipitation combined with mass spectrometry, we identified actin as a new binding partner for Trx-1 (Figure 1A). It has been previously suggested that apoptosis of endothelial cells requires cytoskeletal rearrangements.[28] Thus, an interaction between Trx-1 and actin could be involved in the antiapoptotic function of Trx-1. Using the reciprocal immunoprecipitation approach, we could identify Trx-1 in immunoprecipitates obtained with an anti-γ-actin antibody (Figure 1B). A known interaction partner of Trx-1 is ASK-1. The preservation of the Trx-1/ASK-1 complex is

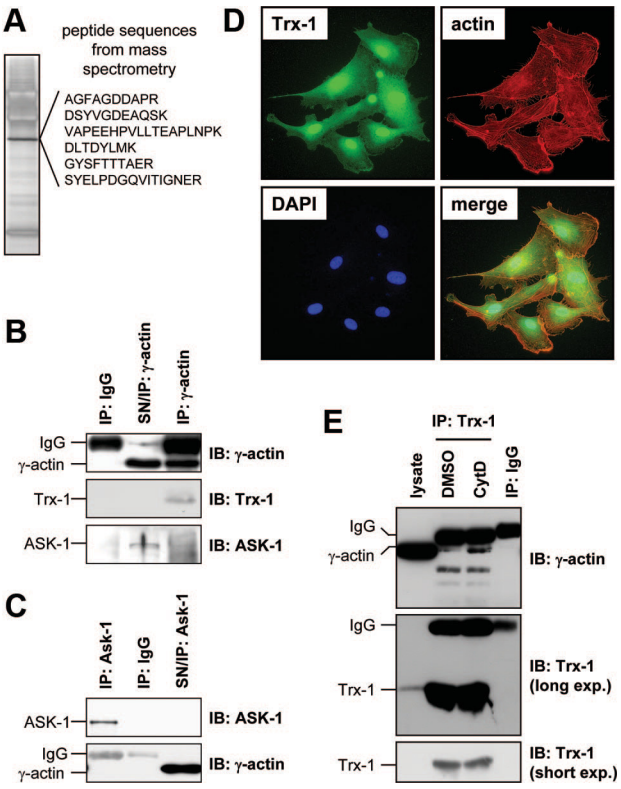


Figure 1. Actin is a new interaction partner of Trx-1. A, Trx-1 was immunoprecipitated out of EC lysates. Immunoprecipitates were subjected to SDS-PAGE, and a dominant band of approximately 37 kDa was extracted. Mass spectrometry revealed 6 peptides completely matching human actin. B, The reciprocal immunoprecipitation was performed using an anti-γ-actin antibody. Trx-1 was coimmunoprecipitated with γ-actin, but ASK-1 did not form a complex with γ-actin. IgG served as a negative control. Top shows an immunoblot with an anti-actin antibody; middle, immunoblot with an anti-Trx-1 antibody; bottom, immunoblot with an anti-ASK-1 antibody. SN/IP indicates supernatant of immunoprecipitation. C, Cell lysates used in B were also subjected to an immunoprecipitation with an anti-ASK-1 antibody. ASK-1 did not form a complex with γ-actin. Top, immunoblot with an anti-ASK-1 antibody; bottom, immunoblot with an anti-actin antibody. IgG served as a negative control. D, Immunostainings were performed to visualize Trx-1 (green); actin was stained with Alexa Fluor 568 phalloidin (red) and nuclei with 4′,6-diamidino-2-phenylindole (DAPI) (blue). The merge revealed that Trx-1 colocalizes with nonpolymerized actin (yellow). E, ECs were incubated with dimethyl sulfoxide (DMSO) as solvent or with 0.1 μmol/L cytochalasin D (CytD) for 30 minutes. Trx-1 was immunoprecipitated out of EC lysates. IgG served as a negative control. Lysate corresponds to untreated total endothelial cell lysates before immunoprecipitation. Top, immunoblot with an anti-actin antibody; middle and bottom, immunoblots with an anti-Trx-1 antibody (long exposure, middle blot; short exposure, lower blot).

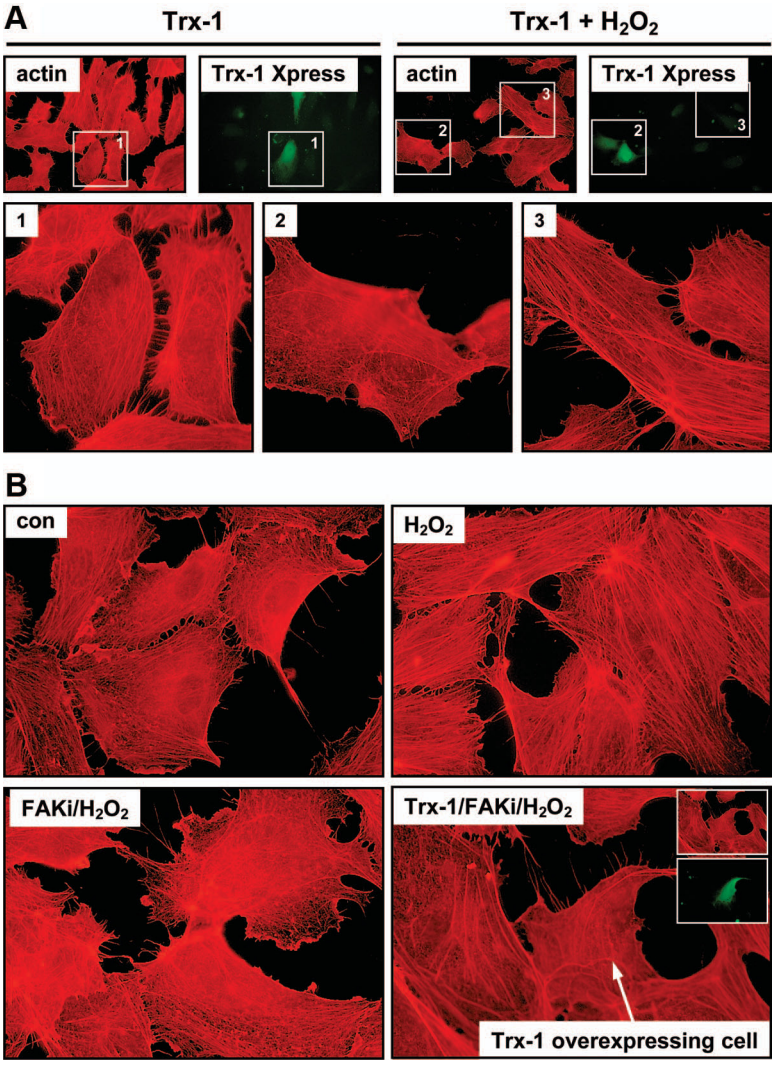


Figure 2. Inhibition of H_2O_2-induced stress fiber formation by Trx-1 and blockage of FAK. ECs were transfected with an expression vector for Xpress-tagged Trx-1 and treated with 200 μmol/L H_2O_2 for 1 hour (Trx-1+H_2O_2) or left untreated (Trx-1). A, Trx-1-overexpressing cells were immunostained with an anti-Xpress antibody (green), and actin was stained with Alexa Fluor 568 phalloidin (red). The enlarged pictures below show the sections marked with the corresponding numbers in the small photographs. Cells overexpressing Trx-1 do not exhibit stress fibers after H_2O_2 treatment. B, Incubations with 40 nmol/L FAK inhibitor PF573228 were done for 6 hours before 1 hour of H_2O_2 treatment (FAKi/H_2O_2). Actin was stained with Alexa Fluor 568 phalloidin (red). Cells transfected with a Trx-1-Xpress expression vector and treated with PF573228/H_2O_2 as before (Trx-1/FAKi/H_2O_2) were counterstained with an anti-Xpress antibody (green).

required to protect endothelial cells from apoptosis.[16] Thus, we wanted to know whether ASK-1 is also part of the Trx-1–actin complex. Interestingly, we did not find ASK-1 associated with actin, demonstrating that at least 2 different pools of Trx-1 exist in endothelial cells (Figure 1B), one binding to actin and the other one to ASK-1. We also performed the reciprocal approach, but we did not find actin in a complex with ASK-1 after immunoprecipitation with an ASK-1 antibody (Figure 1C). In a second approach, we used immunofluorescence and found that Trx-1 colocalized with actin. Interestingly, costaining was observed predominantly with nonpolymerized actin (Figure 1D). To further strengthen this observation, we incubated endothelial cells with cytochalasin D, a known interrupter of actin fiber formation. Indeed, incubation of endothelial cells with cytochalasin D for 30 minutes increased the association of Trx-1 with actin, demonstrating that Trx-1 interacts predominantly with nonpolymerized actin (Figure 1E and Supplemental Figure I, available online at http://atvb.ahajournals.org).

Overexpression of Trx-1 Inhibits Stress Fiber Formation and FAK Phosphorylation

Because it is known that oxidative stress–induced bundle formation of actin leads to actin polymerization and so-called stress fibers, we next investigated whether Trx-1 can inhibit stress fiber formation. Incubation with H_2O_2 induced bundle formation of actin after only 1 hour, and overexpression of Trx-1 completely blocked these cytoskeletal changes (Figure 2A). Because polymerization of actin requires activation of FAK by phosphorylation,[29] we incubated ECs with the FAK inhibitor PF573228 for 6 hours before 1 hour of H_2O_2 treatment. This preincubation inhibited stress fiber formation (Figure 2B). Interestingly, FAK inhibition in Trx-1 overexpressing ECs did not change the phenotype compared with PF573228 treatment (Figure 2B, lower left panel) or overexpression of Trx-1 (Figure 2A) alone. In line with this finding, treatment of ECs with H_2O_2 for 1 hour induced phosphorylation of FAK, which was completely blocked by preincubation with PF573228 for 6 hours (Figure 3A and 3B). Preincubation with exogenous human Trx-1 for 6 hours also inhibited phosphorylation of FAK, but no additive effects were observed when ECs were coincubated with PF573228 and Trx-1 (Figure 3A and 3B), suggesting a common pathway.

Inhibition of FAK Prevents H_2O_2-Induced Trx-1 Degradation and Apoptosis in ECs

We next investigated whether actin bundle formation plays a role in endothelial cell apoptosis induction. H_2O_2 induced

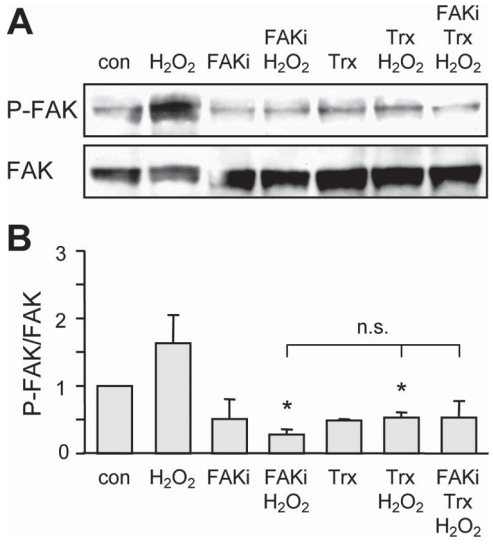


Figure 3. Preincubation with PF573228 and with exogenous human Trx-1 inhibits H_2O_2-induced phosphorylation of FAK. ECs were incubated with 40 nmol/L PF573228, 1 ng/mL recombinant human Trx-1 (Sigma-Aldrich), or both for 6 hours before treatment with 200 μmol/L H_2O_2 for 1 hour. A, FAK phosphorylation was assessed by Western blotting with an anti-phospho-FAK (Tyr397) antibody; total FAK amounts were determined with an anti–total FAK antibody. B, Total FAK and phospho-FAK were evaluated densitometrically, shown is the ratio between phospho-FAK (P-FAK) and total FAK (FAK) (n=3 to 6, *$P<0.05$ versus H_2O_2). n.s. indicates not significant; con, control; FAKi, FAK inhibitor.

apoptosis in ECs ([22,24] and Figure 4A). Preincubation with PF573228 for 6 hours significantly inhibited H_2O_2-induced stress fiber formation and apoptosis (Figure 4A and 4B). We have previously shown that H_2O_2 decreases Trx-1 protein levels and does not change Trx-1 mRNA levels.[22,26] Therefore, we hypothesized that inhibition of nonphysiological, high activation of FAK might stabilize Trx-1. Indeed, preincubation with PF573228 for 6 hours reduced H_2O_2-induced Trx-1 degradation (Figure 4C and 4D). Thus, stress fiber formation seems to be a prerequisite for Trx-1 degradation and endothelial cell apoptosis induction.

