Urokinase receptor in regulation of cellular senescence and DNA damage response: role for the ubiquitin-proteasomal system

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Abstracts:
uPAR regulates vascular smooth muscle cells senescence by interfering with Telomeric Repeat Binding Factor 2 (TRF2) proteasomal degradation. XlIIth international workshop on Molecular & Cellular Biology of Plasminogen Activator, July 9-13, 2011Clare College Cambridge, England

uPAR mediates Telomeric Repeat Binding Factor 2 proteasomal degradation in senescence. Life in motion: Dynamics of Molecules and Systems. 2nd IRB Barcelona PhD Student Symposium. 17-18 November 2011

List of abbreviations

ATM	Ataxia-telangiectasia mutated
BARD	BRCA-associated ring domain
bFGF	Active fibroblast growth factor
BRCA1	Breast cancer type
BRIT1	BRCT-repeat inhibitor of hTERT expression
cAbl	Abelson murine leukemia viral homolog 1
Chk	Checkpoint kinase
CP	Core particle
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DOX	Doxorubicin
DSBs	Double-strand breaks
DSS1	Deleted in split hand/split foot protein 1
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FANCL	Fanconi anemia complementation group L
FPRL	Formyl peptide receptor-like
GPCR	G protein coupled receptor
GPI	Glycosylphosphatidylinositol
HR	Homologous recombination
IRIF	Ionizing radiation-induced foci
JAK	Janus kinase
kDa	Kilodaltons
LRP	Low density lipoprotein receptor-related protein
mAb	Monoclonal antibody
MCPH1	Microcephalin
MDa	Megadalton
Nbs1	Nijmegen breakage syndrome 1
Mdc1	Mediator of DNA-damage checkpoint 1
Mdm2	Murine double minute
MFI	Mean fluorescence intensity
MMP	Matrix metalloproteinase
MRN	Mre11-Rad50-Nbs1
NF-κB	Nuclear factor-kappaB
NHEJ	Nonhomologous end joining
53BP1	p53 binding-protein 1
pAb	Polyclonal antibody
PAC	Proteasome assembly chaperones
PAI-1	Plasminogen activator inhibitor type-1
PAI-2	Plasminogen activator inhibitor type-2
PAS	Plasminogen activation system

PDGFRß	Platelet-derived growth factor receptor beta
PIKK	Phosphoinositide-3-kinase-related protein kinase
PKC	Protein kinase C
POT1	Protection of Telomeres 1
Pro-MMPs	Pro-metalloproteinases
Pro-uPA	Pro-urokinase
RP	Regulatory particle
SAβG	Senescence-associated β-galactosidase
SHFM1	Split hand-split foot malformation 1
SIAH	Seven in absentia homolog
siRNA	Small interfering RNA
SS	Single-stranded
STAT	Signal Transducer and Activator of Transcription
suPAR	Soluble urokinase-type plasminogen activator
TIN2	TERF1-interacting nuclear factor2
TGF-β	Transforming growth factor-beta
TopBP1	Topoisomerase IIβ-binding protein 1
TPP1	TEL patch of telomere protein 1
TRF1	Telomeric-repeat binding factor1
UBC	Ubiquitin-conjugating enzyme
UBE1L2	Ubiquitin-activating Enzyme 1-like 2
UPS	Ubiquitin-proteasome system
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

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Abstract

DNA damage is a relatively common event in the life of cell. Based on type and location of DNA lesion several cellular responses are induced that enables the cell either to repair the damage or to activate a programmed cell senescence or death process. The precise knowledge of the molecular mechanisms that determine DNA damage lesion and downstream signaling response remains, however, elusive.

The uPA/uPAR is a multifaceted system mediating a diverse array of extracellular and intracellular processes. We observed that the level of uPAR expression plays a decisive role in regulation of telomeric repeat binding factor 2 (TRF2) during doxorubicin-induced senescence. TRF2 is the main telomere-associated protein implicated in the maintenance of DNA structure and known to be necessary for proper telomere function. Our results demonstrate that uPAR controls the ubiquitin-proteasome system in vascular smooth muscle cells (VSMC) and regulates doxorubicin-induced TRF2 ubiquitination and proteasomal degradation via this mechanism.

Using repairable DNA damage response model, we found that uPAR also involved in DNA damage repair mechanisms. The underlying pathways involved uPAR-mediated regulation of the proteasome regulatory subunit, Rpn7 and its redistribution to DNA damage foci. We further showed that Rpn7 nuclear translocation requires Rpn7 association with the tyrosine kinase c-Abl. We provide evidence that nuclear c-Abl associates with the tyrosine phosphatase SHP-2, which undergoes acetylation and serves for regulation of DNA repair.

In this thesis we elucidated mechanisms underlying uPAR mediated DNA damage response with particular focus on DNA repair mechanism and senescence as an important consequence of irreparable telomeric lesion. Elucidation of the factors involved in DNA damage response mechanisms may lead to novel therapeutic avenues for age related disease and cancer.

Key words: Urokinase receptor, DNA damage response, TRF2, Ubiquitin proteasome system

Zusammenfassung

Der Umgang mit DNA Schädigung spielt eine große Rolle im Leben einer Zelle. Je nach Ort und Typ der DNA Läsion werden dabei verschiedene zelluläre Antworten induziert, die Zelle entweder dazu befähigen, den Schaden zu reparieren oder zur programmierten Zellseneszenz und Zelltod führen. Über die zugrunde liegenden molekularen Mechanismen und Signalwege der DNA Schädigung und der dadurch induzierten zellulären Antwort ist wenig bekannt.

Das Urokinase-Plasminogen-Aktivator (uPA) / uPA-Rezeptor (uPAR) System reguliert eine Vielzahl von intra- und extrazellulären Prozessen. Wir haben herausgefunden, dass das uPAR Expressionslevel eine entscheidende Rolle in der Regulation des „Telomeric Repeat Binding Factor 2" (TRF2) bei der Doxorubicin-induzierten Zellseneszenz spielt. TRF2 ist als wichtiges Telomer assoziiertes Protein maßgeblich an der Aufrechterhaltung der DNA Struktur beteiligt und notwendig für die regelrechte Telomer Funktion. Unsere Ergebnisse demonstrieren, dass uPAR das Ubiquitin-Proteasom-System in glatten Gefäßmuskelzellen (VSMC) kontrolliert und dadurch die Doxorubicin-induzierte TRF2 Ubiquitinierung, sowie dessen proteosomalen Abbau reguliert.

Durch die Untersuchung von zellulären Antworten auf nicht letale DNA-Schädigung fanden wir heraus, dass die zugrundeliegenden Signalwege die uPAR-vermittelte Regulation der proteosomalen Untereinheit (Rpn7) und deren Rekrutierung in die Regionen der DNA Schädigung beinhalten. Für die nukleäre Translokation von Rpn7 wird die Assoziation mit der Tyrosinkinase c-Abl benötigt. Wir konnten außerdem zeigen, dass die nucleäre c-Abl mit der Tyrosinphosphatase SHP-2 assoziiert ist, die wiederum nach Acetylierung an der Regulation der DNA Reparatur beteiligt ist.

In dieser Doktorarbeit werden die Mechanismen der uPAR-vermittelten Antwort auf DNA Schädigung dargestellt. Der besondere Fokus liegt dabei auf DNA Reparaturprozessen, sowie auf Seneszenz, als einer wichtigen Konsequenz auf irreparable Telomerläsionen. Diese Arbeit kann dazu beitragen, Faktoren zu identifizieren, die im Antwortmechanismus auf DNA Schädigung eine Rolle spielen und somit den Weg zu neuen therapeutischen Strategien gegen alterungsabhängige Krankheit und Krebs eröffnen.

Schlüsselwörter: Urokinase Rezeptor, Antwort auf DNA-Schädigungen, TRF2, Ubiquitin-Proteasom-System

1.Introduction

1.1. Urokinase type plasminogen activator receptor

The urokinase-type plasminogen activator receptor (uPAR) is a glycosyl phosphatidylinositol (GPI)-anchored protein involved in a variety of biological processes including fibrinolysis, inflammation, tissue development, extracellular matrix (ECM) remodelling during wound healing, atherosclerotic plaque formation, angiogenesis, tumor invasion, and metastasis. uPAR is part of a cell surface system that consists of the serine protease urokinase (uPA) and specific inhibitors named plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2).

Through binding with its natural ligand uPA, uPAR regulates proteolytic activity at the cell surface involved in cellular responses in both physiological and pathological conditions. In addition to mediating proteolysis it acts as a signalling receptor that promotes cell migration, proliferation and adhesion [1-3]. The uPAR signalling function is independent of uPA proteolytic activity and in some cases does not require ligand binding [4].

1.1.2. uPAR structure

The uPAR is a member of the lymphocyte antigen 6 family, composed of three domains: the amino terminal D1 domain, the linker D2 domain and carboxy-terminal D3 domain [5]. The domains packed together into a concave structure making a central cleft for uPA domains interactions [6, 7]. This keeps the entire structure of uPAR free for interaction with transmembrane proteins such as integrin, vitronectin, signalling receptors e.g. EGFR, GPCR, FPRL, PDGFR and with different modulators (Fig1) [3, 8, 9].

uPAR binding to the cell membrane through its third domain allows the localization of cell surface proteolytic activity. Cleavage of the GPI anchor by phospholipases or extracellular proteases results in the soluble forms of uPAR (suPAR), which possesses functional activity [10]. Cleavage at the site of linker gives rise to the truncated form of uPAR consisting of the domains D2 and D3 that can be associated with membrane or shed [10-12]. Such cleavage affects the biological activity of uPAR in both extracellular proteolysis and cell signaling.

uPAR is highly glycosylated at residues in all domains and glycosylation pattern of uPAR differs between cell types and can be changed during activation of signalling pathways [13, 14]. It can also affect trafficking and the solubility of uPAR as well as interaction of uPAR with its ligand [14-16].
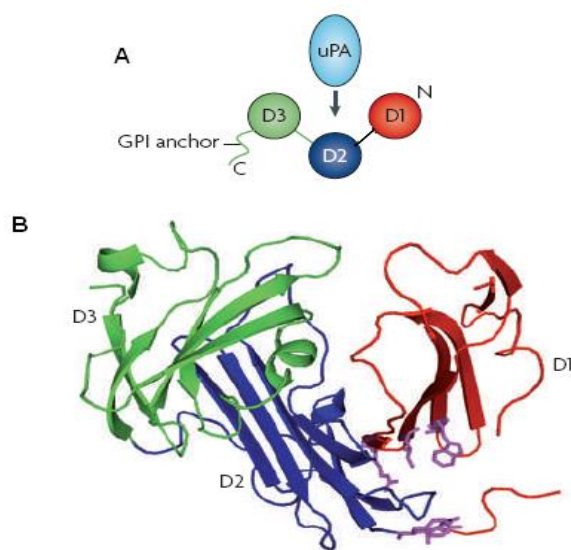
Figure 1.The structure of uPAR .a. The location of the ligand-binding sites and the GPI anchor. b. The three dimensional (3D) structure of uPAR, with domains coloured as in part [17].

1.1.3. uPAR expression

In the physiological condition, uPAR is expressed in various tissues including lungs, kidneys, spleen, vessels, uterus, bladder, thymus, heart, liver, testis and others. The enhanced uPAR expression is observed during ECM remodeling in gestational tissues [18], during embryo implantation, placental development [19] and during epidermal wound healing [20].

Stress, injury and inflammation also induce uPAR expression. It has been documented that uPAR expression is increased in many pathological conditions such as cancer, inflammation and infections. A wide variety of human cancers including solid tumours, leukaemias and lymphomas overexpress uPAR [21, 22] and increased level of suPAR in body fluids correlates with poor prognosis in cancer [21]. Systemic levels of suPAR positively correlate with inflammation in cardiovascular diseases, type-2-diabetes mellitus, immune system activation, cancer and mortality [23, 24].

uPAR expression is increased through many signaling pathways by activating transcription factors that act on the uPAR promoter. Control of uPAR transcription is mediated by extracellular signal-regulated kinase (ERK) through activator protein 1 (AP1), a transcription

factor that contributes to the mitogenic effect of Ras–ERK signalling in tumour cells [25]. Nuclear factor-κB (NF-κB) also activates uPAR expression, through a non-consensus NF-κB-binding site in the uPAR promoter [26]. The expression of uPAR could be also regulated at the post-transcriptional level through the function of mRNA binding proteins [27]. Regulatory proteins that bind *PLAUR* mRNA encoding uPAR can affect its stability or induce mRNA degradation [28].

1.1.4. uPAR functions

1.1.4.1. Proteolytic functions

uPAR regulates extracellular proteolytic cascade of the plasminogen activation system (PAS), which is considered as an important regulator of ECM proteolysis. uPAR binds the inactive zymogen form proenzyme of uPA (pro-uPA), which is then converted to active uPA on cell membrane or in solution. Activated uPA in turn is capable to generate plasmin from plasminogen thus degrading intravascular fibrin and ECM. Generated plasmin triggers a cascade of matrix metalloproteinases (MMP) activation that additionally contributing to the ECM digestion. uPAR mediated ECM degradation is involved in diverse cellular process including proliferation, migration and adhesion. Further activities of plasmin may result in increased level of active fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) as a consequence of ECM degradation, as well as the direct activation of transforming growth factor-beta (TGF-β) [29]. Therefore, the role of uPAR-mediated extracellular proteolysis extends beyond ECM degradation to the control of cell growth and differentiation through growth factor activation or their release from ECM (Fig 2).

1.1.4.2. Non-proteolytic functions

In addition to regulation of extracellular proteolysis, many biological activities of uPAR are independent of the uPA proteolytic activity and can even occur in the absence of uPA. These functions are largely related to the regulation of cell functional behaviour, interactions between the cells and the surrounding ECM. It is well documented that uPAR in spite of lacking the transmembrane and cytoplasmic domains possesses ability for intracellular signaling through its interaction with different transmembrane proteins. By these interactions

uPAR is able to transduce signals and mediate diverse intracellular signalling involved in cell adhesion, migration and proliferation.
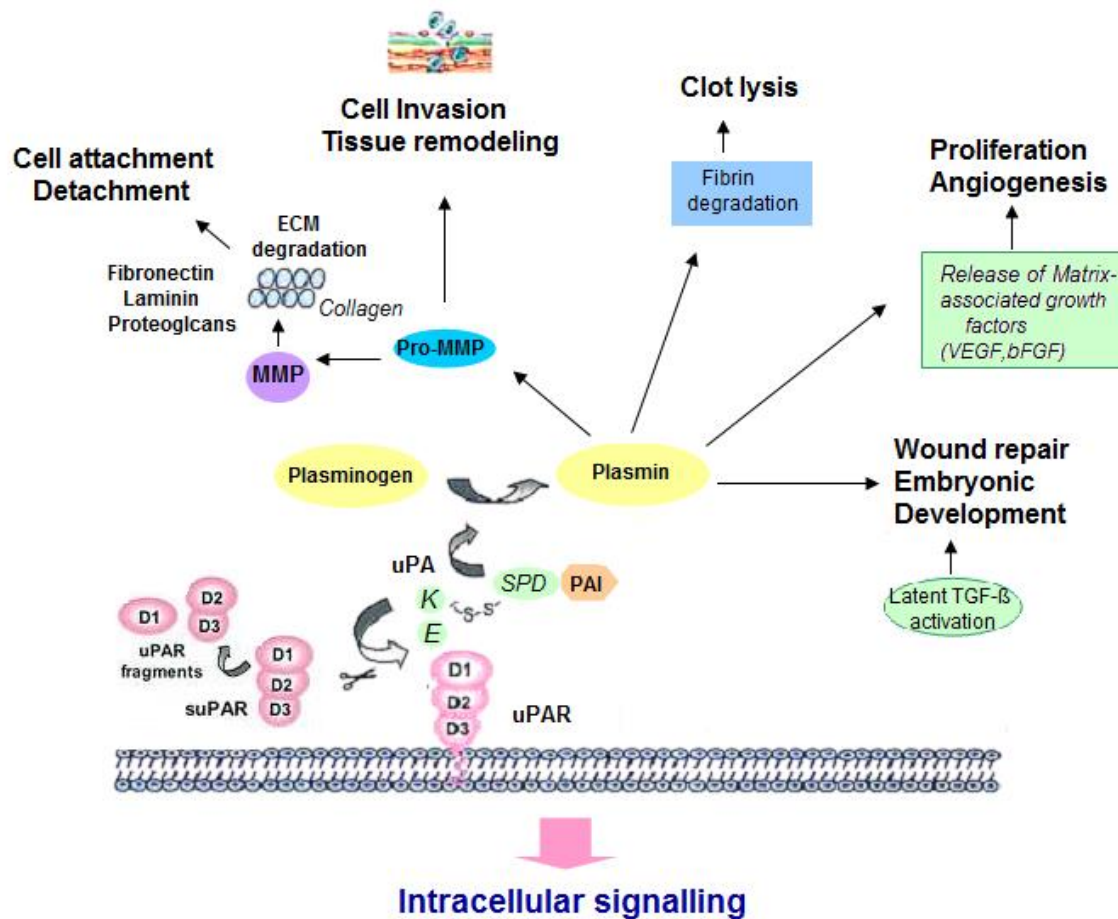

Fig 2. Effect of uPAR proteolytic activity on matrix degradation and growth factor activation. uPAR binds inactive pro-uPA, which is then converted to active uPA leading to conversion of the inactive zymogen plasminogen to active plasmin. This cascade mediates degradation of fibrin and ECM or activation of latent growth factors. Figure adapted and modified from Alfani D., et al.[30]

uPAR interacts with the matrix protein vitronectin via the somatomedin B domain (SMB) of vitronectin [31, 32]. In addition to such a direct interaction with matrix proteins, uPAR interacts with integrin adhesion molecules [33]. Upon binding of uPAR to integrin,

conformational changes occur in α5β1 integrin, which subsequently forms an additional binding site for fibronectin and enhances cell binding to fibronectin [34]. Binding of fibronectin to α5β1 integrin induces focal adhesion kinase (FAK) phosphorylation and activates Ras-ERK signalling pathway, which is necessary for tumor growth in vivo (Fig3).
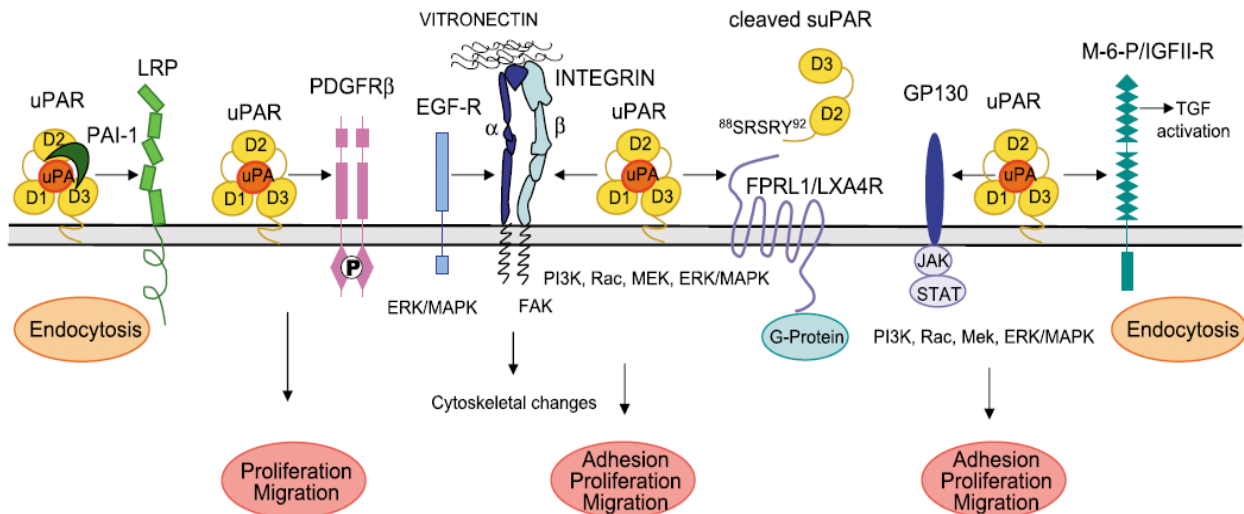

Fig 3. Compilation of important interactions within the uPA systems leading to signalling events [35]

Consistently, disruption of uPAR expression or FAK inhibition leads to tumor dormancy in human carcinoma cells [36, 37].

uPAR is able to transduces signals via interactions with the G protein-coupled receptors (GPCRs). For this interaction a motif between domain 1 and 2 has to be unmasked either by uPA binding to uPAR or by cleavage of domain 1 of uPAR, revealing a chemotactic epitope. Resulting cleaved peptide has chemoattractant activity, induces ERK1/2 phosphorylation and is considered as a ligand for GPCRs [38]. It has been shown that the family of formyl peptide receptor (FPR) is involved in transduction of chemotactic activity of uPA and subsequent cytoskeletal changes [9].

Interaction of uPAR with growth factor receptors is another possible mechanism for signal transduction. As a regulator of cell proliferation, uPAR overexpression constitutively

activates the EGFR pathway in many human cancer cell lines [3]. In these cell lines, uPAR overexpression activates EGFR in the absence of EGF. Co-immunoprecipitation experiments showed that EGFR directly interacts with α5β1 integrin and this interaction is enhanced by uPAR expression, leading to the activation of ERK signaling pathway and cell proliferation [39, 40].

uPAR also associates with platelet-derived growth factor receptor beta (PDGFR-beta), which serves as a transmembrane adaptor for uPAR in vascular smooth muscle cells (VSMC) and mediates uPAR-directed signalling via the Jak/Stat pathway [41, 42]. It has been shown that the tyrosine phosphatase SHP-2 mediates these processes in uPA dependent manner that may contribute to the pathogenesis of the vascular remodeling [43].

uPAR signalling can occur through direct internalization. The lipoprotein receptor-related protein (LRP-1) binds uPAR–uPA–PAI-1 complexes and mediates endocytosis of multiple ligands, transports the uPAR and other membrane proteins into the endosomes and binds intracellular adaptor proteins involve in cell signalling [44].

uPAR internalization also occurs via LRP-independent mechanism leading to subsequent nuclear translocation of uPAR. In this mechanism uPAR can directly affect transcriptional regulation of specific genes [45, 46].

1.1.5. Role of uPAR in vasculature and vascular diseases

Blood vessel growth and formation occur in different ways including vasculogenesis and angiogenesis [47]. Many studies have demonstrated the role of uPAR in these processes under pathological and physiological conditions [21, 48]. Angiogenesis involves a series of tightly regulated cellular processes initiated primarily by the VEGF [49, 50]. Inhibition of uPAR functional activity affects VEGF-mediated signaling and functional responses leading to significant decrease in the invasive potential of endothelial cells during angiogenesis [51, 52]. The uPA/uPAR system has been implicated in a broad spectrum of pathophysiological processes involved in occlusive and age-related cardiovascular diseases such as atherosclerosis, restenosis and aneurysm [53, 54]. It has been shown that both uPA and uPAR are upregulated in atherosclerotic lesions and involved in neointima formation and early lesion development [55-58].

uPA, uPAR and PAI-1 are essential for the regulation of migration and proliferation of leukocytes/macrophages, vascular endothelial- and smooth muscle cells [59-61]. This property of uPAR enables its active participation in wound healing and vascular remodeling. Moreover, uPAR regulates remodeling-related inflammatory responses serving as a modulator of immunocompetent receptors and of lymphocyte recruitment to the site of injury [62-66]. uPAR targeting in human blood vessels in organ cultures and in murine models for neointima formation *in vivo,* leads to strong inhibition of vascular remodeling [67, 68].

1.2. DNA damage response

The DNA damage response (DDR) is a powerful intracellular network that has the potential to repair DNA damage and resolution of DNA replication problems. It is a key factor in the maintenance of genome stability comprised of sensor proteins that recognize damaged DNA; transducer proteins that relay and amplify the damage signal; and effector proteins that control cell cycle progression, DNA repair, apoptosis and senescence [69, 70]. The activation of signaling pathways depends on the type and extent of DNA damage and also the cell type.

To maintain genomic stability, all types of DNA structural alterations including nicks, gaps, double-strand breaks (DSBs) and the alterations that block DNA replication must be detected. Different independent molecular complexes are known to be involved in sensing different types of DNA damage. Of the most importance are the phosphoinositide-3-kinase-related protein kinase (PIKK) family members ataxia-telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related protein (ATR) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs). While ATR activation is associated with single-stranded DNA and stalled DNA replication forks, ATM and DNA-PKcs respond mainly to DSBs. Many mediator complex such as Mre11-Rad50-Nbs1 (MRN), histone acetyltransferase Tip60, Topoisomerase IIβ-binding protein (TopBP1) and the protein phosphatase PP5 have been implicated in ATM, ATR activation and recruitment [71-73]. In mammals, ATR activation in response to DSBs appears to require ATM [74].

Mediators are proteins that act directly downstream of the ATM and ATR kinases. They play role in recruiting additional substrates and serve as a scaffold upon which to assemble complexes. During last few years many mediators such as mediator of DNA-damage

checkpoint 1 (Mdc1), p53 binding-protein 1(53BP1), MRN complex, Claspin, BRCT-repeat inhibitor of hTERT expression (Brit1) and microcephalin (Mcph1) has been discovered.

The histone H2A family (H2Ax) is one of the key mediators, which becomes phosphorylated by ATM, ATR and DNA-PKcs at the site of DNA damage [75]. This phosphorylation then directly recruits Mdc1 followed by subsequent recruitment of many additional factors such as 53BP1, Checkpoint kinase 2 (Chk2) and p53 to the sites of damage leading to the generation of DNA damage foci. DDR foci are intracellular structures where DDR signalling originates. Elucidation of mechanisms of their formation and function is crucial to understand how DDR activities are exerted. Factors that lie upstream in the DDR signalling cascade and function close to the DNA damage site are constituents of DDR foci. 53BP1, a mediator with roles in recombination, Chk2 and p53 activation, is recruited to foci in an H2AX- and Mdc1-dependent manner.

DDR leads to induction of different pathways that are mostly regulated by protein kinases and their phosphorylation substrates. Over the past decade, single protein analysis of ATM and ATR substrate revealed many proteins that are phosphorylated in response to DNA damage agents that have known roles in DNA replication as well as DNA repair process such as nucleotide metabolism, transcription coupled repair, global excision repair, crosslink repair, mismatch repair and homologous recombination (HR) [76, 77]. These connections show the critical role that DDR pathways play in controlling DNA repair and genomic stability beyond their roles in controlling the cell cycle (Fig 5).

Eukaryotes use many different mechanisms to repair chromosomal double-strand breaks (DSBs). In mammalian cells there are two major pathways for DNA-damage repair, namely nonhomologous end joining (NHEJ) and homologous recombination (HR). HR is an accurate form of repair, which requires an undamaged sister chromatid to act as a DNA template and functions only after DNA replication [78]. In contrast, NHEJ is active throughout the cell cycle entails straight forward ligation of DNA ends [79]. In addition to HR and NHEJ, there are increasing evidences for the existence of alternative end-joining pathways that directly ligate DNA ends in the absence of NHEJ [80-82].

9

Studies revealed that genomes are not uniformly reparable and that some genomic loci, such as telomeric tracts, resist DNA-damage repair despite a global cellular competence for DNA repair [83, 84]. The irreparability of telomere may be the direct and unavoidable consequence of their functions in preventing chromosomal end-to-end fusions. The mechanisms seem to be evolutionarily conserved in yeast, rodents and primates [85].
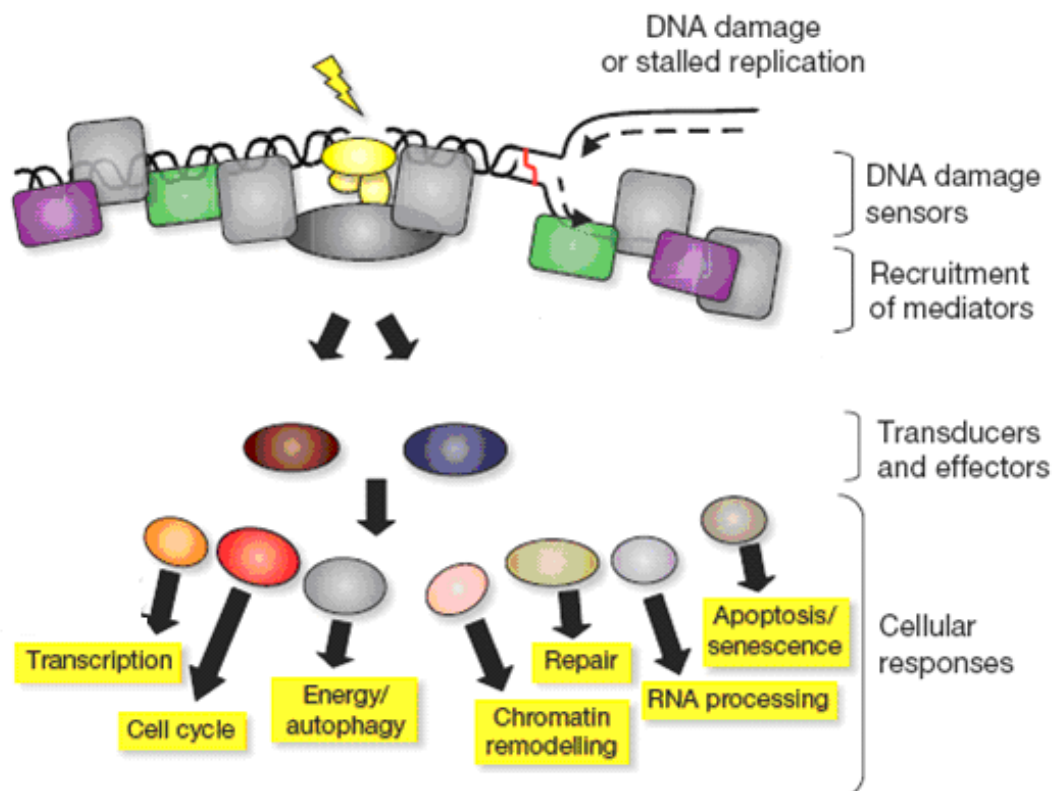

Figure 4. DNA damage response. The presence of a lesion in the DNA is recognized by various sensor proteins. Figure adapted and modified from Jackson, S. P.2009 [86]**.**

1.2.1. Telomere and DNA damage response

Telomeres are specialized nucleo-protein structures form the end part of linear eukaryotic chromosomes [87]. Using cytogenetic approaches, it was shown that natural chromosome termini possess special properties that protected it from chromosomal abnormalities and DNA-damage responses during telomere replication, recombination and erosion [88, 89]. Telomere structures consist of tandem repeat DNA sequences and associated proteins. Mammalian telomeric DNA is composed of G-rich tandem repeats of the (TTAGGG)n sequence (The G-strand overhang), which in humans extends 10-15 kilo bases (Kb) whereas in inbred mouse strains it is approximately 40– 60 Kb [89].