H_2O_2-Induced Stress Fiber Formation Is a Prerequisite for Trx-1 Degradation and Endothelial Cell Apoptosis Induction

To analyze the sequence of events during apoptosis induction, we set up experiments in which stress fiber formation was induced before FAK inhibition. Therefore, we pretreated ECs for 1 hour with H_2O_2, continued incubation for 17 hours in the presence or absence of PF573228, and analyzed stress fiber formation, Trx-1 protein levels, and apoptosis. Interestingly, this postincubation with PF573228 did not inhibit H_2O_2-induced apoptosis (Figure 5A). This was because incubation with PF573228 after H_2O_2 treatment inhibited neither stress fiber formation (Figure 5B) nor degradation of Trx-1 protein (Figure 5C and 5D). Based on our observations that Trx-1 interacts predominantly with nonpolymerized actin (Figure 1D and 1E and Supplemental Figure I), these data suggest that disruption of Trx-1–actin interactions by induction of actin polymerization with H_2O_2 exposes Trx-1 and, thus, enhances its degradation.

Discussion

The data from our present study reveal actin as a new binding partner for Trx-1. Interaction of Trx-1 predominantly occurs with nonpolymerized actin. Inhibition of nonphysiological, high FAK phosphorylation and thus activation before H_2O_2 treatment inhibited stress fiber formation, Trx-1 degradation, and apoptosis induction. In contrast, blockade of FAK activity after preincubation with H_2O_2 did not prevent stress fiber formation, Trx-1 degradation, and apoptosis induction, demonstrating that the interaction with actin protects Trx-1 from degradation and thereby ECs from apoptosis induction.

Trx-1 is a multifunctional protein that has been demonstrated to induce transcriptome changes by interacting with several transcription factors and thereby modulating their functions.[30] With respect to apoptosis induction in vascular cells in vitro and in vivo, it has been shown that Trx-1 interacts with ASK-1 and thereby inhibits its proapoptotic action.[15–17,31] Another binding partner for Trx-1, which has antiproliferative and proinflammatory properties, is TXNIP.[14,18,20,21,31] Inhibition of TXNIP–Trx-1 interaction or reducing TXNIP expression in vascular cells, including endothelial and vascular smooth muscle cells, increases Trx-1 activity and also reduces ASK-1 activation, probably by binding of Trx-1 to ASK-1.[31] One of the recently described pathways to reduce TXNIP expression is steady laminar flow, the most potent antiinflammatory and antiapoptotic stimulus for endothelial cells, which seems to make more reduced Trx-1 available in endothelial cells.[31] Our data now identify actin as a new physiological binding partner for Trx-1 in endothelial cells. Recently, overexpressed Trx-1 has been shown to associate with actin in tumor cells.[32] Interestingly, Trx-1 and actin do not form a trimeric complex with ASK-1 in endothelial cells. These findings suggest that at least 2 different pools of Trx-1 exist, one binding to actin and another one binding to ASK-1. Under conditions of oxidative stress, Trx-1 is released out of both complexes, leading to stress fiber formation and ASK-1 activation, which subsequently result in the induction of endothelial cell apoptosis (findings here and[15]). Actin has been demonstrated to be essential for effects induced by steady laminar flow, as cytoskeletal rearrangements are necessary for signal transduction.[33] On the contrary, formation of rigid actin bundles (so-called stress fibers) is observed during apoptosis.[27] Therefore, it is tempting to speculate that laminar flow increases Trx-1–actin interactions as one new mechanism to inhibit stress fiber formation, to allow cytoskeletal changes and thereby to protect against apoptosis induction. This hypothesis is also underscored by the finding that impaired migratory capacity correlates with robust stress fiber formation,[34] which interferes with the dynamic reorganization of the actin cytoskeleton required for migrating cells.[35,36] Furthermore, under conditions of oxidative stress, misassembled focal adhesion complexes and stress fibers are formed, leading to intense membrane blebbing, a hallmark of apoptosis.[27] These data suggest that drastic actin bundle formation induced by oxidative stress marks cells for the apoptotic process. In this study, we found that treatment with H_2O_2 induces stress fibers, and once the fibers have been formed, the cell will undergo apoptosis. Because Trx-1 interacts predominantly

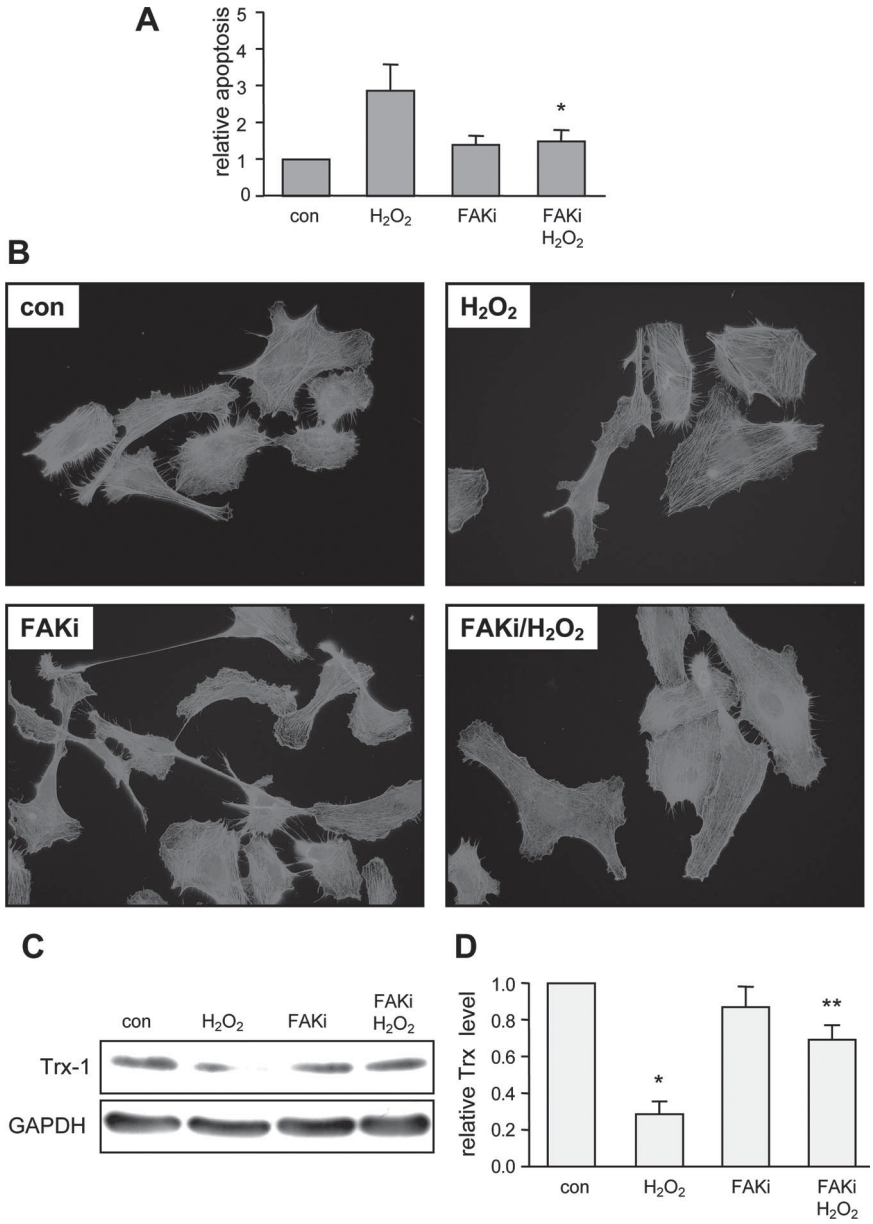


Figure 4. Blocking FAK phosphorylation before H_2O_2 treatment inhibits apoptosis induction, stress fiber formation, and Trx-1 degradation. ECs were preincubated with or without 40 nmol/L PF573228 for 6 hours and incubation was continued in the presence (FAKi/H_2O_2) or absence (FAKi) of 200 μmol/L H_2O_2 for another 18 hours. A, Apoptosis rates were determined by FACS analysis using annexin V–APC binding and 7-amino-actinomycin (7AAD) staining. (n=3, *$P<0.05$ versus H_2O_2). con indicates control. B, Actin was stained with Alexa Fluor 568 phalloidin (red), and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). C, Trx-1 and GAPDH levels were assessed by Western blotting. D, Trx-1 and GAPDH were evaluated densitometrically. Shown is the ratio between Trx-1 and GAPDH (n=4, *$P<0.05$ versus control, **$P<0.05$ versus H_2O_2).

with nonpolymerized actin, one could speculate that Trx-1 protects actin from polymerization by direct interaction. Thus, the Trx-1–actin interaction may protect endothelial cells from stress fiber–dependent apoptosis induction by preventing a misassembly of focal adhesions and, thus, induction of membrane blebbing. Of note, inhibition of stress fiber formation also resulted in reduced Trx-1 degradation. As Trx-1 is degraded by cathepsin D in lysosomes under conditions of oxidative stress,[22] the interaction of Trx-1 with nonpolymerized actin might not allow its transport to these organelles. Therefore, the Trx-1–actin interaction seems to result in a mutual protection of the 2 proteins.

Another potential mechanism of how stress fiber formation could be inhibited is the S-nitros(yl)ation of actin. It has been observed in different cell types that S-nitros(yl)ation of actin reduces its polymerization and thereby leads to shorter actin filaments.[37,38] Because Trx-1 can also be S-nitros(yl)ated in endothelial cells,[24] a transnitros(yl)ation from Trx-1 to actin may occur, which in turn could prevent rigid bundle formation of actin. An involvement of actin in transnitros(yl)ation has already been investigated by Dalle-Donne et al, who reported that S-nitros(yl)ated actin acts as nitric oxide (NO) donor, showing a fast and potent vasodilating activity already at extremely low concentrations, suggesting that NO can indeed shuttle from S-NO to SH groups.[37]

Taking our data together, we present here for the first time evidence that actin, as a new binding partner for Trx-1 in endothelial cells, protects Trx-1 from degradation. Oxidative stress induces stress fiber formation, followed by Trx-1 degradation and apoptosis induction in endothelial cells. Stress fiber formation is required for Trx-1 degradation and apoptosis induction. The Trx-1–interaction protects Trx-1 from degradation and actin from enhanced bundle/stress fiber formation. Thus, maintaining

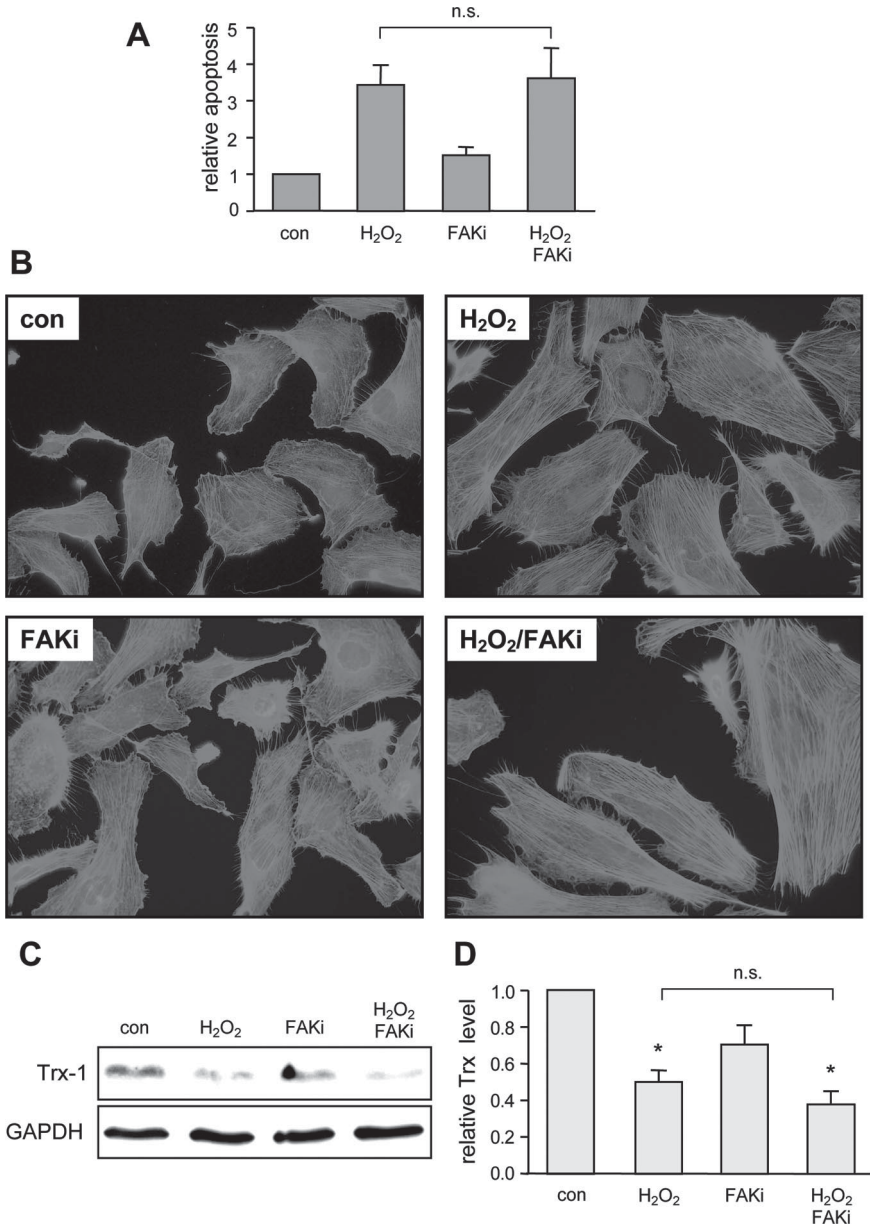


Figure 5. Blocking FAK phosphorylation after H_2O_2 treatment does not inhibit apoptosis induction, stress fiber formation, or Trx-1 degradation. ECs were preincubated with or without 200 μmol/L H_2O_2 for 1 hour, and incubation was continued in the presence or absence of 40 nmol/L PF573228 for another 17 hours. A, Apoptosis rates were determined by FACS analysis using annexin V–APC binding and 7-amino-actinomycin (7AAD) staining (n=3). n.s. indicates not significant; con, control. B, Actin was stained with Alexa Fluor 568 phalloidin (red), and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). C, Trx-1 and GAPDH levels were assessed by Western blotting. D, Trx-1 and GAPDH were evaluated densitometrically. Shown is the ratio between Trx-1 and GAPDH (n=4, *P<0.05 versus control).

the Trx-1–actin interaction is one prerequisite to protect endothelial cells from apoptosis.

Acknowledgments

We thank Diane Schmiegelt for expert technical assistance.

Sources of Funding

This study was supported by the Deutsche Forschungsgemeinschaft (HA-2868/3-2, HA2868/3-3), a start-up grant of the University of Duesseldorf, and the Leducq Transatlantic Network of Excellence (09 CVD_01 to J.H.).

Disclosures

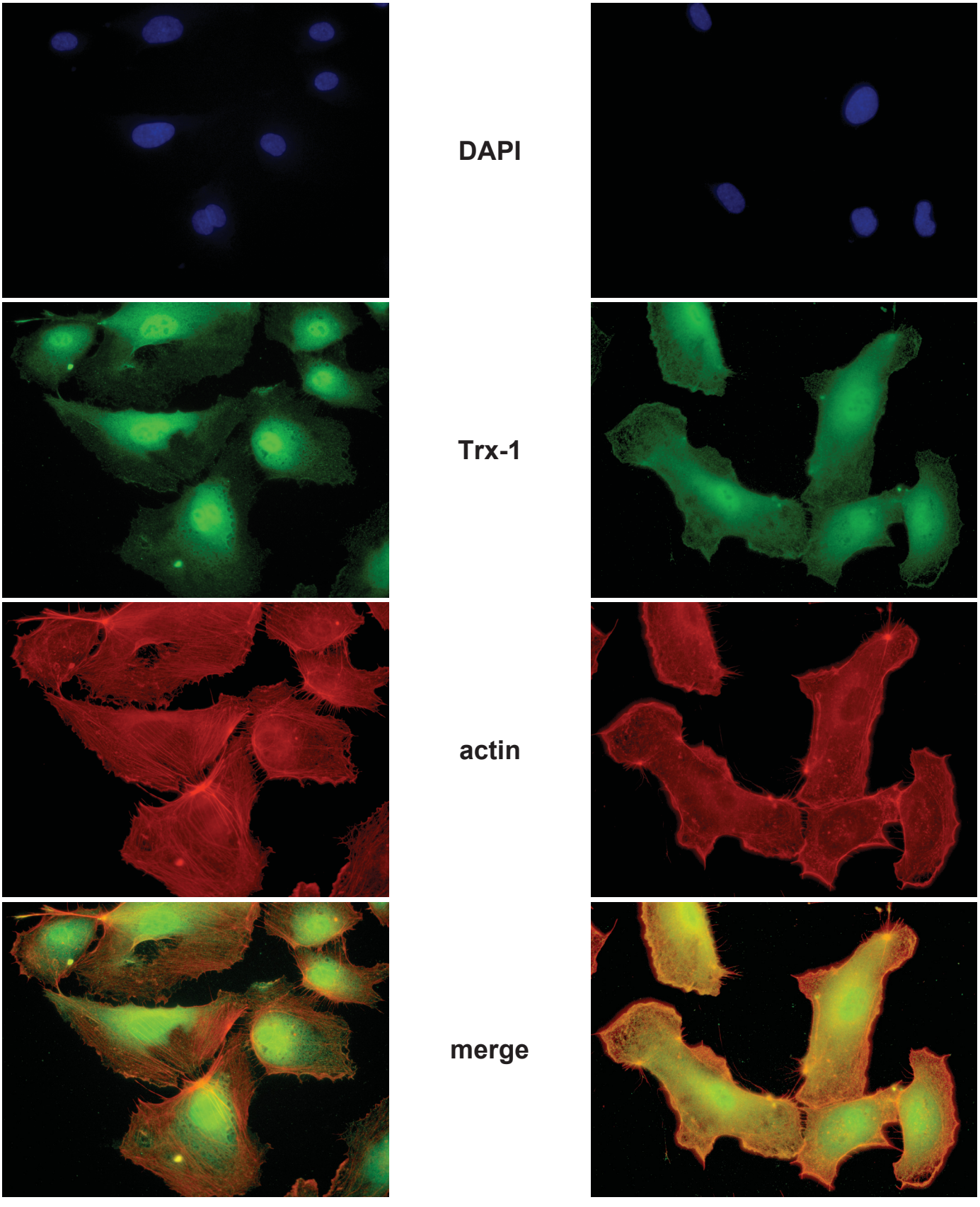
None.

References

1. Holmgren A. Thioredoxin. *Annu Rev Biochem*. 1985;54:237–271.
2. Eklund H, Gleason FK, Holmgren A. Structural and functional relations among thioredoxins of different species. *Proteins*. 1991;11:13–28.
3. Martin JL. Thioredoxin: a fold for all reasons. *Structure*. 1995;3:245–250.
4. Matsui M, Oshima M, Oshima H, Takaku K, Maruyama T, Yodoi J, Taketo MM. Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. *Dev Biol*. 1996;178:179–185.
5. Lillig CH, Holmgren A. Thioredoxin and related molecules: from biology to health and disease. *Antioxid Redox Signal*. 2007;9:25–47.
6. Powis G, Montfort WR. Properties and biological activities of thioredoxins. *Annu Rev Biophys Biomol Struct*. 2001;30:421–455.
7. Nishiyama A, Masutani H, Nakamura H, Nishinaka Y, Yodoi J. Redox regulation by thioredoxin and thioredoxin-binding proteins. *IUBMB Life*. 2001;52:29–33.
8. Ando K, Hirao S, Kabe Y, Ogura Y, Sato I, Yamaguchi Y, Wada T, Handa H. A new APE1/Ref-1-dependent pathway leading to reduction of NF-κB and AP-1, and activation of their DNA-binding activity. *Nucleic Acids Res*. 2008;36:4327–4336.
9. Hirota K, Matsui M, Iwata S, Nishiyama A, Mori K, Yodoi J. AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proc Natl Acad Sci U S A*. 1997;94:3633–3638.
10. Hwang CY, Ryu YS, Chung MS, Kim KD, Park SS, Chae SK, Chae HZ, Kwon KS. Thioredoxin modulates activator protein 1 (AP-1) activity and p27Kip1 degradation through direct interaction with Jab1. *Oncogene*. 2004;23:8868–8875.

11. Kabe Y, Ando K, Hirao S, Yoshida M, Handa H. Redox regulation of NF-κB activation: distinct redox regulation between the cytoplasm and the nucleus. *Antioxid Redox Signal*. 2005;7:395–403.
12. Schroeder P, Popp R, Wiegand B, Altschmied J, Haendeler J. Nuclear redox-signaling is essential for apoptosis inhibition in endothelial cells: important role for nuclear thioredoxin-1. *Arterioscler Thromb Vasc Biol*. 2007;27:2325–2331.
13. Wei SJ, Botero A, Hirota K, Bradbury CM, Markovina S, Laszlo A, Spitz DR, Goswami PC, Yodoi J, Gius D. Thioredoxin nuclear translocation and interaction with redox factor-1 activates the activator protein-1 transcription factor in response to ionizing radiation. *Cancer Res*. 2000;60:6688–6695.
14. Junn E, Han SH, Im JY, Yang Y, Cho EW, Um HD, Kim DK, Lee KW, Han PL, Rhee SG, Choi I. Vitamin D3 up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function. *J Immunol*. 2000;164:6287–6295.
15. Liu H, Nishitoh H, Ichijo H, Kyriakis JM. Activation of apoptosis signal-regulating kinase 1 (ASK1) by tumor necrosis factor receptor-associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin. *Mol Cell Biol*. 2000;20:2198–2208.
16. Liu Y, Min W. Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. *Circ Res*. 2002;90:1259–1266.
17. Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, Ichijo H. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J*. 1998;17:2596–2606.
18. Schulze PC, Liu H, Choe E, Yoshioka J, Shalev A, Bloch KD, Lee RT. Nitric oxide-dependent suppression of thioredoxin-interacting protein expression enhances thioredoxin activity. *Arterioscler Thromb Vasc Biol*. 2006;26:2666–2672.
19. Schulze PC, Yoshioka J, Takahashi T, He Z, King GL, Lee RT. Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. *J Biol Chem*. 2004;279:30369–30374.
20. Yoshioka J, Schulze PC, Cupesi M, Sylvan JD, MacGillivray C, Gannon J, Huang H, Lee RT. Thioredoxin-interacting protein controls cardiac hypertrophy through regulation of thioredoxin activity. *Circulation*. 2004;109:2581–2586.
21. Schulze PC, De Keulenaer GW, Yoshioka J, Kassik KA, Lee RT. Vitamin D3-upregulated protein-1 (VDUP-1) regulates redox-dependent vascular smooth muscle cell proliferation through interaction with thioredoxin. *Circ Res*. 2002;91:689–695.
22. Haendeler J, Popp R, Goy C, Tischler V, Zeiher AM, Dimmeler S. Cathepsin D and H_2O_2 stimulate degradation of thioredoxin-1: implication for endothelial cell apoptosis. *J Biol Chem*. 2005;280:42945–42951.
23. Jakob S, Schroeder P, Lukosz M, Buchner N, Spyridopoulos I, Altschmied J, Haendeler J. Nuclear protein tyrosine phosphatase Shp-2 is one important negative regulator of nuclear export of telomerase reverse transcriptase. *J Biol Chem*. 2008;283:33155–33161.
24. Haendeler J, Hoffmann J, Tischler V, Berk BC, Zeiher AM, Dimmeler S. Redox regulatory and anti-apoptotic functions of thioredoxin depend on S-nitrosylation at cysteine 69. *Nat Cell Biol*. 2002;4:743–749.
25. Abramoff MD, Magelhaes PJ, Ram SJ. Image processing with ImageJ. *Biophotonics Int*. 2004;11:36–42.
26. Haendeler J, Tischler V, Hoffmann J, Zeiher AM, Dimmeler S. Low doses of reactive oxygen species protect endothelial cells from apoptosis by increasing thioredoxin-1 expression. *FEBS Lett*. 2004;577:427–433.
27. Huot J, Houle F, Rousseau S, Deschesnes RG, Shah GM, Landry J. SAPK2/p38-dependent F-actin reorganization regulates early membrane blebbing during stress-induced apoptosis. *J Cell Biol*. 1998;143:1361–1373.
28. Levkau B, Herren B, Koyama H, Ross R, Raines EW. Caspase-mediated cleavage of focal adhesion kinase pp125FAK and disassembly of focal adhesions in human endothelial cell apoptosis. *J Exp Med*. 1998;187:579–586.
29. Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol*. 2005;6:56–68.
30. Lukosz M, Jakob S, Buchner N, Zschauer TC, Altschmied J, Haendeler J. Nuclear redox signaling. *Antioxid Redox Signal*. 2010;12:713–742.
31. Yamawaki H, Pan S, Lee RT, Berk BC. Fluid shear stress inhibits vascular inflammation by decreasing thioredoxin-interacting protein in endothelial cells. *J Clin Invest*. 2005;115:733–738.
32. Wang X, Ling S, Zhao D, Sun Q, Li Q, Wu F, Nie J, Qu L, Wang B, Shen X, Bai Y, Li Y, Li Y. Redox regulation of actin by thioredoxin-1 is mediated by the interaction of the proteins via cysteine 62. *Antioxid Redox Signal*. 2010;13:565–573.
33. Satcher R, Dewey CF Jr, Hartwig JH. Mechanical remodeling of the endothelial surface and actin cytoskeleton induced by fluid flow. *Microcirculation*. 1997;4:439–453.
34. Burridge K, Chrzanowska-Wodnicka M. Focal adhesions, contractility, and signaling. *Annu Rev Cell Dev Biol*. 1996;12:463–518.
35. Nobes CD, Hall A. Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J Cell Biol*. 1999;144:1235–1244.
36. Verma A, Davis GE, Ihler GM. Formation of stress fibres in human endothelial cells infected with *Bartonella bacilliformis* is associated with altered morphology, impaired migration and defects in cell morphogenesis. *Cell Microbiol*. 2001;3:169–180.
37. Dalle-Donne I, Milzani A, Giustarini D, Di Simplicio P, Colombo R, Rossi R. S-NO-actin: S-nitrosylation kinetics and the effect on isolated vascular smooth muscle. *J Muscle Res Cell Motil*. 2000;21:171–181.
38. Thom SR, Bhopale VM, Mancini DJ, Milovanova TN. Actin S-nitrosylation inhibits neutrophil β2 integrin function. *J Biol Chem*. 2008;283:10822–10834.