Cytologically, telomeres in a variety of plants and animals are heterochromatic, implying a high degree of DNA folding [90]. The bulk of telomeric DNA is double stranded, but the extreme terminus of telomeric DNA consists of a 3' overhang of approximately 200 bases. The G-strand overhang is the substrate, to which telomeric repeats are added by telomerase [89].

1.2.2. Telomere binding proteins and their function

The telomeric DNA is associated with a telomere specific protein complex, called shelterin, that functions to protect chromosome ends from all aspects of the DNA damage responds [89]. The components of shelterin were gradually identified over the past 10 years (Fig. 4). Telomeric-repeat binding factor1 (TRF1) was the first mammalian telomeric protein, isolated based on its in vitro specificity for double-stranded TTAGGG repeats typical of vertebrate telomeres [91]. TRF2 was identified, as a TRF1 paralog also possessing DNA-binding activity [92, 93]. Rap1 is an essential constitutive binding partner of TRF2 and depends on TRF2 for its telomeric localization and stability [94, 95]. TRF1-interacting nuclear factor (TIN2), TEL patch of telomere protein 1 (TPP1) and Protection of Telomeres 1 (POT1) are the other components of shelterin that bind to telomeric DNA, involve in telomere function [96-98].
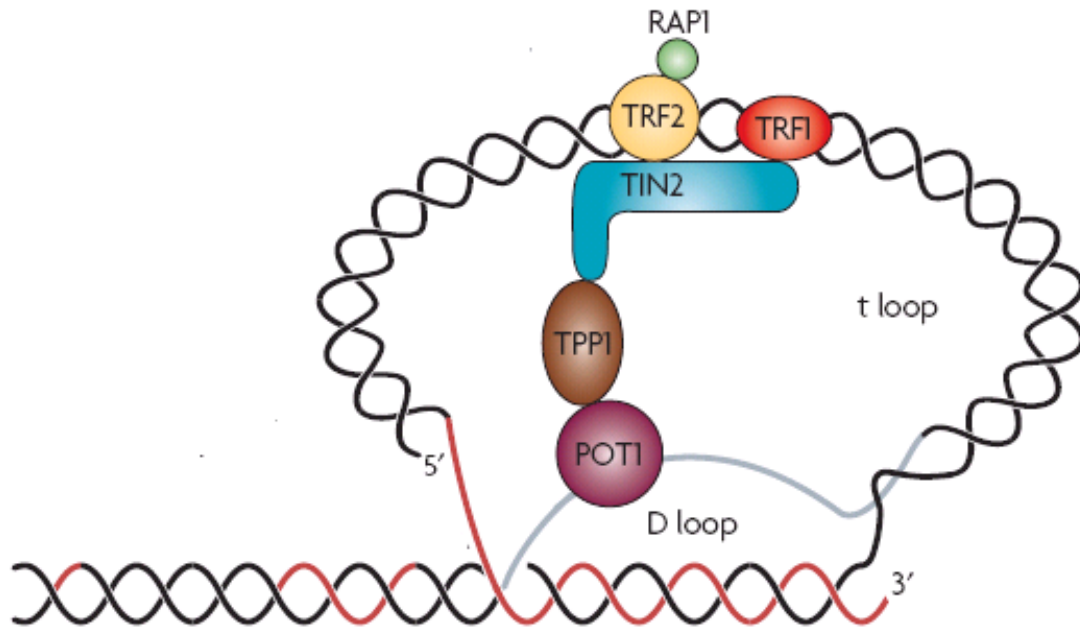
Figure 5. Telomere structure. Telomeres are composed of TTAGGG repetitive sequences that terminate in a 3′ single-stranded (ss) overhang. Telomeric DNA is complexed by the six-shelterin protein, composed TRF1, TRF2, RAP1, TIN2, TPP1 and POT1. The ss overhang can invade the double-stranded region of the telomere to form a protective telomere loop with a ss displacement (D) loop at the invasion site [99].

TRF2 contains a TRFH domain, which directly binds the telomeric double-stranded DNA [93], In addition to telomeric DNA, TRF2 associates with various proteins involved in telosome assembly, telomere-length regulation, DNA replication, repair, end joining, recombination and cell-cycle control [89, 100]. Recent studies have further shown that TRF2 serve as molecular platforms for the recruitment and assembly of the telomere interactome [88, 101]. Homozygous inactivation of either gene resulted in early embryonic lethality in mice [102]. In cultured cells, impairment of TRF2 function (dominant negative expression of TRF2ΔBΔM) led to DNA-damage responses [103, 104]. Studies supported by *in vitro* assays showed that binding of TRF2 to telomeres is reduced by oxidative DNA damage [105]. However, the mechanisms of TRF2-mediated interaction and regulation remain mainly unknown.

1.2.3. The DNA damage hypothesis of telomere dysfunction

In most organisms, telomeres that comprise stretches of short-tandem-DNA repeats are normally generated by the ribonucleoprotein complex telomerase. Excepting specialized cells such as stem cells, human cells generally do not express sufficient telomerase to counteract telomere shortening caused by the inability of the DNA replication machinery to fully replicate chromosomal ends. Thus, human telomeres generally shorten with each cell division [106].

Telomere shortening leads to dysfunctional telomeres and uncapping of chromosomal ends that triggers responses similar to DNA damage signaling involves telomeric foci of phosphorylated histone H2AX and their co-localization with DNA repair and DNA damage checkpoint factors such 53BP1, Mdc1 and Nbs1 and activation of Chk1, Chk2, Mdc1 which are all down stream targets of ATM and p53. The DNA damage signal finally leads to growth arrest senescence or apoptosis [107].

It is notable that telomeres are distinct from the rest of the genome in their DNA repair capacity and have been shown to inhibit non-homologous end joining (NHEJ) as a mechanism to prevent end-to-end chromosome fusions [108]. NHEJ is a major pathway for the repair of DSBs, can be inhibited in vitro by shelterin components, such as TRF2 [109]. For these reasons, it is speculated that telomeric repeats might be the preferential locations for DSBs, following exposure to DNA-damaging agents. The inability to repair DSBs by NHEJ at telomeric regions could be a major contributor to a persistent DDR involves in telomere dysfunction [85].

1.2.4. Senescence as a DNA damage response

Cellular senescence has been defined as a state of permanent replicative arrest. The concept was first described by Hayflick and Moorhead in the early 1960s [110] and was latter attributed to the telomere shortening after several cell replication and lack of telomerase activity [111].

Beside induction of cellular senescence by critical telomere shortening, several other factors including DNA damaging agents and stimulation of several mitogenic pathways can also induce senescence irrespective of telomere length [112, 113]. The ATM checkpoint kinase, a

sensor of DSBs, and the p53 tumor suppressor protein, a substrate of ATM, are central to induction of senescence. Short telomeres and the agents that induce premature senescence all activate the DNA damage pathways; propose a unifying paradigm in which cellular senescence establishment is the result of irreparable telomeric DNA damage generation. However, still the detailed mechanisms of DNA damage induced senescence are not clear.

Cellular senescence accompanied by set of characteristic morphological and physiological features that distinguish senescent cells not only from proliferating cells, but also from arrested quiescent or terminally differentiated cells [114]. Such senescence-associated features typically include irreversible proliferation arrest, enlarged cellular morphology, the activity of the senescence-associated β-galactosidase (SAβG) [115], nuclear hetero-chromatinization [116] and increased numbers of nuclear PML bodies [117], as well as many transcriptional and secretary changes such as up regulation of uPA, PAI-1, p21 cip1, p19 ARF p53, p16 [118, 119]. Importantly, not all the features of cellular senescence are expressed in senescent cell and that none of the listed features are specific or unique to identify senescence cell.

1.2.5. Cellular senescence implication in vasculature

Evidence supporting the hypothesis that age-associated changes in cardiovascular structure and function are risks for cardiovascular disease.

Senescence in the vasculature was first identified by Fenton et al. in an experimental model of neointimal formation, in which balloon endothelial denudation was used as a stimulus to promote vascular cell replication [120]. In this model, SA-β-gal positive cells were found in both the neointima and the media of the injured vessel in endothelial and vascular smooth muscle cells [120]. Vascular senescence seems to be an in vivo phenomenon associated with atherosclerosis. Endothelial and vascular smooth muscle cells in atherosclerotic plaques show morphological characteristics of senescence [121, 122]. Indeed, growing evidence points to human atherosclerosis being characterized by enhanced DNA damage and DDR signaling, leading to senescence of vascular smooth muscle cells and death of other cells to yield atherosclerotic lesions [123].

1.3. Ubiquitin-proteasome system (UPS)

The ubiquitin-proteasome system (UPS) mediates the degradation of most cellular proteins, including short-lived proteins that control cell cycle, transcription, DNA repair, apoptosis and other cellular processes. The system comprises of two main steps: ubiquitination and proteasome-mediated degradation. During ubiquitination, ubiquitin molecules are attached to a substrate protein by a series of ATP dependent enzymatic reactions involving the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin ligase (E3), and occasionally the ubiquitin chain-elongation factor (E4) [124].

1.3.1. Ubiquitination mediating enzymes

The ubiquitin activating enzyme E1 forms a thiol-ester bond between its active site cysteine and the carboxylterminal glycine of ubiquitin. Two E1 enzymes, which are able to activate ubiquitin have been identified in mammalian cells, namely, Ubiquitin-activating Enzyme 1-like 2 (UBE1L2) and ubiquitin-like modifier-activating enzyme 6 (Uba6) [125, 126].

The activated ubiquitin on E1 is transferred to the active site cysteine of an E2 ubiquitin-conjugating enzyme (UBC), by a trans-esterification reaction [124]. The human genome encodes over 40 UBCs, which contain a conserved 150-amino acid core domain that includes the cysteine which accepts an activated ubiquitin from E1 [127].

Ubiquitin ligases E3 family is involved in recognition of the substrates and transfer of ubiquitin from the E2 to the ε-amino group of a substrate lysine. Many E3s are discovered in eukaryotes and they are classified into four types: homologous to E6AP C-terminus (HECT) type, U-box type, single RING-finger type, and multi-subunit RING-finger type. Seven in absentia homolog (SIAH) is a member of RING-finger Ubiquitin E3 enzymes involved in ubiquitination and proteasome-mediated degradation of specific proteins. The activity of this ubiquitin ligase has been implicated in the development of certain disease, the regulation of the cellular response to hypoxia and induction of apoptosis [128, 129]. Recent studies showed that E3 ubiquitin ligase SIAH1 is responsible for p53 mediated TRF2 ubiquitination and degradation related to telomere damage response and cell senescence [130].

1.3.2. 26S proteasome structure

The 26S proteasome is a 2.5 MDa protein complex consisting of two complexes: the catalytic 20S core particle (CP) and the 19S regulatory particle (RP) [131]. The 20S CP is a barrel-shaped structure of a stack of four seven subunit rings in a α7 b7 β7 α7 configuration. Both exterior rings contain one set of seven different α subunits; and both interior rings contain one set of seven different β subunits [132] . The proteolytic activity of the proteasome is found in the β-subunits [133]. The CP performs three types of catalytic activities: chymotrypsin-like, trypsin-like and caspase-like activities [133]. The 19S RP is a multi-subunit complex, which can be divided into two subcomplexes called the base and the lid (Fig. 1). The base consists of six ATPases (Rpt1-6) and three non-ATPase subunits (Rpn1, Rpn2 and Rpn13), whereas the lid includes at least nine non-ATPase subunits (Rpn3, Rpn5-9, Rpn11, Rpn12 and Rpn15/Sem1) [134]. The connection between the lid and the base is stabilized by the Rpn10 subunit. Lack of structural data makes it difficult to fully understand how the RP subunits are arranged in the subcomplexes and how the RP stimulates the opening of the CP gate and facilitates substrate unfolding and translocation (Fig 6).

1.3.3. Regulation of proteasomal activity

Functional proteasomes require a highly regulated assembly of proteasomal subunits. Some associating proteins serve as the molecular scaffolds and chaperones that regulate the assembly of the 20S and 26S proteasomes [135]. Heat shock proteins and intermediate proteins have critical role in the maturation of the proteasome assembly complex [136]. Regulation of proteasomal activity also occurs at expression level through Rpn4 function. Studies showed that Rpn4 subunit of the 26S proteasome binds to the proteasome-associated control element (PACE) activating expression of α, β and 19S subunits [137]. Rpn4 itself is degraded within 2 min by the proteasomes and enables the subunit to dynamically stabilize proteasome levels within the cell [138].

Posttranslational modification of Rpn proteasomal subunits may affect phosphorylation of the ATPase subunits, Rpt6 in particular, by PKA and correlates with increased chymotryptic and tryptic activity. Alternatively, *O*-GlcNAcylation of Rpt2 serves as a master switch, shutting off proteolytic activity upstream of proteasome phosphorylation [139]. Binding of the

activator and inhibitor proteins to the either end of the 20S provides another way to control proteasomal activity [140].
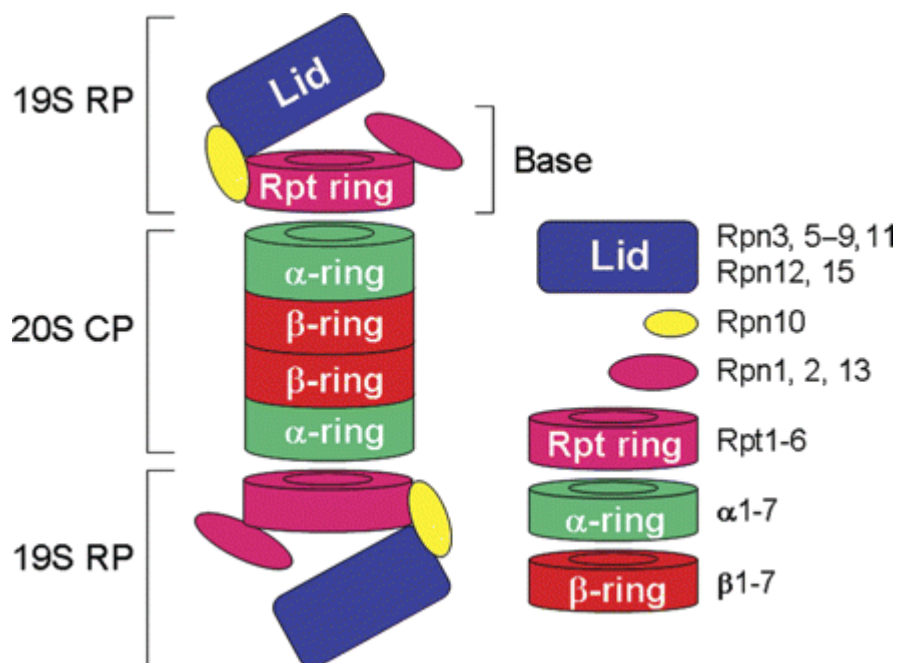

Figure 6.Schematic diagram of the 26S proteasome. The 26S proteasome consists of the 20S core particle and the 19S regulatory particle [141]

1.3.4. Regulatory role of Ubiquitin-proteasome system in DNA damage response

Protein ubiquitination apart from targeting proteins for degradation is emerging as an important regulatory element implicated in many cellular processes as diverse as gene transcription, DDR, receptor trafficking, endocytosis and cell cycle control.

The regulatory role of UPS in DDR in both protein turnover and protein recruitment has been well defined. DDR utilizes multiple classes of ubiquitin-binding motifs to coordinate signaling and repair [142, 143]. Indeed, many of the DDR transducers such as breast cancer type/BRCA-associated ring domain (BRCA1/BARD), Fanconi anemia complementation

group L (FANCL), Rad 18, murine double minute (Mdm2) are enzymes that catalyze ubiquitination of proteins.