Supplementary Figure 1: Zschauer et al.



Supplementary figure 1: Endothelial cells were incubated with 0.05 µM cytochalasin D for 40 min. Cells were fixed and stained with anti-Thioredoxin-1 antibody and with FITC-conjugated secondary antibody. After that cells were incubated with phalloidin directly conjugated to Alexa 594 and DAPI for nuclear staining.

Interacting with Thioredoxin-1 – disease or no disease ?

Zschauer TC, Matsushima S, Altschmied J, Shao D, Sadoshima J, Haendeler J.

Antioxid Redox Signal. 2012; im Druck.

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Von: Shah, Ajay [ajay.shah@kcl.ac.uk]
Gesendet: Montag, 30. April 2012 10:55
An: 'Dr Jojo Haendeler'
Betreff: RE: Antioxidants & Redox Signaling Forum Issue

Dear Professor Haendeler,

Thank you for submitting a revised version of your paper entitled "**Interacting with Thioredoxin-1 – disease or no disease ?** by Zschauer et al for the ARS Forum Issue on "REDOX AND NITROSATIVE REGULATION OF CARDIAC REMODELING" that I am co-editing with David Kass. I am pleased to let you know that you have satisfactorily addressed the points raised by the reviewers and that the manuscript is now acceptable for publication in ARS. You will receive detailed instructions for uploading the manuscript and figures onto the ARS website in due course. In the meantime, please accept my congratulations for this excellent contribution to the Forum Issue.

With kind regards

Ajay Shah

_____
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Forum Review Article

Interacting with Thioredoxin-1 – disease or no disease ?

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Running head: Thioredoxin-1 in the cardiovascular system
Word count (excluding references and figure legends): 4306
Reference numbers: 64
Number of figures: 6 in grayscale

Abstract

Many cardiovascular disorders are accompanied by a deregulated cellular redox balance resulting in elevated levels of intracellular reactive oxygen species (ROS). One major anti-oxidative cellular molecule is Thioredoxin-1 (Trx-1). Its indispensability is demonstrated by the embryonic lethality of Trx-1 deficient mice. Trx-1 is ubiquitously expressed in cells and has numerous, diverse functions. It not only reduces oxidized proteins or, together with peroxiredoxins, detoxifies H_2O_2, but also binds to several proteins and thereby regulates their functions. The interaction partners of Trx-1 differ depending on its localization in the cytosol or in the nucleus. Over the past decade it has become clear that Trx-1 is not only critical for tumor functions, which has resulted in therapeutic approaches targeting this protein, but is also essential for proper functions of the vasculature and the heart. Changes in post-translational modifications of Trx-1 or in its interactions with other proteins can lead to a switch from a physiologic state of cells and organs to diverse pathologies. This review provides insights into the role of Trx-1 in different physiological situations as well as in cardiac hypertrophy, ischemia reperfusion injury, heart failure, atherosclerosis and diabetes mellitus type 2 underscoring the central role of Trx-1 in cardiovascular health and disease. Thus, the manipulation of Trx-1 activity in the heart and/or vasculature, e.g. by small molecules, seems to be a promising therapeutic option in cardiovascular diseases, as general anti-oxidant treatments would not take into account interactions of Trx-1 with other proteins and also eliminate vital ROS.

Introduction

Cardiovascular diseases are the leading cause of death in the world today. There are many factors known increasing the risk for cardiovascular diseases. The major modifiable risk factors include e.g. tobacco use and alcohol abuse, unhealthy diet, obesity, physical inactivity and stress. Cardiovascular complications, such as hypertension, atherosclerosis, pathological hypertrophy, ischemic heart disease and myocardial infarction, result in enormous direct and indirect annual costs (28). Thus, treatment of these complications has a tremendous impact. Therefore, an overall understanding of the underlying causes and mechanism is required to find new possible treatments and enhance those already established.

It is widely accepted that the imbalance of prooxidative and antioxidative systems results in deregulated redox signaling and contributes to the abnormal cellular changes observed in diverse cardiovascular diseases. Redox regulation describes reduction and oxidation events that are responsible for keeping a proper cellular environment. These are essential physiologic processes which include reversible post-translational protein modifications changing the functional properties of the affected molecules, e.g. oxidative inactivation of phosphatases or reductive activation of transcription factors (30,57). They can be found in almost all cells including endothelial cells and cardiomyocytes. If prooxidative systems or situations take the upper hand and antioxidative systems cannot compensate those signals, the cell faces oxidative stress, which can lead to apoptosis. In cardiovascular cells the induction of apoptosis by oxidative stress is predominantly triggered by activation of caspases (6).

In cardiovascular cells in particular there are two major antioxidative defense systems, the glutathione (GSH) and the thioredoxin (Trx) systems. The tripeptide GSH can neutralize reactive oxygen species and reduce oxidized molecules. The reactive glutathione produced in these processes forms glutathione disulfide (GSSG), which is regenerated to GSH by glutathione reductase and NADPH. The Trx system consists of Thioredoxin and the corresponding Thioredoxin Reductase (TR). TR utilizes the electron donor NADPH to reduce and regenerate the dithiol active site in oxidized Trx (for review see (22) and figure 1). Along with Trx-1, which can be found in the cytosol and nucleus, another Trx, Trx-2, exists which is localized in the mitochondria (47). Besides those two ubiquitously expressed Trx systems, a testis-specific Trx system has been described (32). This review, however, will focus on the role of Trx-1 in cardiovascular diseases.

The 12 kD small protein Trx-1 was first discovered in 1964 by Peter Reichard and his group as a hydrogen donor for the ribonucleotide reductase, an essential enzyme involved in DNA

synthesis in *E. coli* (27). Shortly thereafter the amino acid sequence of *E.coli* Trx-1 was determined and the dithiol active site (-Cys-Gly-Pro-Cys-), conserved from bacteria to mammals and essential for the redox-regulatory function, described (21). A few years later the three-dimensional structure of *E.coli* Trx-1 revealed the so-called Trx fold, a structural element of proteins of this class (23). Trx-1 is ubiquitously expressed in mammalian cells and has numerous, diverse functions. Its essential role was demonstrated by genetic targeting leading to severe developmental disorders in the early mouse embryo resulting in embryonic lethality (31). Together with the peroxiredoxins (Prxs) Trx-1 is involved in reducing peroxides, e.g. hydrogen peroxide, by regenerating oxidized Prxs through reduction of their redox-active cysteines (39) (figure 2). Interestingly, Day et al recently demonstrated in fission yeast that the inactivation of the only 2-Cys peroxiredoxin Tpx1 by hydrogen peroxide is required for Trx-1 mediated reduction of oxidized proteins and thus cell survival (9). However, it is not clear whether the same mechanism applies in cardiovascular cells, since they express more than one 2-Cys peroxiredoxin.

Besides this indirect scavenging of peroxides, Trx-1 interacts with several proteins via disulfide bridges thereby modulating protein functions (11). For example, Trx-1 binds to the apoptosis signal-regulating kinase 1 (ASK-1) and prevents apoptosis or to the thioredoxin-interacting protein (Txnip, also called VDUP-1 for vitamin D3-upregulated protein 1 or Tbp-2 for thioredoxin-binding protein 2) inhibiting its functions (41,59). Another study recently showed that in response to physiological amounts of ROS or tumor necrosis factor α (TNFα) the Trx-1/Txnip complex translocates to the plasma membrane and promotes a cell survival signal through VEGFR2 in endothelial cells (56) (figure 2). Upon translocation into the nucleus Trx-1 enhances DNA binding of several transcription factors in concert with APEX nuclease (multifunctional DNA-repair enzyme) 1 (APEX1), including activator protein 1 (AP-1) and nuclear factor κB (NFκB) (17,18,44) (figure 2). Interestingly, cytosolic Trx-1 has an opposite effect on NFκB activation by preventing the degradation of the NFκB inhibitor IκB and thus the translocation of NFκB into the nucleus (18,43). Most of these examples demonstrate that Trx-1 facilitates the reduction of proteins in the cytosol as well as in the nucleus by cysteine thiol-disulfide exchange reaction.

Over the past decade it has become clear that Trx-1 is not only critical for tumor functions where Trx-1 is often upregulated, which has resulted in therapeutic approaches targeting this protein (for review see (37)), but is also essential for proper functions of the vasculature and the heart. Therefore, this review will focus on the role of Trx-1 in different physiological situations and pathophysiological changes within the cardiovascular system.

Cardiac hypertrophy

Cardiac hypertrophy is characterized by a significant increase in the size of cardiomyocytes together with increased ventricular chamber size and a thickening of the ventricular wall. Healthy cardiac hypertrophy results from normal physiological stimuli such as athletic training or pregnancy and is the normal adaptive response to the enhancement in working load. This increase in heart muscle mass and pumping ability is not accompanied by a long-term disease pattern. Pathological hypertrophy, in contrast, is characterized as the response of the heart to stress such as chronic hypertension or myocardial infarction, both of which are associated with pressure or volume overload leading to contractile dysfunction and heart failure. Many of those pathological changes are associated with elevated ROS levels, which induce damage to proteins, lipids and DNA, whereas in physiological hypertrophy ROS act as second messengers and influence central signaling pathways without inducing damage (for review see (48)).

To study the role of Trx-1 in cardiac hypertrophy, mice were generated overexpressing wildtype or a dominant negative Trx-1 mutant, in which the cysteines of the catalytic center Cys 32 and Cys 35 are exchanged to serines, in the heart (58). Animals with cardiac-specific overexpression of wildtype Trx-1 were protected against lipid peroxidation, did not exhibit hypertrophy at baseline and showed reduced hypertrophy after aortic banding. In contrast, expression of the dominant negative mutant leads to cardiac hypertrophy under baseline conditions and in response to pressure overload. These mice were also characterized by increased lipid peroxidation, DNA damage, oxidized glutathione, extracellular signal-regulated kinase 1/2 (ERK1/2), Ras and Raf-1 activation. An involvement of oxidative stress in baseline cardiac hypertrophy was demonstrated by administration of an antioxidant (58).

Other studies showed that not only detoxification of ROS is involved in protection against baseline hypertrophy, but also the interaction of Trx-1 with other proteins. One prominent interaction partner of Trx-1 is Txnip (45,60). However, studies in Txnip deficient mice revealed a transient attenuation of pressure overload induced hypertrophy not accompanied by changes in Trx-1 expression and activity, suggesting a Trx-1 independent protective mechanism for Txnip in cardiac hypertrophy (62).

In contrast, Ras induced hypertrophy is Trx-1 dependent. Upon oxidative stress exerted through hypertrophic stimuli, Trx-1 seems to keep cysteine 118 of Ras in a reduced state thereby preventing a Ras-mediated hypertrophic response in cardiac myocytes (36) (figure 3). This was shown by overexpression of Trx-1 in these cells, which prevented alpha adrenergic receptor induced, Ras-mediated hypertrophy. In this situation, ROS levels were not decreased, however, Ras activation was reduced. Involvement of a Trx-1 dependent redox regulatory process was substantiated by TR1 inhibition with azelaic acid, which potentiated protein synthesis leading to hypertrophy (26).

Another known interaction partner of Trx-1 is ASK-1. Reduced Trx-1 binds to ASK-1 via cysteines 32 and 35 in its catalytic center and thus inactivates the enzyme (41). Free ASK-1 itself is well described to play a major role in cardiac hypertrophy and also in promoting apoptosis of cardiomyocytes (19,25). Reactive oxygen species may disrupt the Trx-1/ASK-1 interaction through oxidative modifications of the Trx-1 catalytic center, which in turn releases ASK-1 subsequently leading to the aforementioned changes (figure 3).

Mammalian class II histone deacetylases (HDACs) have been reported to play a crucial role in the regulation of cardiac hypertrophy through repression of target genes of several transcription factors, such as serum response factor (SRF), nuclear factor of activated T cells (NFAT) and myocyte enhancer factor 2 (MEF2) (7,8,15). Translocation of class II HDACs from the nucleus to the cytoplasm occurs in response to G protein-coupled receptor signaling and subsequent phosphorylation by Ca^{2+} calmodulin-dependent kinases, e.g. CaMKIIδ, and other kinases like protein kinase C δ (for review see (35)). Besides this known shuttle of class II HDACs, a novel phosphorylation-independent and redox-sensitive nuclear export of HDAC4 has been proposed. Upon hypertrophic stimuli HDAC4 is oxidized under formation of a disulfide bond between Cys-667 and Cys-669 and exported from the nucleus possibly through unmasking a nuclear export signal within its C-terminal region. In the cytosol Trx-1 partially exerts its anti-hypertrophic capacity by reducing oxidized HDAC4 in a multiprotein complex with Txnip and DnaJb5, thereby allowing HDAC4 to re-enter the nucleus. At first Trx-1 reduces an intramolecular disulfide bond between cysteines 274 and 276 in DnaJb5 to allow binding to HDAC4, which is then reduced by Trx-1 after its regeneration by TR1 (figure 4) (1). Even more, CaM kinases, like e.g. CaMKII may also be activated through oxidation of methionine residues in response to angiotensin II, possibly leading to phosphorylation of HDACs. Trx-1 is capable of reducing Methionine sulfoxide reductases, which in turn can inactivate CaMKII. Thus, it is possible that Trx-1 may negatively regulate CaMKII, which could contribute to the protective effect of Trx-1 in cardiac hypertrophy.

In summary, Trx-1 has a general protective role in cardiac hypertrophy not only through antioxidative mechanisms, but also via direct interactions with several proteins. A direct interaction and thereby a reduction of the target proteins by Trx-1 has been shown for Ras and HDAC4 preventing pro-hypertrophic responses. In the case of ASK-1 Trx-1 functions as a scavenger molecule precluding the pro-apoptotic activity of the kinase.

Ischemia / Reperfusion

Ischemia is a period of restricted or even no blood supply to an organ, e.g. after rupture of an atherosclerotic plaque and subsequent arterial occlusion leading to an infarcted heart. Once the blood flow is re-established the affected part of the organ is subjected to reperfusion. Paradoxically, this reperfusion phase generally results in lethal tissue damage due to e.g.

inflammation and/or oxidative stress rather than physiological recovery. This so called lethal reperfusion injury can be alleviated by a process called preconditioning. Herein, repeated short-term non-lethal ischemic and reperfusion periods ultimately protect the myocardium from a consecutive potential lethal ischemia (33).

In a model of working isolated rat hearts it was shown that in response to ischemia/reperfusion Trx-1 is slightly downregulated whereas preconditioning increases Trx-1 expression, decreases infarct size, cardiomyocyte apoptosis and oxidative stress. These cardioprotective effects were abrogated in response to cisplatin, which has been described as a non-specific inhibitor of Trx-1 and therefore should be regarded with caution due to possible side effects. Furthermore, in the same study, it was demonstrated that transgenic mouse hearts overexpressing Trx-1 exhibited significantly improved post-ischemic ventricular recovery and reduced myocardial infarct size in comparison to wildtype hearts after ischemia/reperfusion (53). Along the same line, infusion of recombinant human Trx-1 (rhTrx-1) shortly before reperfusion of the ischemic myocardium significantly reduced apoptosis and myocardial infarct size. Immunohistochemical analysis confirmed the uptake of rhTrx-1 throughout the ischemic/reperfused myocardium. However, it is not clear from this study whether rhTrx-1 is taken up in its reduced or oxidized form. S-nitros(yl)ation of rhTrx-1 prior to administration potentiated those protective effects whereas a bacterial isoform isolated from *E.coli* (eTrx) lacking cysteine 69 showed similar effects as non-modified rhTrx-1, indicating an important role of this post-translational modification in cardioprotection without being a prerequisite. Mechanistically this anti-apoptotic impact to some extent appears to be carried out through diminished p38-mitogen activated protein kinase (MAPK) activation, a known downstream target of ASK-1 (50) (figure 5). This observation is in accordance with data describing an increase in the anti-apoptotic function of Trx-1 if S-nitros(yl)ated on cysteine 69 in endothelial cells (12). It is known that there is a significant increase in the content of nitrated proteins under various pathological conditions. This is in agreement with data presented by Yin et al. who demonstrated that Trx-1 is nitrated in ischemia/reperfusion subsequently leading to its inactivation. Administration of nitrated Trx-1 in working mouse hearts prior to reperfusion did not result in a significant reduction in infarct size (figure 5) (61). In agreement with this observation is the decrease in ischemia/reperfusion induced caspase-3 activation in a similar experimental setting by administration of unmodified Trx-1 or a non-nitratable mutant Trx-1 (Y49F) in contrast to nitrated Trx-1. Of note, nitration of Trx-1 in the ischemic/reperfused cardiac tissue diminished the binding of Trx-1 to ASK-1 and enhanced p38-MAPK activation (51,63) (figure 5). Trx-1 may also carry out its protective influence through antagonizing ion channel remodeling in the post myocardial infarcted heart leading to arrhythmia and contractile dysfunction. Especially the expression of ventricular K^+ channels seems to be negatively regulated by the impaired cardiac Trx-1 system after

ischemia/reperfusion through intensified ASK-1- Jun-N-terminal kinase (JNK)-p38-MAPK signaling (49). Finally, there is evidence that overexpression of Trx-1 induces genes coding for parts of the oxidative phosphorylation machinery and the citric acid cycle in mitochondria (2). The downregulation of Trx-1 in ischemia/reperfusion might thus contribute to the well-described damage of these organelles under these conditions.

Overall, during ischemia/reperfusion the protective effects of Trx-1 do not only rely on its binding partners, including transcription factors, which change gene expression programs, but also on different post-translational modifications of Trx-1 itself, which can result in opposing activation states.

Heart failure

The inability of the heart to supply sufficient blood flow to the whole body is generally defined as heart failure. Myocardial infarction, ischemic heart diseases and cardiomyopathies can often lead to heart failure. As described above, Trx-1 is protective against ischemia/reperfusion and cardiac hypertrophy. Thus, these beneficial effects may also prevent the progression of heart failure. In a therapeutic setting this would require long-term treatment with Trx-1. Therefore, the development of small molecules which can increase Trx-1 activity in the heart or mimick its action would provide an excellent long term treatment for heart failure.

Atherosclerosis

Atherosclerosis is a chronic inflammatory response in the vessel wall leading to the formation of atherosclerotic plaques that are characterized by accumulation of immune cells, lipids and cellular debris covered by a fibrous cap (40). Upon rupture of the plaque sudden thrombotic occlusion of the artery can occur, which in the heart leads to myocardial infarction. One of the earliest steps in the development of atherosclerosis involves the loss of integrity of the endothelium, the innermost cellular layer in the vessel, which is in part due to oxidative stress and apoptosis (4,54).

Trx-1 is one important anti-apoptotic protein in endothelial cells (EC) and mediates this effect through different mechanisms, which depend on post-translational modifications, subcellular localization and different binding partners of Trx-1. The crucial role of Trx-1 in EC apoptosis has been shown by overexpression and downregulation leading to protection or an increased sensitivity towards programmed cell death, respectively (12,14). The protective effects are mediated by different mechanisms, among them translocation of Trx-1 into the nucleus under physiological concentrations of ROS. There, Trx-1 activates transcription factors binding to the antioxidant response element (ARE) culminating in an anti-oxidative gene expression program. Well-described examples are the upregulation of Glutathione S-transferase P1, the

promoter of which contains several AREs (44), and the reduction of nuclear factor erythroid 2–related factor 2 (Nrf-2), enabling its binding to these regulatory elements (16). The latter is also evident in the Nrf-2 knockout mouse, in which one prominent phenotypical feature is the apoptosis of endothelial cells (38). A potent anti-apoptotic and therefore anti-atherosclerotic stimulus is the blood flow itself. It has been shown that in areas of human carotid atherosclerotic plaques with low or turbulent flow, apoptosis rates of EC are significantly higher than in those areas with normal, laminar blood flow (52). This laminar blood flow, also called shear stress, results in the downregulation of Txnip, releasing Trx-1 thus enhancing its binding to ASK-1. This interaction not only inhibits apoptosis through preventing homodimerization of ASK-1, a prerequisite for its activation, and making ASK-1 prone to ubiquitination and degradation (29), but also plays a role in preventing a pro-inflammatory response. Degradation of ASK-1 prevents activation of JNK and p38-MAPK thereby inhibiting TNFα induced expression of vascular cell-adhesion molecule 1 (VCAM1), a surface molecule important for the interaction of EC with T cells and monocytes in the early inflammatory response leading to atherosclerotic plaque formation (29,60). Along the same lines Trx-1 downregulates monocyte chemoattractant protein-1 (MCP-1) expression and secretion (5), thus, suppressing monocytes/macrophages recruitment and adhesion. For monocytes themselves it was shown that Trx-1 plays a role in preventing apoptosis (24). Interestingly, treatment of monocyte-derived macrophages with a synthetic peroxisome proliferator-activated receptor gamma (PPARγ) agonist led to upregulation of Txnip and elevated apoptosis rates (3). Taken together, this indicates that the Trx-1/Txnip interaction may contribute to monocyte apoptosis regulation.

In addition, laminar blood flow was demonstrated to enhance the activity of Trx-1 in EC through increasing the amount of S-nitros(yl)ated Trx-1 (20). Interestingly, apoptosis protection by Trx-1 involves not only activation of anti-oxidative gene programs and inhibition of pro-apoptotic signaling cascades, but also interactions with cytoskeletal components. We recently demonstrated that the binding of Trx-1 to γ-actin is a new, ASK-1 independent anti-apoptotic mechanism. Challenging the actin cytoskeleton with H_2O_2 leads to aberrant rearrangements and formation of actin stress fibers. This is accompanied by a reduction in Trx-1 protein levels possibly through Cathepsin D mediated degradation and increased EC apoptosis (13,64). Since overexpression of Trx-1 prevents stress fiber formation and inhibition of actin bundle formation blocks Trx-1 degradation, this interaction seems to mutually protect both proteins from oxidative stress (64) (figure 6).

Taken together, Trx-1 may exerts its protective role in atherosclerosis through preventing endothelial cell apoptosis at several levels, thereby ensuring the integrity of the vessel wall and preventing inflammatory processes.

Diabetes mellitus

Diabetes mellitus is a metabolic disease, which is characterized by prevailing hyperglycaemia in the blood. Type 1 diabetes mellitus, also known as juvenile diabetes, is considered an autoimmune disease in which the insulin producing beta-cells of the pancreas are destroyed resulting in very low levels of insulin and leading to high blood sugar levels. On the other hand, in type 2 diabetes mellitus, also called adult-onset diabetes, the responses of cells to insulin and the uptake of glucose are impaired resulting in high blood sugar levels. The prevalence of type 2 diabetes is ever increasing becoming the major form of diabetes diagnosed in patients and is considered to be a major risk factor for cardiovascular diseases, e.g. atherosclerosis or myocardial infarction (10).