Ubiquitin conjugation via alternative lysine residues mediates specific protein-protein interactions. Therefore proteins can be modified not only with ubiquitin polymers composed of single isopeptide linkages but also with heterogeneous ubiquitin chains highlighting the vast potential for multiple levels of regulation [101]. Still much more remains to be discovered about how ubiquitin modification in DDR controls protein function, activity and recruitment.

The most common function of UPS is protein degradation through the proteasomal activity. Suppressor of an exocyst mutant (Sem1), deleted in split hand/split foot protein 1 (DSS1) and split hand-split foot malformation (SHFM1) protein are subunits of the 19S proteasome in both yeast and human cells that are recruited with the 19S and 20S proteasomes to DNA DSB in vivo and are required for efficient repair of DSB through HR and NHEJ mechanisms [144]. Human DSS1/SHFM1 physically binds to BRCA2/FANCD1 and is required for its stability and function [16, 145].

It is shown that proteasome inhibitors inhibit both monoubiquitination and nuclear foci formation of DNA damage–signaling processes, such as foci formation of phosphorylated ATM, 53BP1, NBS1, BRCA1, and RAD51 [86].

2. Objectives

Objectives of the thesis

There are two general aims of this thesis; the first was to understand the role of uPAR in DNA damage induced senescence as an irreparable consequence of telomeric dysfunction and the second was to obtain better information on the involvement of uPAR in repair mechanism of DNA damage response.

In the first part, the focus was on TRF2, the main component of telomere structure and its regulation during drug induced senescence. It was aimed to study uPAR possible role in TRF2 regulation and to elucidate the underlying mechanism. This part of the thesis was motivated by previous evidence suggesting the implication of uPA/uPAR system in senescence process. In the second part, the focus was on involvement of uPAR in DNA repair mechanisms, based on its regulatory role in UPS. This part was motivated by the findings resulted from the first part of study on the role of uPAR in UPS and also by several clues from different studies reporting the involvement of uPA/uPAR in pathways triggered by DNA damage.

The specific aims of these manuscripts are summarized concisely below:

Manuscript 1

1. Evaluate the effect of low doses of the anti-cancer drug Doxorubicin on VSMC
2. Evaluate the effect of Doxorubicin on TRF2 during senescence response
3. Elucidate the uPA/uPAR system requirement for senescence response
4. Investigate the mechanism of uPAR mediated TRF2 proteasomal degradation
5. Investigate the mechanism of uPAR mediated TRF2 ubiquitinaton

Manuscript 2

1. Evaluate the effect of uPAR depletion in DNA damage repair mechanisms and cell survival
2. Evaluate the functional behavior of Rpn7 proteasomal compartment during DNA damage response and implication of uPAR
3. Identify the regulatory factors involved in Rpn7 regulation during uPAR mediated DNA damage response

4. Evaluate the significance of SHP-2 in uPAR mediated DNA damage response

Elucidation of regulatory factors and pathways involved in DNA damage response mechanisms may lead to novel therapeutic avenues for age related diseases and cancer.

3. Publications and manuscripts

3.1. Manuscript 1

Urokinase receptor mediates Doxorubicin-induced vascular smooth muscle cell senescence via proteasomal degradation of TRF2

Journal of **Vascular Research**

Urokinase Receptor Mediates Doxorubicin-Induced Vascular Smooth Muscle Cell Senescence via Proteasomal Degradation of TRF2

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Abstract

The anthracycline doxorubicin is a widely used effective anti-cancer drug. However, its application and dosage are severely limited due to its cardiotoxicity. The exact mechanisms of doxorubicin-induced cardiotoxic side effects remain poorly understood. Even less is known about the impact of doxorubicin treatment on vascular damage. We found that low doses of doxorubicin induced a senescent response in human primary vascular smooth muscle cells (VSMC). We observed that expression of urokinase receptor (uPAR) was upregulated in response to doxorubicin. Furthermore, the level of uPAR expression played a decisive role in developing doxorubicin-induced senescence. uPAR silencing in human VSMC by means of RNA interference as well as uPAR knockout in mouse VSMC resulted in abrogation of doxorubicin-induced cellular senescence. On the contrary, uPAR overexpression promoted VSMC senescence. We further found that proteasomal degradation of telomeric repeat binding factor 2 (TRF2) mediates doxorubicin-induced VSMC senescence. Our results demonstrate that uPAR controls the ubiquitin-proteasome system in VSMC and regulates doxorubicin-induced TRF2 ubiquitination and proteasomal degradation via this mechanism. Therefore, VSMC senescence induced by low doses of doxorubicin may contribute to vascular damage upon doxorubicin treatment. uPAR-mediated TRF2 ubiquitination and proteasomal degradation are further identified as a molecular mechanism underlying this process.

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Introduction

Clinical use of doxorubicin, a powerful anti-cancer drug of the anthracycline family, is limited because of its acute and chronic cardiovascular side effects manifested by cardiomyopathy [1, 2]. Molecular mechanisms underlying doxorubicin cardiotoxicity remain, despite intensive studies, poorly understood. Correspondingly, clinical approaches to prevent and minimize its toxic effects on the heart are still missing. A growing body of evidence indicates that cancer treatment with anthracyclines results, beyond cardiomyopathy and refractory congestive heart failure, in further forms of cardiovascular diseases

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affecting the vascular wall [3]. However, little is known about its vascular toxicity. A couple of reports documented damage to the vascular endothelium in response to doxorubicin [4], which is most likely related to doxorubicin-induced apoptosis of endothelial cells [5, 6]. Even less is known about the effects of doxorubicin treatment on vascular smooth muscle cells (VSMC), though deregulation of these cells is one of the key events contributing to negative vascular remodeling and dysfunction [7].

It is believed that the cardiotoxic effects of doxorubicin are related to increased oxidative stress and reactive oxygen species (ROS) generation [1, 2]. Increasing evidence indicates, however, that the use of antioxidants failed to prevent anthracycline-induced cardiovascular side effects both in clinical trials and animal models, and thus further, still unknown essential mechanisms may be involved [2]. Moreover, these mechanisms seem to be different depending on the doxorubicin dose. Though treatment with both low and high doses of doxorubicin culminates in cardiovascular disorders, their cellular effects are not the same [8]. A number of recent studies report that doxorubicin can activate the ubiquitin-proteasome system (UPS) and mediate by this way degradation of transcription factors and other key proteins possibly underlying doxorubicin cardiotoxicity [9]. We have recently demonstrated that the multifunctional urokinase (uPA)/urokinase receptor (uPAR) system, which is an important regulator of VSMC in health and disease [10], mediates the specific ubiquitination and proteasomal degradation of proteins determining VSMC functional behavior. We further found that uPAR deficiency resulted in decreased proteasomal activity in blood vessels of uPAR–/– mice [11].

In this study, we examined the effects of low doses of doxorubicin on cellular and functional properties of human VSMC. We investigated the contribution of UPS to these effects. We hypothesized that uPAR may orchestrate the outcome of doxorubicin-induced VSMC responses by regulating UPS. We demonstrate that doxorubicin induces VSMC senescence that may contribute to vascular damage. We further show that uPAR controls this process via the regulation of telomeric repeat binding factor 2 (TRF2) ubiquitination and proteasomal degradation.

Methods

Cell Culture and Doxorubicin Treatment
Human primary umbilical artery VSMC were isolated from the umbilical artery using an explant technique in VascuLife SMC culture medium (CellSystems®; Biotechnologie Vertrieb GmbH, St. Katharinen, Germany). The procedure conforms to the Decla-

ration of Helsinki and was approved by the local ethics committee. First-passage fibroblasts were removed from the culture by cell separation using monoclonal anti-fibroblast antibodies (anti-CD90; Dianova GmbH, Hamburg, Germany) and magnetic Dynabeads® goat anti-mouse IgG (Invitrogen, Carlsbad, Calif., USA). VSMC were used between passages 2 and 4. Aortic VSMC were isolated from male uPAR–/– mice and uPAR+/+ (wild-type, WT) mice as controls (all on C57/BL6 background, age 10–12 weeks). All animal experiments were carried out according to the European Commission guidelines and were approved by the Ethics Committee of the Hannover Medical School. Animals were euthanized by intravenous injection of 200 µl of 2% Avertin solution. The aortas were dissected, cut into pieces that were 1–2 mm on a side and subjected to enzymatic digestion as described [12]. VSMC were cultivated in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal bovine serum. For induction of senescence, VSMC and umbilical artery SMC (UASMC) were exposed to doxorubicin (Sigma) treatment at doses of 0.25, 0.5 and 1 µM doxorubicin for 3 h in medium containing no serum. Cells were then washed 3 times with PBS and incubated in growth medium.

BAY 11-7085, a nuclear factor (NF) κB inhibitor, was purchased from Enzo Life Sciences (Lausen, Switzerland). VSMC were pretreated with 10 µM BAY 11-7085 prior to doxorubicin treatment. After cell washing and medium exchange, BAY was added to the growth medium. The proteasome inhibitor MG132 was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). Cells were pretreated with 10 µM MG132 prior to doxorubicin treatment. After cell washing and medium exchange, MG132 was added to the growth medium.

Senescence-Associated β-Galactosidase Activity
Senescence-associated β-galactosidase (SA-Gal) staining was performed 3 days after doxorubicin treatment according to the methods described previously [13]. Cells were counterstained with 0.1 µg/ml DAPI solution for 5 min. The numbers of β-Gal-positive cells were scored under bright field per total cell number in the same field using a Leica DM LB fluorescence microscope (Leica Microsystems). Each experiment was carried out in triplicate and at least 500 cells were scored in total in 5 different random fields.

Alternatively, fluorogenic substrate (C12FDG; Molecular Probes, Invitrogen) was used for measuring β-Gal activity using flow cytometry as described [14]. FACS analysis was performed on FACSCalibur (BD Biosciences). Summit software (Dako) was used for analyzing the study data.

Apoptosis
Cell apoptosis characterized by accumulation of phosphatidylserine on the extracellular surface of the membrane was studied using the annexin V apoptosis detection kit (Santa Cruz Biotechnology, Santa Cruz, Calif., USA). Cells were stained according to the manufacturer's instructions and subsequently analyzed by flow cytometry (FACSCalibur).

Cell Proliferation
Cell proliferation was quantified based on the measurement of BrdU (5-bromo-2-deoxyuridine) incorporation during DNA synthesis using a colorimetric cell proliferation ELISA kit (Roche Applied Bioscience).

Plasmid Construction and Nucleofection

Small interfering RNA (siRNA) for downregulation of uPAR, TRF2 and Siah1, and control silencing RNA was obtained from Santa Cruz Biotechnology and transfected with human UASMC using Amaxa Nucleofector™ (Lonza). A basic primary smooth muscle cell nucleofector kit (Lonza) was used according to the manufacturer's instructions.

For green fluorescent protein (GFP)-uPAR overexpression, the lentivirus pDEST-lenti transfer vector was generated by blunt ligation gateway cassette rfa-verB (Invitrogen) at *Pme*I and *Sma*I sites of the pLV-tRKRAB-Red vector (Tronolab). Entry clones for the transfer of GFP-uPAR were produced by cloning the PCR products in the pENTR/D TOPO plasmid (Invitrogen). The GFP-uPAR vector (kindly provided by N. Sidenius) served as template for PCR [15]. For GFP-uPAR overexpression, pEXPR clones were generated by site-specific recombination between pDEST-lenti and pENTR/D TOPO-M-CSFR by Gateway LR Clonase enzyme mix (Invitrogen). (GFP-uPAR sense primer: 5′-tcagatctcgagctgcc-ctc and GFP-uPAR anti-sense primer: 5′-aggtccagaggagagtgcct.) For experiments with GFP-uPAR constructs, pCMV-dR8.74, pMD.2G (Tronolab) and pEXPR plasmids were co-transfected (using a 3:2:1 ratio of pEXPR:pCMV-dR8.74:pMD2G) into 293T cells by PerFectin transfection reagent (Genlantis, San Diego, Calif., USA), as recommended by the manufacturer. Forty-eight hours after transfection, the viral particles containing cell supernatants were harvested, filtered, concentrated and stored at –70°C for future use. TRF2-IRESS-enhanced GFP plasmid was a gift from Dr. de Lange [16] (Addgene plasmid No. 19798).

Immunostaining

Cells grown on coverslips were fixed with 2% formaldehyde, permeabilized in 0.1% Triton X-100 for 3 min and blocked with 3% (w/v) BSA/PBS at 4°C overnight. After 24 h, cells were labeled with monoclonal mouse anti-TRF2 (Imgenex) and subsequently with corresponding Alexa Fluor® 488- or Alexa Fluor 594-conjugated secondary antibody (Invitrogen) for 1 h at room temperature. DRAQ5 (BioStatus) was applied as nuclear stain. Cells were then mounted with mounting medium (Aqua-Poly-Mount; Polysciences) and analyzed on a Leica TCS-SP2 AOBS confocal microscope. For immunostaining of mouse aortic VSMC, cells were incubated with 5% mouse serum in PBS followed by 1 h incubation with 5% normal goat serum.

Preparation of Cell Lysates, Immunoprecipitation and Western Blotting

Cultured cells were lysed in RIPA buffer containing 1 mM PMSF, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mM Na_3VO_4 and 1 mM NaF and incubated for 10 min at 4°C. The lysates were centrifuged at 10,000 rpm for 10 min. TRF2 was immunoprecipitated from 600 μg of total cell lysate with 4 μg of specific polyclonal antibodies against TRF2 (Santa Cruz). After 3 h, immunocomplexes were precipitated with A/G PLUS-agarose beads. Precipitates were washed 3 times in PBS containing protease inhibitors and subjected to SDS electrophoresis. The membranes were developed with antibodies against p53, p21, p16 (Santa Cruz), uPAR (R&D Systems). Antibody against ubiquitin for detecting ubiquitination of immunoprecipitated TRF2 was purchased from Santa Cruz.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA was isolated from UASMC using the QiaSpin miniprep kit (Qiagen) according to the manufacturer's protocol. TaqMan analysis was performed on a LightCycler® 480 real-time PCR system using LightCycler 480 RNA Master Hydrolysis probes (Roche Applied Sciences). The following primers were used: uPAR (human); sense 5′-ACCACCAAATGCAACGAGG-3′; antisense 5′-GTAACACTGGCGGCCATTCT-3′; probe 6-FAM-CAATCCTGGAGCTTGAAAATCTGCCG-TAMRA, GAPDH (human); sense 5′-GAAGGTGAAGGTCGGAGTC-3′; antisense 5′-GAAGATGGTGATGGGATTC-3′; 6-FAM-CAAGCTTCCC-GTTCTCAGCC-TAMRA probe; TRF2 (human); sense 5′-GGA-GGAGGCGGGAGTAGC-3′; antisense 5′-ACTTGAGCACCCA-GCGATTG-3′; probe 6-FAM-TGCCTCTTCCAGCCGTGCCT-CC-TAMRA.

Proteasomal Activity Assay

Total proteasomal activity in cell lysates was measured using the 20S proteasomal assay kit (Cayman Chemical Company, Ann Arbor, Mich., USA) as described by the manufacturer. In brief, UASMC were grown in a 96-well plate and treated with different concentrations of doxorubicin according to the stated protocol. The plate was then centrifuged at 500 *g* for 5 min and the cells were washed with 20S proteasome assay buffer. Lysis buffer was added to each well, and the plate was shaken for 30 min at room temperature. The plate was then centrifuged at 1,000 *g* for 10 min, and supernatant from each well was transferred to the corresponding wells in a black 96-well plate. Thereafter, 10 μl of assay buffer and 10 μl of the substrate (SUC-LLVY-AMC) were added. After 1 h of incubation at 37°C, the plate was read using a Magellan GENIOUS (Tecan, Männedorf, Switzerland) at 360 (excitation) and 480 nm (emission). The enzymatic activity was normalized to the protein concentration. The results are reported as means ± SD.