The diabetic disease state with hyperglycemic conditions has been associated with an increase in ROS. The elevated ROS levels seem to be generated in the mitochondria by a one electron transfer to oxygen. This mitochondrial superoxide overproduction may result in protein kinase C (PKC) and NFκB activation and an increase in advanced glycation end-products (AGEs) forming methylglyoxal, which in turn harms the endothelial monolayer (34). The intensified oxidative potential is challenging for the cellular antioxidative systems and may lead to deregulated protein interactions as mentioned earlier. Indeed, in an animal model of streptozotocin-induced diabetes in rats, ROS were increased and Trx-1 activity was significantly decreased without a change in expression or protein levels in the diabetic animals in comparison to untreated littermates. The diminished activity of Trx-1 is thought to be due to an induction of Txnip expression through high glucose-induced activation of p38-MAPK signaling. Elevated levels of Txnip would result in more inhibitory binding to Trx-1 and explain the reduced Trx-1 activity. Of note, insulin treatment reduced high glucose-induced Txnip expression and rescued Trx-1 activity (46). One may speculate that this glucose-dependent upregulation of Txnip also disturbs the Trx-1/ASK-1 axis, since the enhanced inhibitory binding of Txnip to Trx-1 releases ASK-1, which then induces EC apoptosis. In terms of clinical approaches there are recent studies that show a protective effect of Trx-1 after myocardial infarction in streptozotocin-induced animal models for diabetes. Adenoviral gene therapy with Trx-1 after myocardial infarction in diabetic rats reduced fibrosis, oxidative stress and apoptosis and enhanced capillary and arteriolar density (42). Administration of recombinant human Trx-1 in a comparable experimental setting in mice attenuated apoptosis, reduced infarct size and improved cardiac function (61). This cumulative evidence suggests that Trx-1 may be a suitable therapeutic to decrease heart damage after myocardial infarction in diabetic patients.

Another mechanistic explanation for reduced Trx-1 activity could be glycative inhibition by methylglyoxal, a byproduct of metabolic pathways, which is elevated in diabetic patient plasma. In the setting of an ischemia/reperfusion model of H9c2 cardiomyoblasts, cells

preincubated with methylglyoxal had lower Trx-1 activity accompanied by an enhanced p38-MAPK activation and reduced binding of Trx-1 to ASK-1 (55).

In conclusion, diabetes is a disease closely correlated with cardiovascular morbidity and mortality, which is accompanied by excessive ROS formation and a modified Trx-1 system. Animal models suggest that functional improvement after cardiovascular insults maybe obtained by Trx-1 delivery. One has to keep in mind, that increased Trx-1 activity is present in nearly all tumors, such that therapeutic interventions of this kind bear an inherent danger. However, approaches targeting elevated mitochondrial ROS production or trying to increase mitochondrial Trx-2 activity to support classical diabetes therapies could be envisioned in the future.

Conclusion and future directions

Trx-1 plays a central role in the physiology of the cardiovascular system. Inactivation or loss of Trx-1 has been demonstrated in multiple cardiovascular diseases. Therefore, Trx-1 has been used in animal models to protect against cardiovascular diseases. To understand the protective effects of Trx-1 in the cardiovascular system, several studies have investigated its anti-oxidative capacity, its interaction with several proteins and its potential in modifying gene expression programs. It has become clear that Trx-1 protects against cardiovascular diseases through multiple pathways. However, important unsolved problems are 1) how to increase the concentration of Trx-1 in the heart and vessels or 2) how to administer Trx-1 to the heart. To increase intracellular Trx-1 levels, it could be envisioned to either inhibit its degradation or to upregulate its transcription. However, this alone might not be sufficient, because Trx-1, in order to execute its multiple functions, has to be kept in a reduced form, which requires TR1 and NADPH. Moreover, the threshold levels of Trx-1 necessary to exert its protective functions in the heart and vasculature are unknown and require further investigation. Another option would be to design small molecule drugs enhancing Trx-1 activity. An important issue when using systemically acting compounds is the expression of Trx-1 in tumors. This has been addressed in therapeutic approaches targeting this protein in cancer patients. However, all systemic approaches improving Trx-1 functions should be treated with caution, as they might foster tumor development and/or progression. Conversely, reciprocal considerations have to been taken into account, when trying to treat tumors by interfering with Trx-1 functions, because this might lead to severe cardiovascular dysfunction. Therefore, molecules, which could be targeted to specific tissues and cellular compartments would be the premier option.

Acknowledgments

This study was supported by the Deutsche Forschungsgemeinschaft (HA2868/3-3), a Start-Up grant of the University of Duesseldorf, the Leducq Transatlantic Network of Excellence 09 CVD_01 to J.H. and J.S.) and U.S. Public Health Service Grants HL59139, HL67724, HL69020, HL91469, HL102738, AG27211 (to J.S.).

Abbreviations

AP-1 - Activator protein-1; APEX1 - APEX nuclease (multifunctional DNA-repair enzyme) 1; ARE – antioxidant response element; ASK-1 – apoptosis signal-regulating kinase 1; EC – endothelial cells; ERK1/2 - extracellular signal-regulated kinase 1/2 (ERK1/2); GSH – glutathione; GSSG – glutathione disulfide; HDAC – histone deacetylase; JNK – Jun-N-terminal kinase; MAPK – mitogen activated protein kinase; MCP -1 Monocyte chemoattractant protein-1; MEF2 – myocyte enhancer factor 2; NFAT – nuclear factor of activated T-cells; NFκB - Nuclear factor κB; Nrf-2 - Nuclear factor erythroid 2–related factor 2; Prx – Peroxiredoxin; PPARγ - peroxisome proliferator-activated receptor gamma); ROS – reactive oxygen species; SRF – serum response factor; TNFα **-** tumor necrosis factor α; TR – Thioredoxin-1 Reductase, Trx-1 - Thioredoxin-1; Txnip - thioredoxin-interacting protein; VCAM1 - vascular cell-adhesion molecule 1

Author Disclosure Statement

The authors have nothing to disclose.

References

1. Ago T, Liu T, Zhai P, Chen W, Li H, Molkentin JD, Vatner SF, Sadoshima J. A redox-dependent pathway for regulating class II HDACs and cardiac hypertrophy. *Cell* 133: 978-93, 2008.
2. Ago T, Yeh I, Yamamoto M, Schinke-Braun M, Brown JA, Tian B, Sadoshima J. Thioredoxin1 upregulates mitochondrial proteins related to oxidative phosphorylation and TCA cycle in the heart. *Antioxid Redox Signal* 8: 1635-50, 2006.
3. Billiet L, Furman C, Larigauderie G, Copin C, Page S, Fruchart JC, Brand K, Rouis M. Enhanced VDUP-1 gene expression by PPARgamma agonist induces apoptosis in human macrophage. *J Cell Physiol* 214: 183-91, 2008.
4. Busse R, Fleming I. Endothelial dysfunction in atherosclerosis. *J Vasc Res* 33: 181-194, 1996.
5. Chen B, Guan D, Cui ZJ, Wang X, Shen X. Thioredoxin 1 downregulates MCP-1 secretion and expression in human endothelial cells by suppressing nuclear translocation of activator protein 1 and redox factor-1. *Am J Physiol Cell Physiol* 298: C1170-9, 2010.

6. Clarke M, Bennett M, Littlewood T. Cell death in the cardiovascular system. *Heart* 93: 659-64, 2007.
7. Dai YS, Xu J, Molkentin JD. The DnaJ-related factor Mrj interacts with nuclear factor of activated T cells c3 and mediates transcriptional repression through class II histone deacetylase recruitment. *Mol Cell Biol* 25: 9936-48, 2005.
8. Davis FJ, Gupta M, Camoretti-Mercado B, Schwartz RJ, Gupta MP. Calcium/calmodulin-dependent protein kinase activates serum response factor transcription activity by its dissociation from histone deacetylase, HDAC4. Implications in cardiac muscle gene regulation during hypertrophy. *J Biol Chem* 278: 20047-58, 2003.
9. Day AM, Brown JD, Taylor SR, Rand JD, Morgan BA, Veal EA. Inactivation of a peroxiredoxin by hydrogen peroxide is critical for thioredoxin-mediated repair of oxidized proteins and cell survival. *Mol Cell* 45: 398-408, 2012.
10. Eckel RH, Wassef M, Chait A, Sobel B, Barrett E, King G, Lopes-Virella M, Reusch J, Ruderman N, Steiner G, Vlassara H. Prevention Conference VI: Diabetes and Cardiovascular Disease: Writing Group II: pathogenesis of atherosclerosis in diabetes. *Circulation* 105: e138-43, 2002.
11. Fu C, Wu C, Liu T, Ago T, Zhai P, Sadoshima J, Li H. Elucidation of thioredoxin target protein networks in mouse. *Mol Cell Proteomics* 8: 1674-87, 2009.
12. Haendeler J, Hoffmann J, Tischler V, Berk BC, Zeiher AM, Dimmeler S. Redox regulatory and anti-apoptotic functions of thioredoxin depend on S-nitrosylation at cysteine 69. *Nat Cell Biol* 4: 743-9, 2002.
13. Haendeler J, Popp R, Goy C, Tischler V, Zeiher AM, Dimmeler S. Cathepsin D and H2O2 stimulate degradation of thioredoxin-1: implication for endothelial cell apoptosis. *J Biol Chem* 280: 42945-51, 2005.
14. Haendeler J, Tischler V, Hoffmann J, Zeiher AM, Dimmeler S. Low doses of reactive oxygen species protect endothelial cells from apoptosis by increasing thioredoxin-1 expression. *FEBS Lett* 577: 427-33, 2004.
15. Han A, He J, Wu Y, Liu JO, Chen L. Mechanism of recruitment of class II histone deacetylases by myocyte enhancer factor-2. *J Mol Biol* 345: 91-102, 2005.
16. Hansen JM, Watson WH, Jones DP. Compartmentation of Nrf-2 redox control: regulation of cytoplasmic activation by glutathione and DNA binding by thioredoxin-1. *Toxicol Sci* 82: 308-17, 2004.
17. Hirota K, Matsui M, Iwata S, Nishiyama A, Mori K, Yodoi J. AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proc Natl Acad Sci U S A* 94: 3633-8., 1997.

18. Hirota K, Murata M, Sachi Y, Nakamura H, Takeuchi J, Mori K, Yodoi J. Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-kappaB. *J Biol Chem* 274: 27891-7, 1999.
19. Hirotani S, Otsu K, Nishida K, Higuchi Y, Morita T, Nakayama H, Yamaguchi O, Mano T, Matsumura Y, Ueno H, Tada M, Hori M. Involvement of nuclear factor-kappaB and apoptosis signal-regulating kinase 1 in G-protein-coupled receptor agonist-induced cardiomyocyte hypertrophy. *Circulation* 105: 509-15, 2002.
20. Hoffmann J, Dimmeler S, Haendeler J. Shear stress increases the amount of S-nitrosylated molecules in endothelial cells: important role for signal transduction. *FEBS Lett* 551: 153-8, 2003.
21. Holmgren A. Thioredoxin. 6. The amino acid sequence of the protein from escherichia coli B. *Eur J Biochem* 6: 475-84, 1968.
22. Holmgren A. Antioxidant function of thioredoxin and glutaredoxin systems. *Antioxid Redox Signal* 2: 811-20., 2000.
23. Holmgren A, Soderberg BO, Eklund H, Branden CI. Three-dimensional structure of Escherichia coli thioredoxin-S2 to 2.8 A resolution. *Proc Natl Acad Sci U S A* 72: 2305-9, 1975.
24. Imen JS, Billiet L, Cuaz-Perolin C, Michaud N, Rouis M. The regulated in development and DNA damage response 2 (REDD2) gene mediates human monocyte cell death through a reduction in thioredoxin-1 expression. *Free Radic Biol Med* 46: 1404-10, 2009.
25. Izumiya Y, Kim S, Izumi Y, Yoshida K, Yoshiyama M, Matsuzawa A, Ichijo H, Iwao H. Apoptosis signal-regulating kinase 1 plays a pivotal role in angiotensin II-induced cardiac hypertrophy and remodeling. *Circ Res* 93: 874-83, 2003.
26. Kuster GM, Pimentel DR, Adachi T, Ido Y, Brenner DA, Cohen RA, Liao R, Siwik DA, Colucci WS. Alpha-adrenergic receptor-stimulated hypertrophy in adult rat ventricular myocytes is mediated via thioredoxin-1-sensitive oxidative modification of thiols on Ras. *Circulation* 111: 1192-8 Epub 2005 Feb 21, 2005.
27. Laurent TC, Moore EC, Reichard P. Enzymatic Synthesis of Deoxyribonucleotides. Iv. Isolation and Characterization of Thioredoxin, the Hydrogen Donor from Escherichia Coli B. *J Biol Chem* 239: 3436-44, 1964.
28. Leal J, Luengo-Fernandez R, Gray A, Petersen S, Rayner M. Economic burden of cardiovascular diseases in the enlarged European Union. *Eur Heart J* 27: 1610-9, 2006.
29. Liu Y, Min W. Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. *Circ Res* 90: 1259-66, 2002.