ROS Detection

ROS were detected fluorescently using 5 (and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA; Invitrogen). VSMC were loaded with 5 μM of the dye for 30 min at 37°C, then washed 3 times with cell culture medium and treated with different concentrations of doxorubicin for 3 h. Fluorescence was measured after 3, 6 and 24 h.

Chromatin Immunoprecipitation and Telomere Detection

Telomeric association of TRF2 was assessed using chromatin immunoprecipitation (ChIP) followed by DNA isolation, dot blot, hybridization and chemiluminescence detection of telomeric DNA. ChIP was performed according to a previously described protocol [17]. Briefly, cells were fixed with 1% formaldehyde for 10 min at 18°C. Paraformaldehyde was quenched by glycine (125 mM). Cells were washed and scraped in RIPA buffer. DNA was sheared by sonication. Lysates were incubated overnight with anti-TRF2 antibody and salmon sperm DNA-saturated protein A/G-agarose to immunoprecipitate TRF2. DNA was purified using Chelex-100 resin. Input DNA samples were purified along with the immunoprecipitates. Isolated DNA samples were transferred to the HyBond N+ membrane (Amersham) under vacuum. Hybridization and detection of telomeric DNA was performed using the Telo TAGGG telomere length assay kit (Roche Applied Biosciences) according to the manufacturer's protocol.

All experiments were performed at least three times. 'n' represents the number of independent experiments. For Western blotting, one representative blot out of at least three is shown. Statistical significance ($p < 0.05$) was analyzed using Student's t test. Values of $p < 0.05$ were regarded as statistically significant.

Results

Doxorubicin Induces VSMC Senescence

High doses of doxorubicin lead to apoptotic cell death, as shown for cardiomyocytes and endothelial cells [5, 18, 19]. When exposed to low doxorubicin doses, cardiomyocytes revealed a senescence-like phenotype [20]. Since nothing is known about the effects of doxorubicin treatment on VSMC function, we first examined dose-dependent cell responses. We observed that low doxorubicin concentrations induced cellular senescence in VSMC. Thus, VSMC expressed β-Gal activity that serves as a marker for senescence, as determined by microscopy (fig. 1a) and flow cytometry (fig. 1b). Doxorubicin induced β-Gal activation in a dose-dependent manner at a concentration range of 0.25–1 μM (fig. 1c). Induction of the senescent phenotype is generally characterized by impaired proliferative responses. In agreement with these observations, VSMC proliferation was inhibited in response to senescence-inducing doxorubicin concentrations (fig. 1d). Similar to cellular responses of other cell types, high doses of doxorubicin resulted in VSMC apoptosis (fig. 1e). Senescence-associated proteins such as p53 and p21 were dose-dependently upregulated in VSMC following doxorubicin treatment, whereas expression of p16 did not differ after doxorubicin treatment (fig. 1f).

uPAR Mediates Doxorubicin-Induced Senescence in VSMC

uPAR is an important regulator of functional responses of VSMC, such as proliferation, migration and differentiation [21–23]. However, whether or not uPAR is involved in the propagation of the senescence signal in these cells is not known. uPAR expression in VSMC was upregulated in response to doxorubicin in a dose-dependent manner. This increase in uPAR expression was noted at both protein and mRNA levels, as reflected by Western blotting and TaqMan analysis (fig. 2a, b). On the contrary, uPA expression was downregulated by doxorubicin treatment (fig. 2c). Further experimental settings, namely confocal microscopy, confirmed uPAR upregulation in VSMC following low-dose doxorubicin (fig. 2d). Expression of uPAR is regulated by several signaling pathways and transcription factors. NFκB is an important regulator of uPAR expression [24]. Since doxorubicin treatment often leads to NFκB activation, we next tested whether uPAR expression is upregulated by doxorubicin via the NFκB pathway. As shown in figure 2e, VSMC treatment with an NFκB inhibitor abrogated doxorubicin-induced upregulation of uPAR expression.

To elucidate whether the observed upregulation of uPAR may be involved in the molecular machinery underlying doxorubicin-induced VSMC senescence, uPAR downregulation was performed by means of interfering RNA combined with cell nucleofection. This approach provides uPAR silencing with a high efficiency rate (fig. 3a). VSMC nucleofected with control siRNA responded to doxorubicin with a senescent phenotype, which was strongly abrogated in uPARsi cells (fig. 3b). A similar effect was observed in mouse VSMC. Thus, WT VSMC responded to doxorubicin treatment with much higher senescence than VSMC from uPAR–/– mice (fig. 3c). By contrast, uPAR overexpression by VSMC lentiviral infection (fig. 3d) resulted in increased cellular senescence (fig. 3e). These data suggest that uPAR is rather an active regulator of the senescence process in VSMC than just a marker of the senescent cell phenotype.

Doxorubicin Induces uPAR-Mediated Proteasomal Activity and Ubiquitination of TRF2 in VSMC

Next, we addressed molecular mechanisms underlying uPAR-mediated senescence in VSMC in response to low-dose doxorubicin. Our data showed that doxorubicin at concentrations of up to 5 μM did not induce any significant oxidative stress in human VSMC (data not shown). Only cell treatment with 10 μM doxorubicin resulted in increased ROS production (1.21 ± 0.20 times in SiCo VSMC and 1.4 ± 0.31 times in uPARsi VSMC). There was no statistically significant difference in 10-μM doxorubicin-induced ROS production by SiCo and uPARsi VSMC. Therefore, uPAR-mediated VSMC senescence induced by doxorubicin doses <1 μM is mediated by other mechanisms than oxidative stress induction.