30. Lukosz M, Jakob S, Buchner N, Zschauer TC, Altschmied J, Haendeler J. Nuclear redox signaling. *Antioxid Redox Signal* 12: 713-42, 2010.
31. Matsui M, Oshima M, Oshima H, Takaku K, Maruyama T, Yodoi J, Taketo MM. Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. *Dev Biol* 178: 179-85., 1996.
32. Miranda-Vizuete A, Ljung J, Damdimopoulos AE, Gustafsson JA, Oko R, Pelto-Huikko M, Spyrou G. Characterization of Sptrx, a novel member of the thioredoxin family specifically expressed in human spermatozoa. *J Biol Chem* 276: 31567-74., 2001.
33. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74: 1124-36, 1986.
34. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404: 787-90, 2000.
35. Oka S, Ago T, Kitazono T, Zablocki D, Sadoshima J. The role of redox modulation of class II histone deacetylases in mediating pathological cardiac hypertrophy. *J Mol Med (Berl)* 87: 785-91, 2009.
36. Pimentel DR, Adachi T, Ido Y, Heibeck T, Jiang B, Lee Y, Melendez JA, Cohen RA, Colucci WS. Strain-stimulated hypertrophy in cardiac myocytes is mediated by reactive oxygen species-dependent Ras S-glutathiolation. *J Mol Cell Cardiol* 41: 613-22, 2006.
37. Powis G, Kirkpatrick DL. Thioredoxin signaling as a target for cancer therapy. *Curr Opin Pharmacol* 7: 392-7, 2007.
38. Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, Yamamoto M, Petrache I, Tuder RM, Biswal S. Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. *J Clin Invest* 114: 1248-59, 2004.
39. Rhee SG, Chae HZ, Kim K. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic Biol Med* 38: 1543-52, 2005.
40. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med* 340: 115-26, 1999.
41. Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, Ichijo H. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *Embo J* 17: 2596-606., 1998.
42. Samuel SM, Thirunavukkarasu M, Penumathsa SV, Koneru S, Zhan L, Maulik G, Sudhakaran PR, Maulik N. Thioredoxin-1 gene therapy enhances angiogenic

signaling and reduces ventricular remodeling in infarcted myocardium of diabetic rats. *Circulation* 121: 1244-55, 2010.

43. Schenk H, Klein M, Erdbrugger W, Droge W, Schulze-Osthoff K. Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-kappa B and AP-1. *Proc Natl Acad Sci U S A* 91: 1672-6., 1994.
44. Schroeder P, Popp R, Wiegand B, Altschmied J, Haendeler J. Nuclear redox-signaling is essential for apoptosis inhibition in endothelial cells--important role for nuclear thioredoxin-1. *Arterioscler Thromb Vasc Biol* 27: 2325-31, 2007.
45. Schulze PC, De Keulenaer GW, Yoshioka J, Kassik KA, Lee RT. Vitamin D3-upregulated protein-1 (VDUP-1) regulates redox-dependent vascular smooth muscle cell proliferation through interaction with thioredoxin. *Circ Res* 91: 689-95, 2002.
46. Schulze PC, Yoshioka J, Takahashi T, He Z, King GL, Lee RT. Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. *J Biol Chem* 279: 30369-74 Epub 2004 May 5, 2004.
47. Spyrou G, Enmark E, Miranda-Vizuete A, Gustafsson J. Cloning and expression of a novel mammalian thioredoxin. *J Biol Chem* 272: 2936-41., 1997.
48. Sugden PH, Clerk A. Oxidative stress and growth-regulating intracellular signaling pathways in cardiac myocytes. *Antioxid Redox Signal* 8: 2111-24, 2006.
49. Tang K, Li X, Zheng MQ, Rozanski GJ. Role of apoptosis signal-regulating kinase-1-c-Jun NH2-terminal kinase-p38 signaling in voltage-gated K+ channel remodeling of the failing heart: regulation by thioredoxin. *Antioxid Redox Signal* 14: 25-35, 2011.
50. Tao L, Gao E, Bryan NS, Qu Y, Liu HR, Hu A, Christopher TA, Lopez BL, Yodoi J, Koch WJ, Feelisch M, Ma XL. Cardioprotective effects of thioredoxin in myocardial ischemia and reperfusion: role of S-nitrosation [corrected]. *Proc Natl Acad Sci U S A* 101: 11471-6, 2004.
51. Tao L, Jiao X, Gao E, Lau WB, Yuan Y, Lopez B, Christopher T, RamachandraRao SP, Williams W, Southan G, Sharma K, Koch W, Ma XL. Nitrative inactivation of thioredoxin-1 and its role in postischemic myocardial apoptosis. *Circulation* 114: 1395-402, 2006.
52. Tricot O, Mallat Z, Heymes C, Belmin J, Leseche G, Tedgui A. Relation between endothelial cell apoptosis and blood flow direction in human atherosclerotic plaques. *Circulation* 101: 2450-3, 2000.
53. Turoczi T, Chang VW, Engelman RM, Maulik N, Ho YS, Das DK. Thioredoxin redox signaling in the ischemic heart: an insight with transgenic mice overexpressing Trx1. *J Mol Cell Cardiol* 35: 695-704, 2003.
54. Vanhoutte PM. Endothelial dysfunction and atherosclerosis. *Eur Heart J* 18 Suppl E: E19-29, 1997.

55. Wang XL, Lau WB, Yuan YX, Wang YJ, Yi W, Christopher TA, Lopez BL, Liu HR, Ma XL. Methylglyoxal increases cardiomyocyte ischemia-reperfusion injury via glycative inhibition of thioredoxin activity. *Am J Physiol Endocrinol Metab* 299: E207-14, 2010.
56. World C, Spindel ON, Berk BC. Thioredoxin-interacting protein mediates TRX1 translocation to the plasma membrane in response to tumor necrosis factor-alpha: a key mechanism for vascular endothelial growth factor receptor-2 transactivation by reactive oxygen species. *Arterioscler Thromb Vasc Biol* 31: 1890-7, 2011.
57. Xu D, Rovira, II, Finkel T. Oxidants painting the cysteine chapel: redox regulation of PTPs. *Dev Cell* 2: 251-2, 2002.
58. Yamamoto M, Yang G, Hong C, Liu J, Holle E, Yu X, Wagner T, Vatner SF, Sadoshima J. Inhibition of endogenous thioredoxin in the heart increases oxidative stress and cardiac hypertrophy. *J Clin Invest* 112: 1395-406, 2003.
59. Yamanaka H, Maehira F, Oshiro M, Asato T, Yanagawa Y, Takei H, Nakashima Y. A possible interaction of thioredoxin with VDUP1 in HeLa cells detected in a yeast two-hybrid system. *Biochem Biophys Res Commun* 271: 796-800, 2000.
60. Yamawaki H, Pan S, Lee RT, Berk BC. Fluid shear stress inhibits vascular inflammation by decreasing thioredoxin-interacting protein in endothelial cells. *J Clin Invest* 115: 733-8, 2005.
61. Yin T, Hou R, Liu S, Lau WB, Wang H, Tao L. Nitrative inactivation of thioredoxin-1 increases vulnerability of diabetic hearts to ischemia/reperfusion injury. *J Mol Cell Cardiol* 49: 354-61, 2010.
62. Yoshioka J, Imahashi K, Gabel SA, Chutkow WA, Burds AA, Gannon J, Schulze PC, MacGillivray C, London RE, Murphy E, Lee RT. Targeted deletion of thioredoxin-interacting protein regulates cardiac dysfunction in response to pressure overload. *Circ Res* 101: 1328-38, 2007.
63. Zhang H, Tao L, Jiao X, Gao E, Lopez BL, Christopher TA, Koch W, Ma XL. Nitrative thioredoxin inactivation as a cause of enhanced myocardial ischemia/reperfusion injury in the aging heart. *Free Radic Biol Med* 43: 39-47, 2007.
64. Zschauer TC, Kunze K, Jakob S, Haendeler J, Altschmied J. Oxidative stress-induced degradation of thioredoxin-1 and apoptosis is inhibited by thioredoxin-1-actin interaction in endothelial cells. *Arterioscler Thromb Vasc Biol* 31: 650-6, 2011.

Figure Legends

Figure 1: The Trx-1 system. One important function of Trx-1 is to catalyze redox reactions. Upon reduction of a target protein, Trx-1 itself is oxidized. Regeneration of oxidized Trx-1 is sustained through the TR1 which itself is restored in a NADPH-dependent manner.

Figure 2. The multiple functions of Trx-1. Trx-1 is capable of reducing proteins and thus restoring their functions. Together with the Prx system Trx-1 detoxifies peroxides. Furthermore, binding of Trx-1 to cytosolic proteins, e.g ASK-1, protects against apoptosis and reductive activation of transcription factors in the nucleus together with APEX1 results in transcriptome changes. Upon stress, translocation to the plasma membrane together with Txnip leads to a cell survival signal.

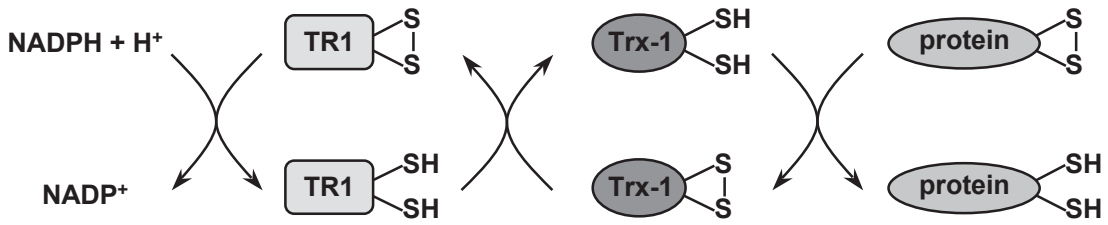
Figure 3: Multiple roles of Trx-1 in cardiac hypertrophy. Trx-1 exerts anti-hypertrophic actions through interaction with and reduction of different proteins. Hypertrophic stimuli such as ROS result in oxidation and nuclear export of HDAC4. In a multi-protein complex Trx-1 reduces oxidized HDAC4 which then re-enters the nucleus and represses SRF-, NFAT-, MEF2-target genes involved in hypertrophy. Protective binding of Trx-1 to ASK-1 is disrupted through ROS; free ASK-1 then promotes apoptosis and hypertrophy in cardiovascular cells. Moreover, Trx-1 prevents a Ras-mediated hypertrophic response through keeping Ras in a reduced state.

Figure 4: Protective interaction of Trx-1 with HDAC4 in the hypertrophic response. In the nucleus reduced HDAC4 plays a crucial role in prevention of cardiac hypertrophy through repression of transcription. Hypertrophic stimuli lead to ROS formation and oxidation of HDAC4, which then is exported out of the nucleus. In the cytosol Trx-1 exerts in part its anti-hypertrophic effect through reducing HDAC4 in a multi-protein complex with Txnip and the heat shock protein DnaJb5. To form this reducing complex Trx-1 first reduces DnaJb5 allowing HDAC4 to bind. After regeneration by TR, Trx-1 is capable of reducing HDAC4, which can then re-enter the nucleus.

Figure 5: Post-translational modifications of Trx-1 in ischemia/reperfusion injury. Sequestration of ASK-1 by Trx-1 is protective during ischemia/reperfusion in part through diminished p38-MAPK activation. S-nitros(yl)ation of cysteine 69 of Trx-1 potentiates the protective effect whereas nitration of tyrosine 49 of Trx-1 leads to a dissociation of the Trx-1/ASK-1 complex resulting in enhanced p38-MAPK activation and thus loss of protection.

Figure 6: The interaction of Trx-1 with γ-actin in EC. Under conditions of normal blood flow Trx-1 binds to γ-actin, which results in a mutual protection of both proteins against degradation and stress fiber formation, respectively and thus the EC itself. ROS occurring in areas of turbulent or no blood flow partly result in thick actin bundle formation leading to a dissociation of Trx-1 from actin and subsequently to Trx-1 degradation most likely exerted

through Cathepsin D and stress fiber formation. Both events precede EC apoptosis and thus to the onset of cardiovascular diseases like e.g atherosclerosis.



Zschauer et al. figure 1
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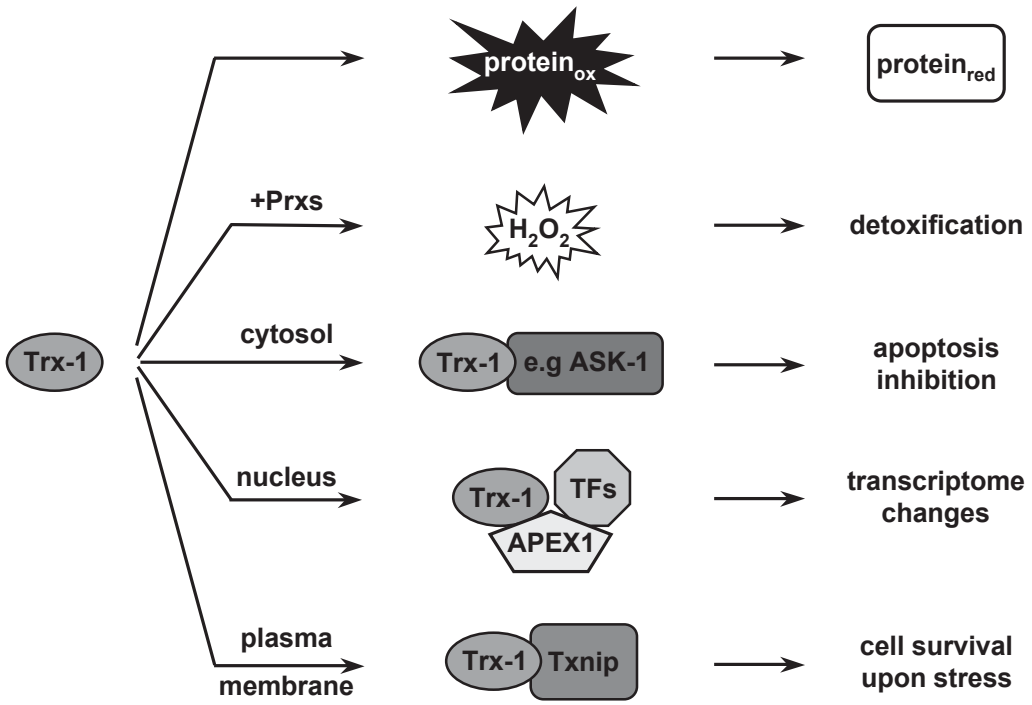

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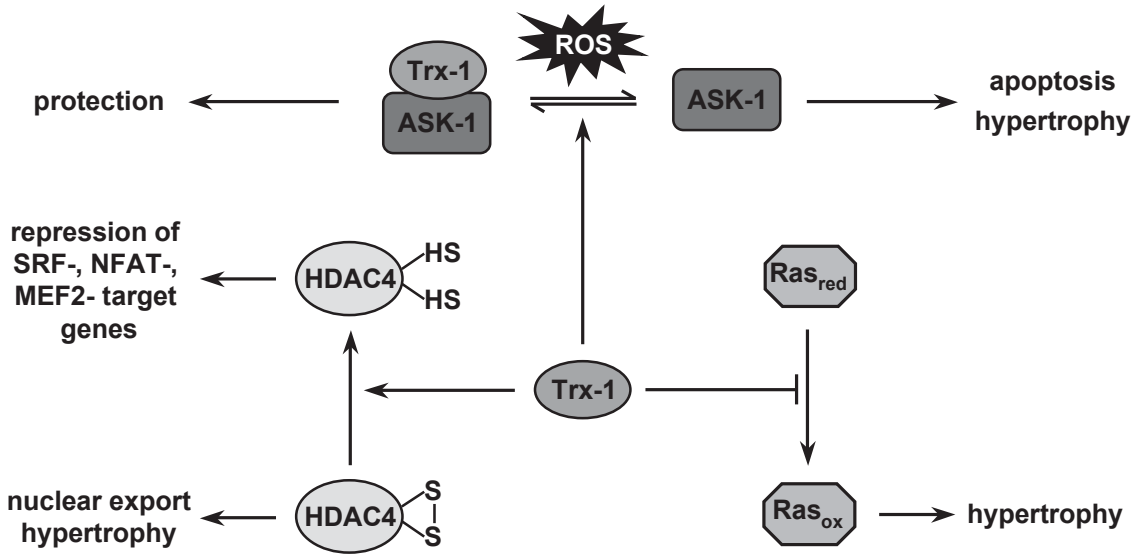

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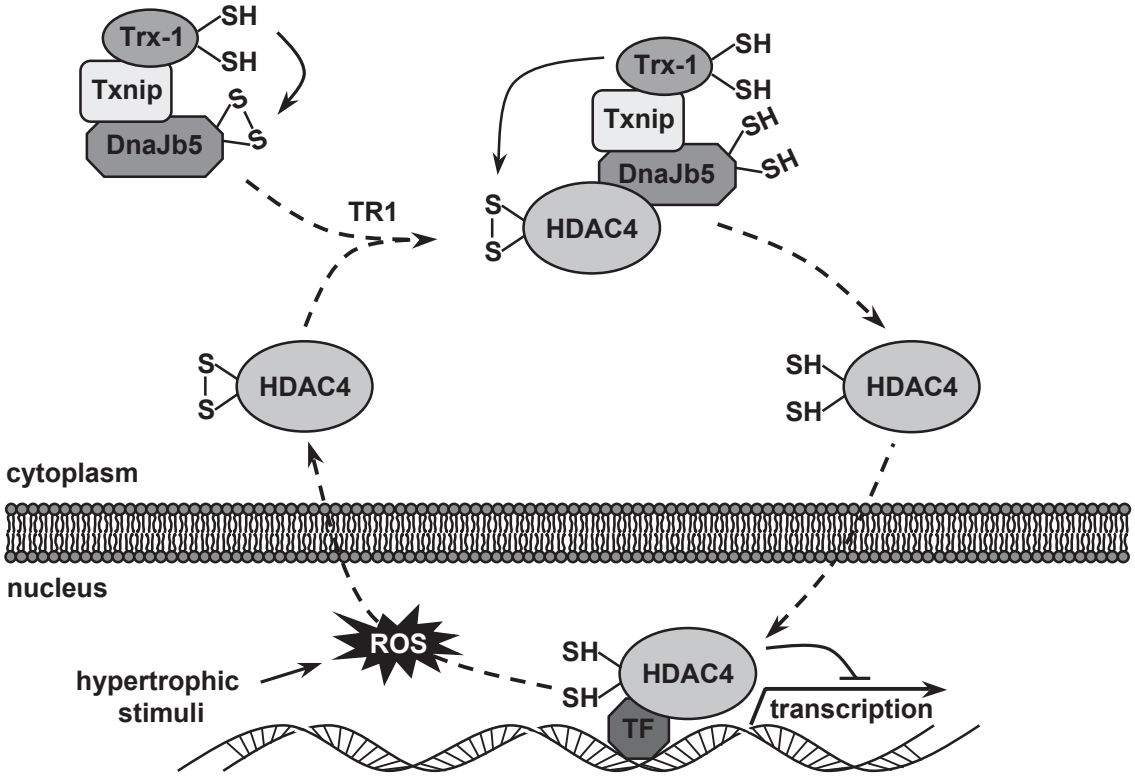

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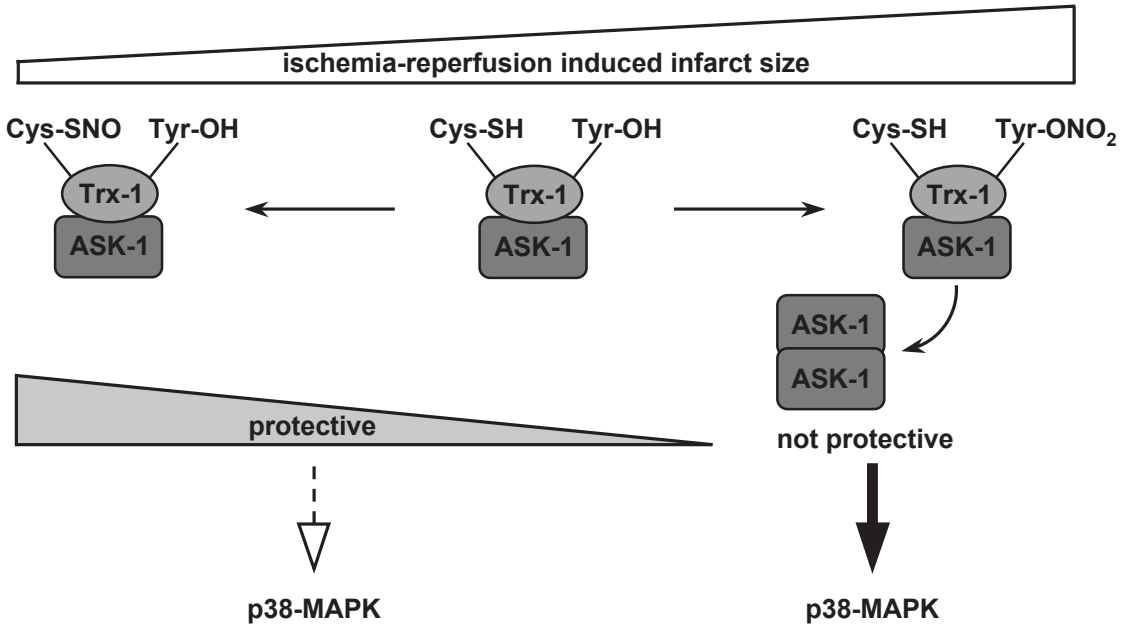

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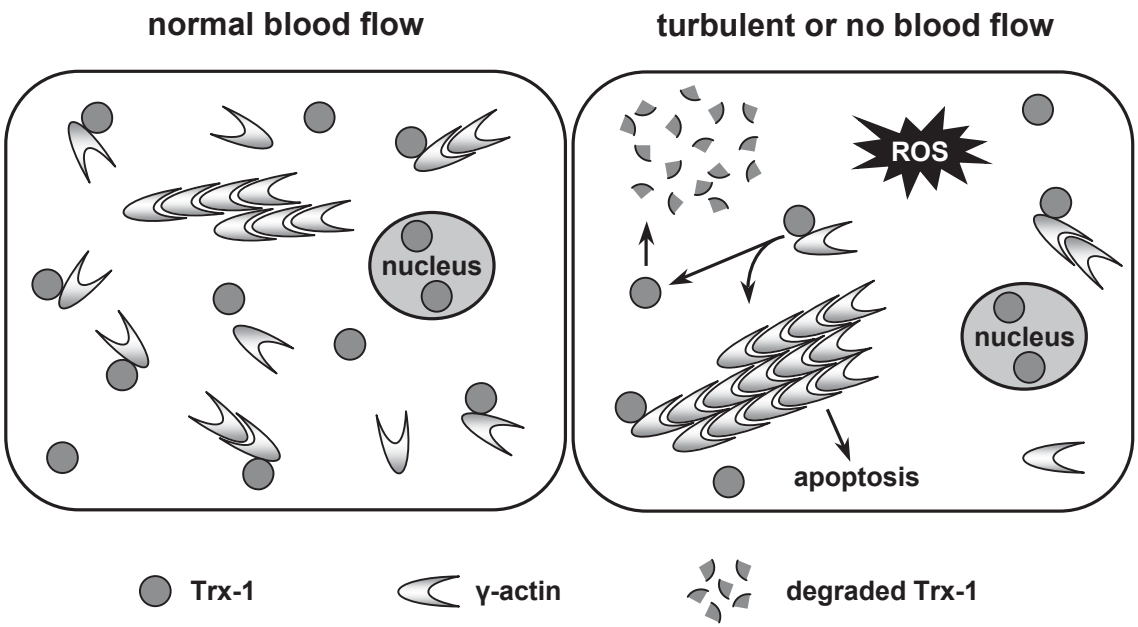

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Danksagung

Mein besonderer Dank geht an Frau PD Dr. Jojo Haendeler für die Bereitstellung des interessanten Promotionsthemas. Ebenso möchte ich meinen Dank aussprechen für die hervorragende Betreuung sowie die zahlreichen Möglichkeiten an nationalen sowie internationalen wissenschaftlichen Tagungen teilzunehmen.

Bei Herrn Prof. Dr. William Martin bedanke ich mich für die Übernahme des Korreferats.

Ebenso geht ein großes Dankeschön an Herrn PD Dr. Yogi Altschmied für die angenehme Betreuung und das stets kritische Auge bei der Durchsicht meiner Ergebnisse, Poster und Vorträge.

Auch möchte ich mich bei meiner Büromitbewohnerin Frau Dr. Anna Eckers für die kritischen Blicke bei der Durchsicht meine Arbeit bedanken sowie für die höchst lustige Büroatmosphäre.

Bei meiner gesamten Arbeitsgruppe möchte ich mich für die kollegiale Umgebung bedanken und das sie meine Launen immer mal wieder ertragen haben.

Bei meinen Arbeitskollegen bedanke ich mich für die schöne gemeinsame Zeit.

Maren, Dir danke ich von ganzem Herzen (auch mit +1), für alles was war und was noch sein wird. K & L!

Auch möchte ich meinen Eltern und meiner Schwester von ganzem Herzen dafür danken, dass sie mir meinen bisherigen Lebensweg ermöglicht haben und immer für mich da sind. Danke für Euer Verständnis und Unterstützung.