We then provided prove for the hypothesis that these mechanisms may be related to uPAR interfering with doxorubicin-directed effects on UPS. In agreement with observations done on other cell types, doxorubicin treatment induced an increase in proteasomal activity in VSMC. However, in uPARsi cells, this doxorubicin-induced effect was abrogated (fig. 4a). Similarly, uPAR–/– mouse VSMC failed to upregulate proteasomal activity
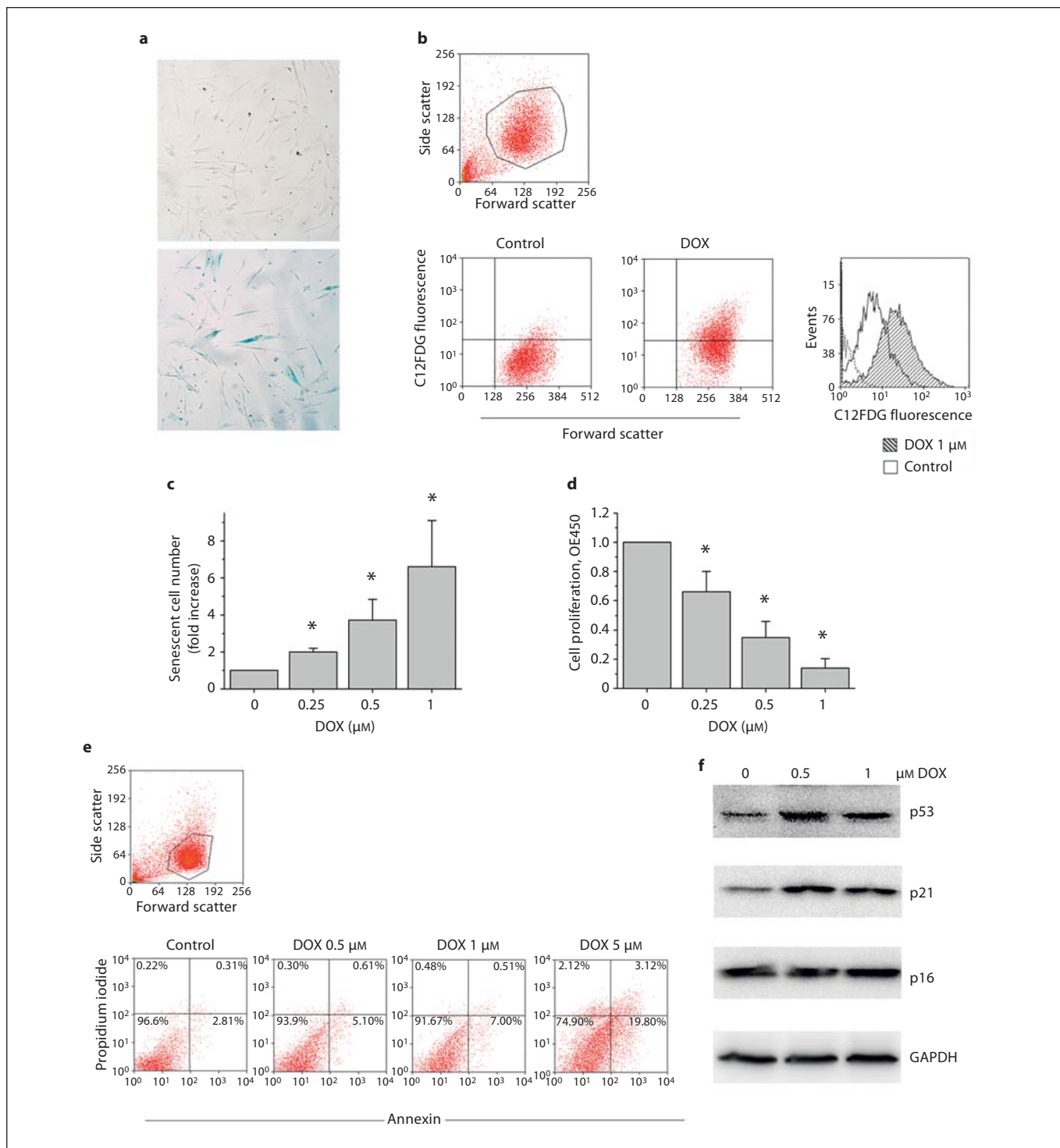
Fig. 1. Low concentrations of doxorubicin (DOX) induce senescence of human VSMC. **a** Control (upper panel) and DOX-treated (1 μM, lower panel) VSMC were stained for β-Gal to visualize senescent cells. **b** DOX-induced VSMC senescence was analyzed by FACS as described in the Methods section. **c** Quantification of senescent cells after VSMC treatment with different concentrations of DOX assessed by β-Gal staining as described in the Meth-ods section (n = 6). **d** Proliferation of VSMC after treatment with different concentrations of DOX was measured by BrdU assay (n = 3). **e** VSMC apoptosis after low and high concentrations of DOX assessed by FACS using annexin V/propidium iodide staining. **f** Expression of p53, p21 and p16 after VSMC treatment with DOX was assessed by Western blotting. GAPDH served as a loading control.
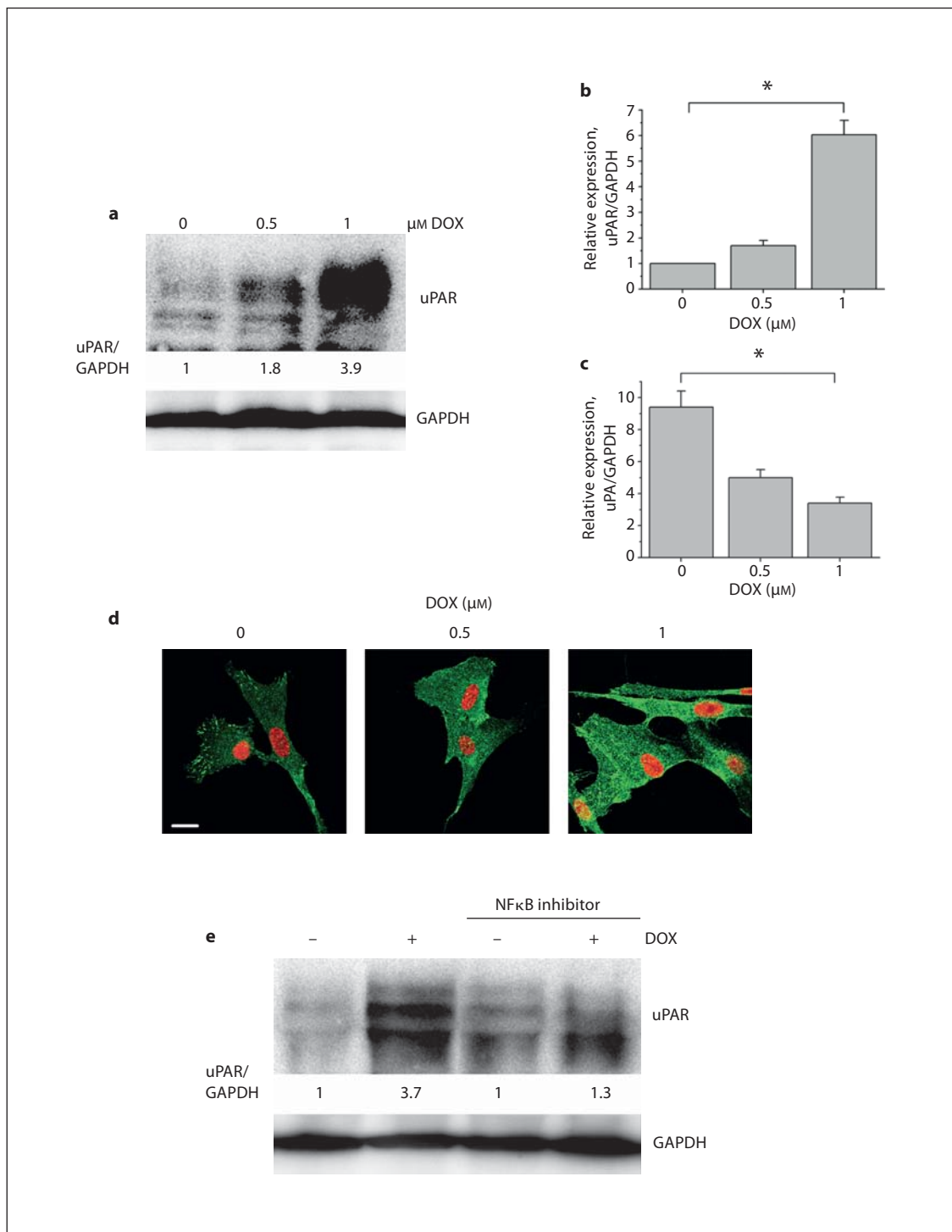
Fig. 2. uPAR expression is upregulated in doxorubicin (DOX)-treated VSMC. **a** uPAR protein expression 24 h after VSMC treatment with the indicated concentrations of DOX assessed by Western blotting. **b** uPAR mRNA expression 24 h after DOX treatment was analyzed by TaqMan (n = 3). **c** uPA mRNA expression 24 h after DOX treatment analyzed by TaqMan (n = 3). **d** DOX-treated VSMC were fixed and stained for uPAR (Alexa 488) 24 h after DOX treatment. DraQ5 was used as nuclear stain. Scale bar = 10 μm. **e** VSMC were pre-treated with NFκB inhibitor prior to addition of DOX. uPAR expression was analyzed 24 h after DOX treatment.
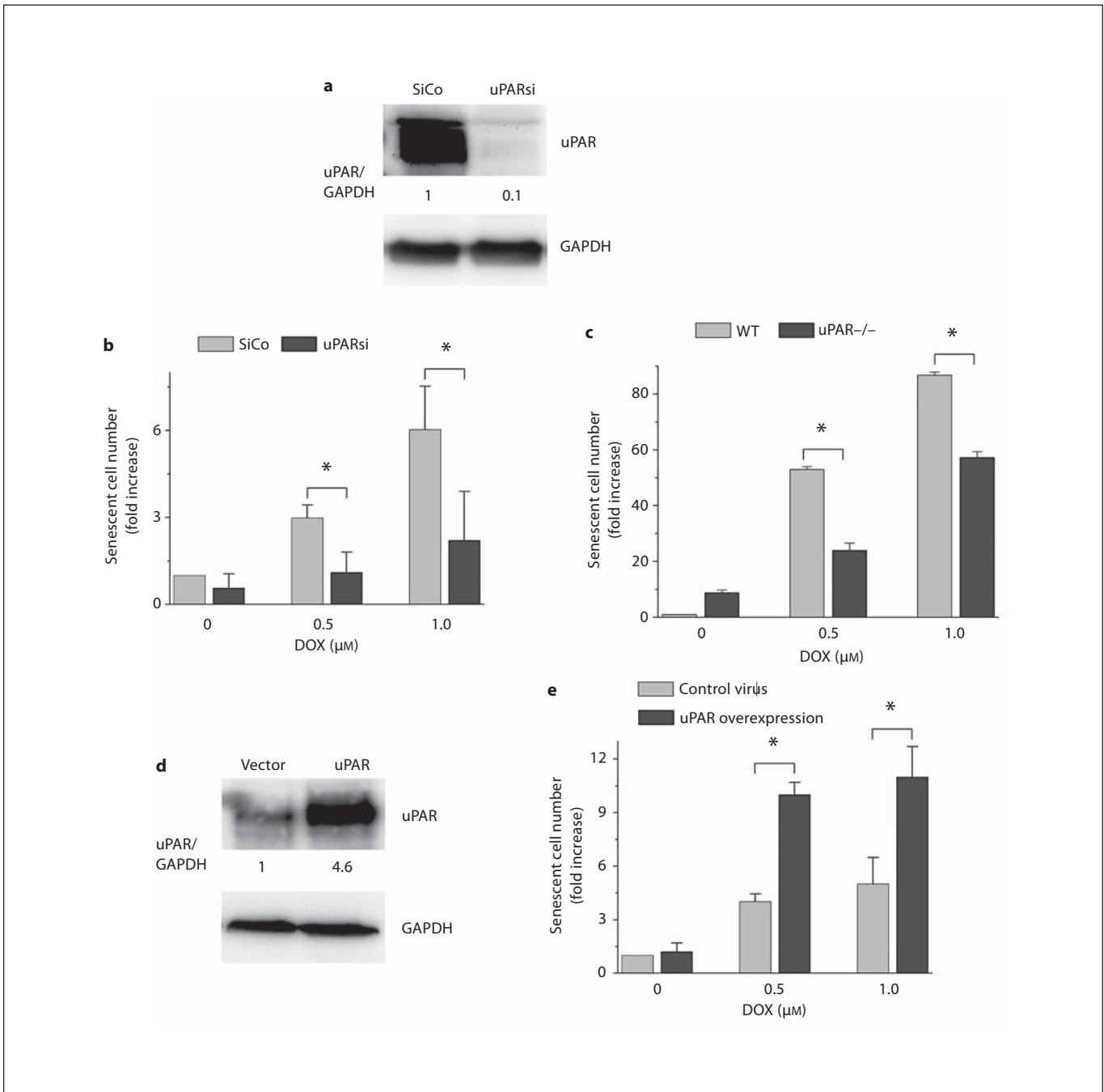
Fig. 3. uPAR regulates doxorubicin (DOX)-induced VSMC senescence. **a** uPAR expression after cell nucleofection with uPARsi RNA assessed by Western blotting. **b** DOX-induced senescence of SiCo- and uPARsi-nucleofected VSMC was quantified by β-Gal staining (n = 6). **c** DOX-induced senescence of WT and uPAR–/– mouse VSMC was quantified by β-Gal staining (n = 3). **d** uPAR expression was upregulated by VSMC lentivirus infection. **e** DOX-induced senescence of control and uPAR-overexpressing virus-infected VSMC was quantified by β-Gal staining (n = 3).
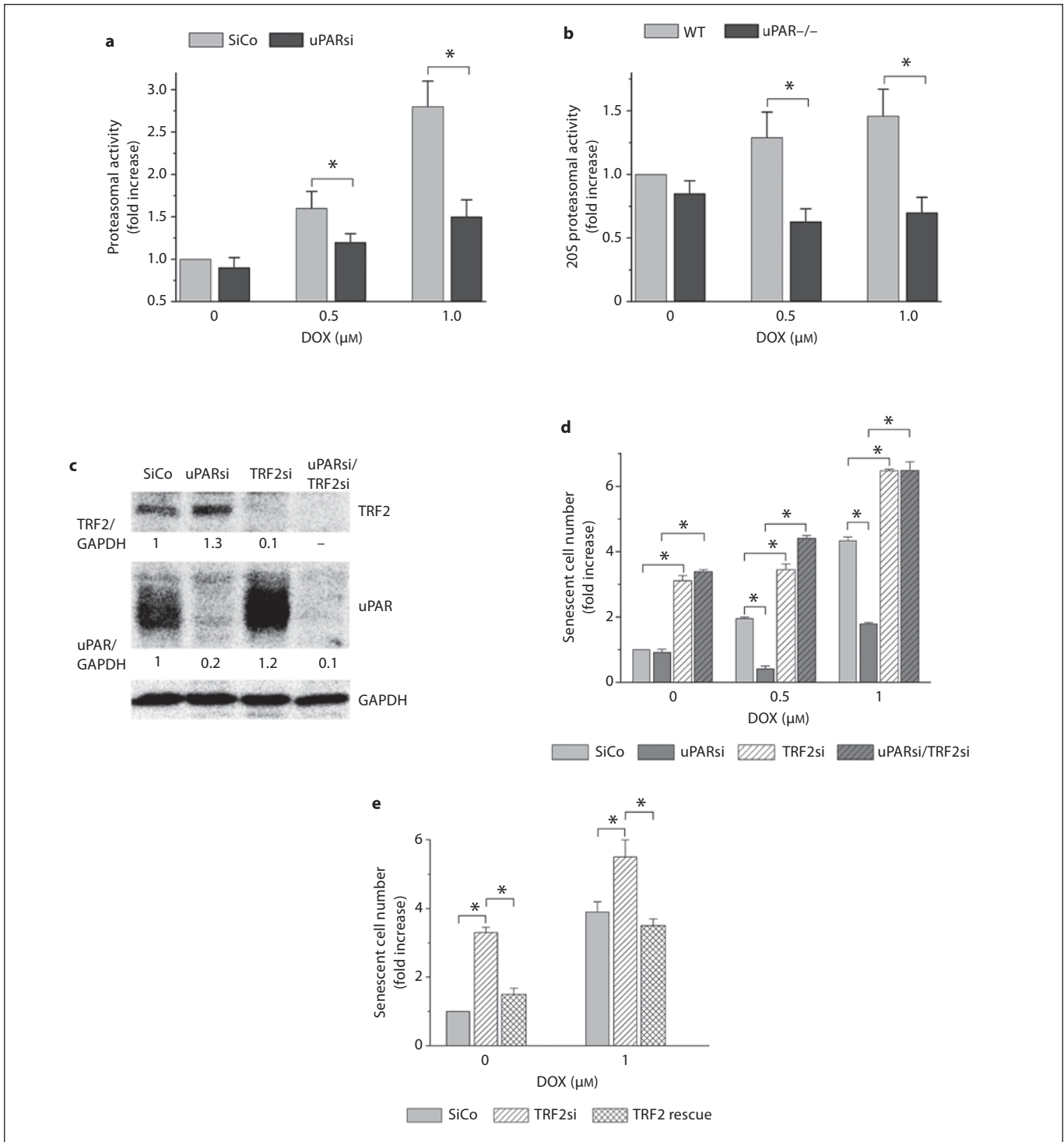
Fig. 4. Doxorubicin (DOX)-induced proteasomal activity and the role of TRF2 in DOX-induced VSMC senescence. **a** DOX-induced 20S proteasomal activity in SiCo- and uPARsi-nucleofected human VSMC was assessed fluorimetrically as described in the Methods section (n = 4). **b** DOX-induced 20S proteasomal activity was measured fluorimetrically in VSMC isolated from WT and uPAR–/– mice (n = 3). **c** Downregulation of expression of TRF2, uPAR and TRF2 and uPAR concomitantly was achieved in human VSMC by cell nucleofection with siRNA. **d** DOX-induced senescence of VSMC nucleofected to downregulate uPAR, TRF2, or both was quantified by β-Gal staining (n = 3). **e** VSMC were nucleofected with TRF2si RNA or TRF2si RNA simultaneously with TRF2-IRESS-enhanced GFP plasmid to replenish TRF2 expression. DOX-induced VSMC senescence was assessed by β-Gal staining.
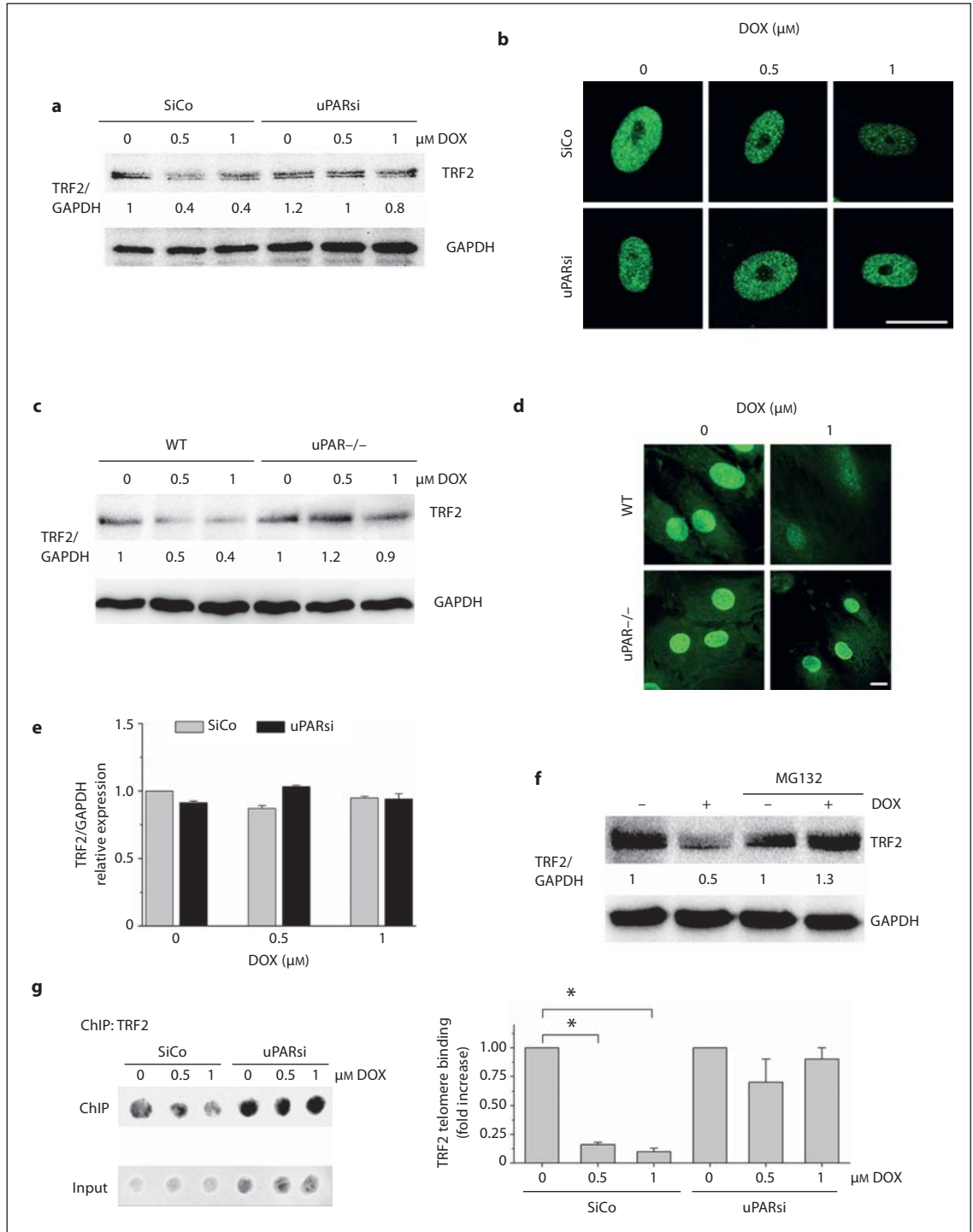
Fig. 5. Doxorubicin (DOX) treatment induced TRF2 proteasomal degradation in an uPAR-dependent manner. **a** TRF2 degradation 24 h after DOX treatment in SiCo- and uPARsi-VSMC was assessed by Western blotting. **b** VSMC were fixed and stained for TRF2 (Alexa 488) 24 h after DOX treatment. Scale bar = 10 μm. DOX-induced TRF2 degradation in VSMC isolated from WT and uPAR–/– mice assessed by Western blotting (**c**) and immunocytochemistry (**d**, scale bar = 10 μm). **e** TRF2 mRNA ex-pression 24 h after DOX treatment was assessed by TaqMan analysis. **f** VSMC were pre-treated with proteasome inhibitor MG132 prior to DOX (1 μM) treatment. TRF2 protein level was assessed 24 h after DOX treatment by Western blotting. **g** TRF2 association with telomeric DNA in DOX-treated SiCo- and uPARsi-VSMC was analyzed by ChIP. The lower panel shows input DNA quantity. The right panel shows quantification of ChIP data (n = 3).

a

IP: TRF2 WB: ubiquitin

WB: TRF2

b

IP: TRF2 WB: ubiquitin

WB: TRF2

c

IP: TRF2 WB: ubiquitin
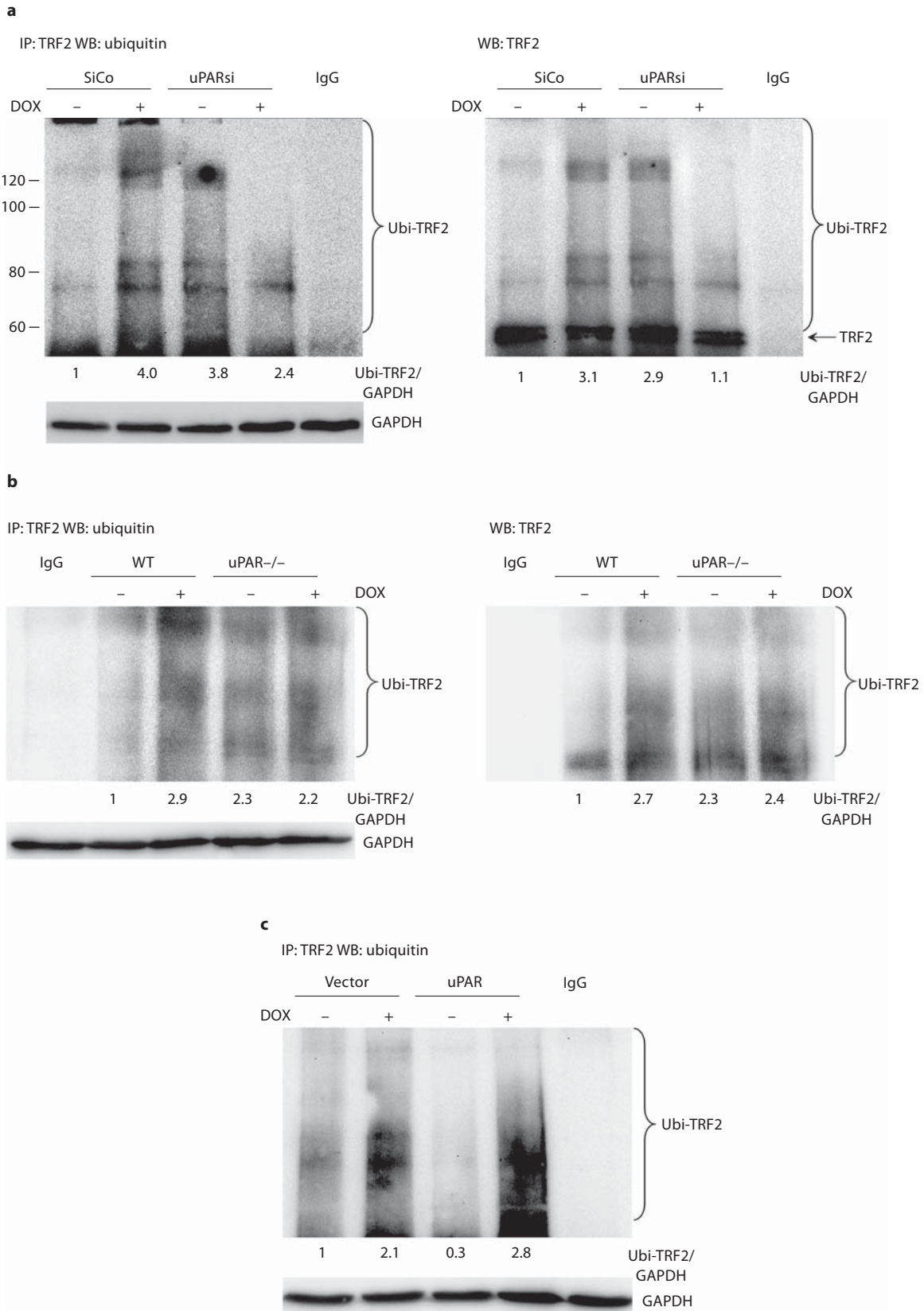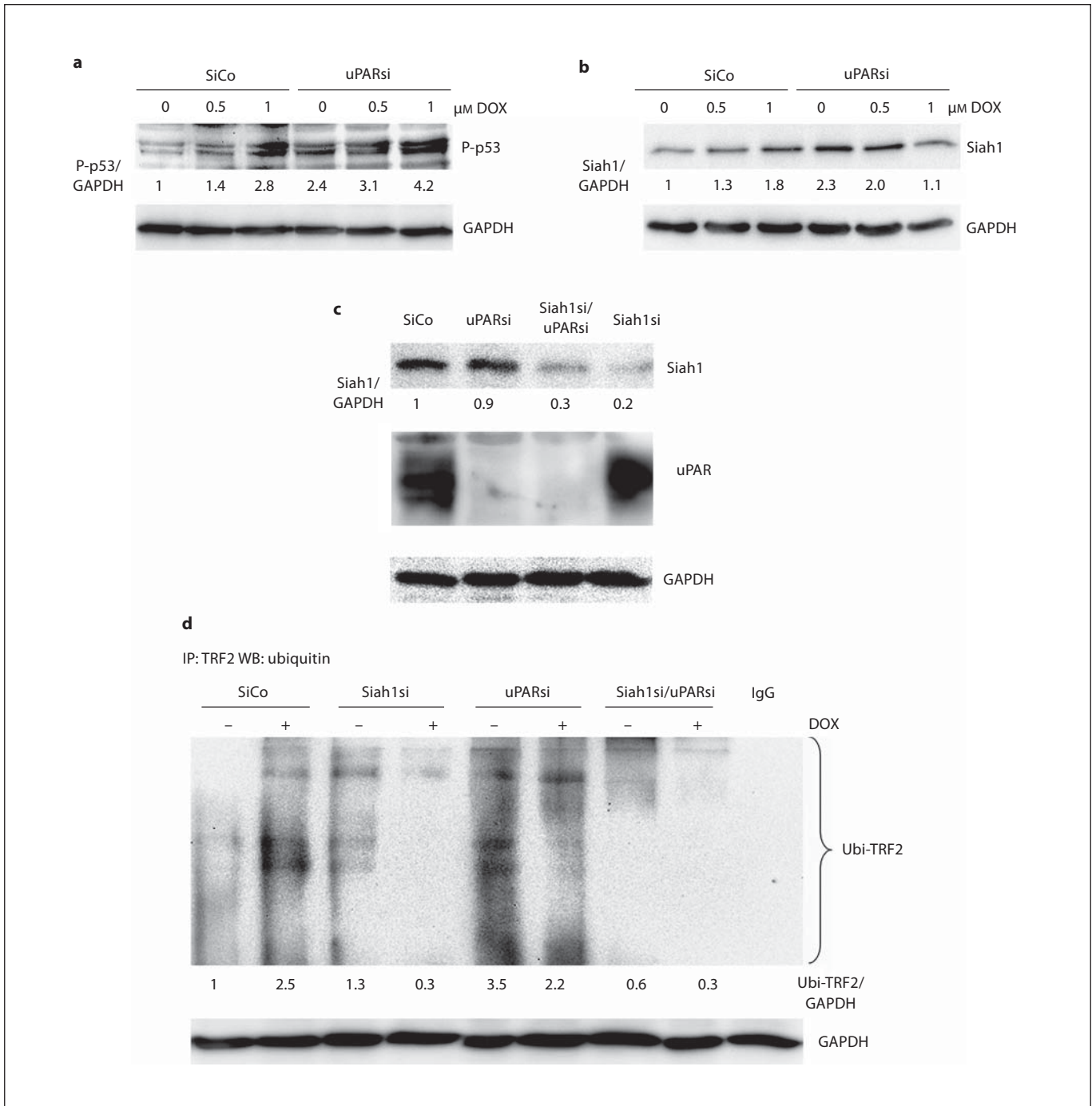
Fig. 6. uPAR regulates TRF2 ubiquitination (Ubi). **a** Doxorubicin-induced TRF2 ubiquitination in SiCo- and uPARsi-VSMC was assessed by immunoprecipitation. The right panel shows the membrane developed with TRF2 antibody after stripping. GAPDH was used to demonstrate a similar amount of protein used in each immunoprecipitation reaction. **b** Doxorubicin-induced TRF2 ubiquitination in WT and uPAR–/– mouse VSMC was assessed by immunoprecipitation similar to **a**. **c** TRF2 ubiquitination in uPAR-overexpressing VSMC measured by immunoprecipitation.

Fig. 7. Siah1 ubiquitin ligase mediates uPAR-dependent TRF2 ubiquitination. **a** p53 (Ser[15]) phosphorylation in doxorubicin (DOX)-treated SiCo and uPARsi VSMC. **b** Siah1 expression in DOX-treated VSMC. **c** Siah1 and uPAR expression in VSMC nucleofected with Siah1 siRNA, uPARsi RNA and, concomitantly, Siah1 and uPAR siRNA. **d** TRF2 ubiquitination in VSMC nucleofected for Siah1si, uPARsi or double-nucleofected for Siah1si/uPARsi assessed by immunoprecipitation.

uPAR in VSMC Senescence

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in response to doxorubicin treatment (fig. 4b). These data suggest a regulatory role for uPAR in UPS control in VSMC in response to doxorubicin.

Recent reports suggest that telomere dysfunction has an impact on apoptotic cell death and senescence [25]. In particular, telomere binding factors (TRF1 and TRF2) attract more and more attention as key regulators of the senescence program in cancer and aging [26]. Therefore, we aimed to determine whether TRF2 might serve as a substrate for uPAR-related ubiquitination and proteasomal degradation and thus may trigger doxorubicin-induced, uPAR-controlled senescence in VSMC. To get an idea about the functional relevance of TRF2 for doxorubicin-induced VSMC senescence, TRF2 downregulation by means of siRNA was performed (fig. 4c). TRF2 was downregulated either alone or concomitant with uPARsi. Decreased protein expression was ascertained 24 h after cell nucleofection. At that time point, cells were treated with doxorubicin to induce senescence. Doxorubicin-induced senescence was significantly increased in TRF2si cells, providing evidence for TRF2 involvement in this process (fig. 4d). Further downregulation of uPAR in TRF2si cells failed to rescue cells from doxorubicin-induced senescence (fig. 4c, d). To confirm the specificity of the TRF2 downregulating effect on doxorubicin-induced senescence, we have performed a rescue experiment by expressing TRF2 from the TRF2-IRESS-enhanced GFP plasmid [16] in cells nucleofected with TRF2si RNA. Control cells were co-nucleofected with empty vector. As shown in figure 4e, replenishing TRF2 abrogates doxorubicin-induced senescence. Together, these observations confirm that TRF2 is causative for doxorubicin-induced senescence and that uPAR and TRF2 are parts of the same senescence pathway in VSMC.

uPAR Controls Nuclear Accumulation and DNA Binding of TRF2

To provide further evidence for uPAR-directed regulation of TRF2, we determined the TRF2 level in VSMC and found decreased TRF2 protein expression in response to doxorubicin. This effect was, however, abolished by uPAR downregulation in uPARsi cells (fig. 5a). Consistent with these observations, immunocytochemical studies documented a decreased amount of TRF2 in cell nuclei in doxorubicin-treated cells. Similar to the Western blotting data, this effect was prevented by uPAR silencing (fig. 5b). To further substantiate these findings, we performed additional experiments using uPAR–/– mouse VSMC. The decrease in TRF2 protein and in its nuclear accumulation in response to doxorubicin was abolished in uPAR–/– cells (fig. 5c, d) consistent with the observations done on human VSMC with downregulated uPAR.

Decreased TRF2 protein expression might be achieved either by inhibition of its expression or proteasomal degradation of TRF2. We performed TaqMan analysis to determine TRF2 mRNA expression in doxorubicin-treated cells. As shown in figure 5e, the TRF2 mRNA level did not change, suggesting involvement of proteasomal degradation. Indeed, inhibition of proteasome with MG132 abrogated doxorubicin-induced TRF2 degradation (fig. 5f).

We next analyzed if TRF2 is associated with the corresponding telomeric DNA in VSMC after doxorubicin treatment using ChIP. ChIP analysis revealed a dose-dependent inhibition of the TRF2/DNA complex in response to cell stimulation with doxorubicin. This effect was, however, completely abolished in cells with downregulated uPAR (fig. 5g). These data suggest that in uPARsi VSMC doxorubicin-induced degradation of TRF2 is impaired and TRF2 remains bound to telomeric DNA. It might explain the mechanism of uPAR interference with the program of doxorubicin-induced VSMC senescence.

Protein ubiquitination is a general process preceding and controlling protein degradation by UPS [27]. Therefore, we analyzed TRF2 ubiquitination in VSMC in response to doxorubicin and the effect of uPAR downregulation on this process. Immunoprecipitation of TRF2 followed by immunoblotting with anti-ubiquitin antibody was performed. Indeed, increased ubiquitination of TRF2 was noted in doxorubicin-treated cells that was abolished in uPARsi VSMC (fig. 6a). Interestingly, the basal level of TRF2 ubiquitination was markedly increased in uPARsi VSMC. The membrane was then stripped and developed with anti-TRF2 antibodies to distinguish ubiquitinated TRF2 from other ubiquitinated proteins that can associate with TRF2 in immunoprecipitation. The data confirmed that ubiquitination of TRF2 itself is induced by doxorubicin in uPAR-dependent manner (fig. 6a, right panel). Similar results were obtained when WT and uPAR–/– mouse VSMC were used for the immunoprecipitation assay (fig. 6b). On the contrary, when uPAR expression was upregulated by means of VSMC lentiviral infection, TRF2 ubiquitination in untreated cells was decreased and doxorubicin-induced ubiquitination of TRF2 was present (fig. 6c). Thus, downregulation of uPAR expression has dual effects on the level of TRF2 ubiquitination. On the one hand, it markedly induces basal TRF2 ubiquitination. On the other hand, downregulation of

uPAR prevents doxorubicin-induced ubiquitination and degradation of TRF2. Increased basal TRF2 ubiquitination in the absence of doxorubicin treatment might result from impaired proteasomal degradation of proteins that we observed in uPAR knockout cells and changed activity of TRF2-ubiquitinating and -deubiquitinating enzymes.

In a previous study, Siah1, a p53-inducible E3 ubiquitin ligase, was implicated in TRF2-mediated senescence [28]. Next, we tested if uPAR influences TRF2 ubiquitination via the p53-Siah1 pathway. First, Ser[15] phosphorylation of p53 in doxorubicin-treated VSMC was assessed. As illustrated in figure 7a, p53 phosphorylation is increased after doxorubicin treatment. Again, in uPARsi VSMC, baseline p53 phosphorylation was higher than in control cells. Siah1 expression was also upregulated in uPARsi VSMC (fig. 7b). These data confirm other observations done in this study and might explain increased TRF2 ubiquitination in uPARsi VSMC. Further, we downregulated Siah1 expression alone and concomitant with uPAR (fig. 7c) and performed an immunoprecipitation assay. As shown in figure 7d, in the absence of Siah1, the level of ubiquitinated TRF2 is decreased even in uPARsi VSMC. The doxorubicin-induced response is also abrogated. These data show that uPAR downregulation influences ubiquitination of TRF2 via both Siah1-mediated ubiquitination and downregulation of proteasomal degradation. Whether TRF2 deubiquitination is also affected by uPAR remains to be elucidated.

Discussion

TRF2 is a telomere-binding factor of the telomere-capping protein-complex shelterin and plays a critical role in cancer and aging. TRF2 maintains the structure of telomere termini and is essential to prevent the activation of factors triggering a DNA-damaging response that may lead to the induction of apoptosis or cellular senescence [26, 29]. Though decisive functions of TRF2 and its related proteins in controlling genetic stability are well recognized, nothing is known, despite intense research, about what factors provide TRF2 regulation in physiological and pathophysiological conditions. It has been suggested that elucidation of these factors may lead to novel therapeutic avenues for cancer and aging [26]. Our study identifies uPAR as one of the factors regulating TRF2 in human VSMC in response to low doses of doxorubicin and suggests that uPAR may be a target to affect cardiovascular side effects of doxorubicin treatment.

uPAR is a multifunctional receptor that – in addition to plasminogen activation – initiates intracellular signaling and regulates cell migration, invasion and proliferation [30, 31]. Little is known, however, about the involvement of uPAR in cellular senescence. Recent studies report on uPAR-mediated mechanisms underlying senescence-associated cellular events in a cancer cell line [32]. Interestingly, in contrast to our results, in those cells, uPAR loss by means of siRNA resulted in increased cell senescence related to decreased activity in the FAK/PI3K/Akt signaling pathway. Cell type specificity of uPAR-related cellular mechanisms is well known. Whether and how the uPA/uPAR system interferes with doxorubicin treatment in cancer cells remains to be determined. Solitary studies report increased expression of uPA, but not uPAR, in human lung carcinoma cells in response to higher doxorubicin concentrations [33, 34]. On the other hand, increased uPAR expression was also associated with a senescence-associated secretory phenotype in different cell lines [35]. uPAR was upregulated in VSMC treated with low doxorubicin concentrations. We provide evidence that increased uPAR expression in cells undergoing senescence was not a coincidence but rather a requirement for the senescence program propagation in response to doxorubicin. Thus, VSMC senescence was abrogated in uPARsi cells and cells from uPAR–/– mice, whereas uPAR overexpression increased senescence dramatically.

We have recently elucidated a previously unknown role for uPAR in the regulation of UPS with critical effects on the outcome of cell function [11]. Accumulating evidence indicates that the post-translational modification of proteins by ubiquitination followed by their proteasomal degradation regulates key processes of cell function. Moreover, these studies suggest that therapeutic agents that target UPS might be of clinical importance [36]. It has been demonstrated that UPS-mediated degradation of transcription and cell survival factors in cardiomyocytes are upregulated by doxorubicin treatment and may contribute to doxorubicin cardiotoxicity [2, 9]. Our findings indicate that in addition to cardiotoxicity, doxorubicin-induced UPS may initiate vascular toxicity via induction of cell senescence in VSMC and thus provide new insights regarding side effects of anthracyclines in anti-cancer therapy. Our results demonstrate that low concentrations of doxorubicin affect two main processes of UPS in VSMC, namely ubiquitination and proteasomal-mediated degradation. This mechanism initiates doxorubicin-induced ubiquitination and proteasomal degradation of TRF2 that culminates in VSMC senescence.

These processes were abrogated after uPAR silencing in human cells and in VSMC from uPAR-deficient mice. Together with the observation that uPAR is upregulated in VSMC in response to doxorubicin, our data suggest an important and novel role for uPAR in the regulation of doxorubicin-induced vascular damage.

TRF2 degradation upon telomere damage signaling and cellular senescence in human fibroblast strains has been demonstrated recently. Fijuta et al. [28] reported that TRF2 ubiquitination and proteasomal-related degradation during replicative cell senescence were mediated by the tumor suppressor p53 and the p53-induced E3 ubiquitin ligase Siah1. Other studies addressing doxorubicin cardiotoxicity confirmed a key role for TRF2 and TRF1 in cellular senescence and apoptosis induced by low and high doses of doxorubicin in cardiomyocytes [8, 20, 25]. It was documented that doxorubicin induced downregulation of TRF2 expression in an MAPK-dependent fashion; whether or not UPS-mediated degradation contributed to TRF2 changes under that specific cellular conditions has not been explored [20].

Protein ubiquitination and degradation of ubiquitinated proteins by UPS is a complex and highly coordinated process [27]. How many steps of the UPS machinery are orchestrated remains uncertain. Our results evidence that uPAR was required to mediate both TRF2 ubiquitination and proteasomal degradation. The p53-Siah1 pathway is active in uPARsi VSMC leading to increased TRF2 ubiquitination even in the absence of doxorubicin treatment. The mechanism of how uPAR can affect p53 phosphorylation in VSMC is not yet known. Recently, it was reported that α_v integrin signaling inactivates p53 [37]. Given that uPAR can activate α_v integrins, it is possible that uPAR influences p53 phosphorylation in VSMC by interfering with α_v signaling. Thus, our data suggest that uPAR influences ubiquitination of TRF2 via Siah1-mediated ubiquitination and downregulation of proteasomal degradation. Additionally, protein-deubiquitinating enzymes may influence the amount of ubiquitinated proteins in the cell. Whether and which deubiquitinating enzymes might be affected by uPAR remains to be clarified. The proteasome is a 2.5-MDa protease composed of 20S core particle with protease activity and the 19S regulatory particle [27]. Further studies should be conducted to elucidate how uPAR may affect proteasome activity or/and proteasome assembly. Our findings suggest that uPAR may have multiple functions in the regulation not only of cell surface-related but also intracellular proteolysis.

In summary, the present study provides evidence that already low doses of doxorubicin initiate vascular damage via induction of cellular senescence in VSMC that may have a profound impact on the outcome of cancer therapy by anthracyclines. Our findings demonstrate the underlying mechanism and reveal a novel role for uPAR, which controls telomere function mediated by TRF2.

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3.2. Manuscript 2

Loss of urokinase receptor sensitizes cells to DNA damage and delays DNA repair .Yulia Kiyan, Mahshid Hodjat, Hermann Haller, and Inna Dumler

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Loss of urokinase receptor sensitizes cells to DNA damage and delays DNA repair

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Abstract

DNA damage induced by numerous exogenous or endogenous factors may have irreversible consequences for the cell. The DNA damage response (DDR) is powerful signaling machinery triggered in response to DNA damage, to provide DNA damage recognition, signaling and repair. DDR is a highly coordinated mechanism involving posttranslational changes of repair proteins, their recruitment onto DNA damage foci followed by disassembly and protein degradation. The precise knowledge of the molecular mechanisms determining DNA foci formation, resolution and related signaling remains, however, elusive. In this study, we identified the multifunctional urokinase receptor (uPAR) as a novel factor that determines DDR of different cell types, such as primary human and murine vascular smooth muscle cells (VSMC) and MDA-MB 231 cancer cell line. We observed that uPAR-deficient cells are sensitized to DNA damage and reveal a decreased survival as a result of impaired DNA repair. We found that the underlying pathways involve uPAR-mediated regulation of the proteasome subunit Rpn7 and its redistribution to DNA damage foci. We further show that Rpn7 nuclear translocation requires Rpn7 association with the tyrosine kinase c-Abl. We provide evidence that nuclear c-Abl associates with the tyrosine phosphatase SHP-2, which undergoes acetylation and serves for regulation of DNA repair. Our findings demonstrate an unusual but common, cell type independent uPAR-directed mechanism utilized by cells to modulate DDR and optimize cellular response to DNA damage.

Introduction

Genomic instability resulting from damaged DNA causes many diseases such as cancer, cardiovascular and neurodegenerative disorders, immune deficiencies and metabolic syndrome(Jackson and Bartek, 2009). Both exogenous factors like ultraviolet light, ionizing radiation, environmental chemicals and endogenous sources like reactive oxygen species can induce DNA damage. Moreover, many drugs used to treat cancer, psoriasis, and some other disorders have been identified as DNA-damaging agents (Espinosa et al., 2003; Lebwohl et al., 2005). To combat DNA damage, cells evolved the DNA damage response (DDR), which represents highly coordinated signaling mechanisms aiming at recognition DNA lesions, signaling their appearance, and providing efficient repair. Deficiency and failures in DDR mechanisms lead to increased cell sensitivity to DNA-damaging factors. Though DDR involves a wide range of cellular events, which are regulated at different molecular and cellular levels, many of these pathways occur by a common program in response to different classes of DNA lesions. The main molecular mechanisms underlying DDR are changes in transcriptional control and posttranslational modifications (Jackson and Bartek, 2009). Recent studies have revealed that the ubiquitin-dependent proteasomal degradation system (UPS) is essential to coordinate DDR after DNA damage (Ramadan and Meerang, 2011). A growing body of evidence indicates that UPS is indispensable to mediate and tightly control disassembly, removal, and degradation of DDR proteins recruited to DNA lesions such as DNA double strand breaks (DSB). Data coming from genetic, biochemical and biological approaches propose the UPS as a central element in the DDR orchestration at the sites of DSB. They further suggest the major proteasome assembly, the 26S proteasome as a constitutive and conserved part of the DSB repair mechanism (Jung et al., 2009; Ramadan and Meerang, 2011). The 26S proteasome predominantly mediates the second step of the UPS-directed proteolysis, namely degradation of ubiquitinated proteins. The 26S proteasome consists of the 19S regulatory particles and the 20S catalytic core particle with protease activity. Molecular organization and assembly of 26S proteasome subunits are crucial for regulation of proteasomal activity (Ranek and Wang, 2009). How functional properties of 26S proteasome are regulated and orchestrated upon DDR remains, however, poorly explored.

The multifunctional urokinase (uPA)/urokinase receptor (uPAR) system plays a central role in the molecular events coordinating functional behavior and cell fate in health and disease (Binder et al., 2007; Pillay et al., 2006). Though uPA/uPAR interference with DDR has not been proved experimentally, several clues from different studies suggest that uPA/uPAR might also be involved in at least some pathways triggered by DNA damage. Thus, in different cell types this system regulates main cellular functions related to DDR, such as proliferation, cell cycle, senescence, and apoptosis (Blasi and Carmeliet, 2002; Smith and Marshall, 2010). Others and we have demonstrated recently that uPAR possesses transcriptional activity and may undergo nuclear translocation and regulate cellular events at nuclear level that further strengthens uPAR implication in DDR-related processes (Asuthkar et al., 2012; Kiyan et al., 2012). Our recent studies revealed one novel function for uPAR, which may be additionally relevant to DDR mechanisms. We found that uPAR deficiency resulted in decreased proteasomal activity in tissues of uPAR-/- mice and that uPAR mediates specific ubiquitination and proteasomal degradation of proteins determining cell functional behavior (Hodjat et al., 2012; Kiyan et al., 2012). In the present study we demonstrate that uPAR serves as an active participant in DDR signaling events in a general, cell type independent fashion. uPAR-deficient cancer and smooth muscle cells are sensitized to DNA damage and reveal decreased survival as a result of impaired DNA repair. Consistent with our previous observations, we further show that underlying pathways involve uPAR-mediated regulation of the 26S proteasome subunit Rpn7 and the tyrosine phosphatase SHP-2. We provide evidence for a critical role of the tyrosine kinase c-Abl in these processes.

Results

uPAR downregulation affects activation of DDR proteins

To get a first evidence for uPAR interference with DDR, we analyzed main marker proteins involved in DDR, such as checkpoint kinase 2 (Chk-2) and γ-histone 2 (γH2AX). To induce DNA damage, cells were treated with H_2O_2 for different periods of time. We used VSMC isolated from uPAR-deficient mice and human MDA-MB cells where uPAR downregulation was effectively achieved by nuclefection with the corresponding siRNA.

Activation of Chk-2 and γH2AX was monitored by their phosphorylation in response to H_2O_2. For both cell types we observed that already basal phosphorylation of these proteins was significantly increased in uPAR-deficient cells. Correspondingly, activation of Chk-2 and γH2AX in response to H_2O_2 in cells lacking uPAR, though being reliable, was less effective than in control cells thus pointing to uPAR requirement for properly regulated DDR process (Fig. 1A-D). Similar data were obtained for human VSMC with downregulated uPAR (data not shown).

To further confirm these observations, we performed immunocytochemical studies. In agreement with the biochemical data, uPAR-deficient cells revealed higher basal level for phosphorylated Chk-2, γH2AX, and ataxia talengiectasia mutated (ATM) kinase and showed deregulated response to DNA damage compared to control cells (Fig. 1E,F, shown for human VSMC; similar effects for MDA-MB cells not shown).

uPAR-deficient cells reveal decreased survival and impaired DNA repair

To elucidate potential function for uPAR in DDR related signaling and cell fate, we examined cell death sensitivity to DNA damage in cells with downregulated uPAR. Cell survival was documented 24 hrs after H_2O_2 treatment. Apoptotic cell death was quantified using Cell Death Elisa kit (Roche). We found that both VSMC and MDA-MB cells lacking uPAR were much less resistant to DNA damage and revealed impaired survival (Fig. 2A) and significantly increased cell death (Fig. 2B). To analyze whether these functional changes may result from accumulated damaged DNA in uPAR-deficient cells, single cell gel electrophoresis assay (comet assay) was performed. Comet tail moment reflecting the number of DNA breaks was calculated 4 hrs after inducing DNA damage. We observed that uPAR deficiency independently of cell type indeed lead to delayed DNA repair (Fig. 2C-E).

uPAR mediates intracellular redistribution of the proteasomal subunit Rpn7 and its recruitment to DDR foci

Based on the aforementioned critical role of UPS in DDR and on our recent findings demonstrating uPAR ability to control at least some of UPS functions, we next tested whether the observed sensitization of uPARsi cells to DNA damage and impaired DNA repair in these cells might be related, at least in part, to UPS deregulation. Pilot experiments based on

proteasome purification by immunoprecipitation followed by mass spectrometry of selected bands revealed deregulation of the Rpn7 regulatory particle in uPARsi cells subjected to DNA damage (data not shown). Rpn7 is known to stabilize DNA damage foci upon genotoxic stress (Tsolou et al., 2012) and therefore we next explored Rpn7 intracellular distribution and recruitment to DDR foci in uPAR-deficient and control cells. Indeed, we observed that in control cells subjected to DNA damage Rpn7 revealed effective translocation to cell nucleus, recruitment to foci and colocalization with phosphorylated ATM, a major component of DNA damage foci. In contrast, these processes were significantly impaired in cells with downregulated uPAR (Fig. 3A-C). In uPAR-silenced cells Rpn7 revealed some nuclear staining already in the absence of any treatment and DNA-damaging challenge did not significantly increase Rpn7 nuclear redistribution any further. This was even more pronounced in VSMC from uPAR-/- mice (Fig. 3B,C). To substantiate results of our immunocytochemical studies, we performed cell fractionation and determined Rpn7 in nuclear fractions. As shown in Fig. 3C, the results of these biochemical experiments were in a good agreement with those of immunocytochemistry pointing to uPAR requirement for a functional Rpn7 response to DNA damage.

c-Abl tyrosine kinase is required for uPAR-dependent Rpn7 nuclear import

uPAR is a multifunctional receptor interacting with diverse transmembrane and nuclear signaling proteins to trigger and propagate numerous signaling cascades (Smith and Marshall, 2010). We were next interested in elucidating specific signaling pathways mediating uPAR-dependent Rpn7 nuclear import and recruitment to DNA damage foci. Several recent reports point to an important role in DDR for the non-receptor tyrosine kinase c-Abl, which undergoes strong activation in response to various stimuli including DNA damage (Maiani et al., 2011; Wang et al., 2011). To verify our hypothesis implying a possible involvement of c-Abl in the observed effects, we performed c-Abl inhibition using its specific inhibitor imatinib. Rpn7 nuclear redistribution in response to DNA damage was strongly inhibited by this treatment independently of cell type providing clear evidence for c-Abl necessity in DDR-related Rpn7 nuclear import (Fig. 4A). To gain further insight into c-Abl interference with Rpn7 upon DNA damage, we performed several rounds of immunoprecipitations. Similar to the previous experimental settings, both VSMC and cancer cells subjected to DNA damage

were used. We found that c-Abl was effectively co-precipitated with Rpn7 in control cells and that DNA damage stimulated this reversible association. In uPAR-deficient cells Rpn7 association with c-Abl was much less pronounced and no impact of DNA damage on this association was found (Fig. 4B). We also observed that c-Abl phosphorylation on tyrosine residues in response to DNA-damaging signal was abolished by loss of uPAR (Fig. 4C). c-Abl is known to serve as a cytoplasmic-nuclear shuttle protein and its cellular redistribution is related to functional activity (Gonfloni et al., 2012). In cell fractionation experiments we found that within 60 min of DNA-damaging treatment c-Abl was enriched in nuclear fraction of control but not uPAR-deficient cells (Fig. 4D,E).

The tyrosine phosphatase SHP-2 associates with c-Abl and is involved in the uPAR-dependent DNA repair

c-Abl is a Src homology (SH) 2 and SH3 domain-containing tyrosine kinase. This structure enables c-Abl interaction and association with a number of signaling and repair proteins implying complex roles for c-Abl in DDR (Shaul and Ben-Yehoyada, 2005). Several recent studies provide evidence for a functional interference of c-Abl and the tyrosine phosphatase SHP-2, which is a natural substrate for c-Abl, induced by the DNA damage (Yuan et al., 2005; Yuan et al., 2003). We have demonstrated previously that SHP-2 plays an important role controlling uPAR-dependent signaling and functions in human VSMC (Kiyan et al., 2009). It was therefore tempting to suppose that c-Abl, beyond regulation of Rpn7, might mediate further uPAR-dependent cellular pathways upon DNA damage, such as those related to SHP-2. Indeed, we found in our immunoprecipitation experiments that c-Abl was associated in nuclear fractions with SHP-2 in uPAR- and DNA damage- dependent fashion (Fig. 5A). To define a potential contribution of SHP-2 to uPAR-directed and c-Abl-dependent DDR mechanisms, we examined SHP-2 activation in response to DNA damage in uPAR-lacking and control cells and functional consequences of SHP-2 deficiency for DNA repair. As expected, we observed pronounced, time-dependent SHP-2 activation in control cells subjected to DNA damage. However, in uPAR-deficient cells level of phosphorylated SHP-2 was negligible and showed no change after DNA damaging treatment (Fig. 5B). Efficient DNA repair pathways require different post-translational modifications at lysine residues of histone and non-histone proteins, such as acetylation, methylation, ubiquitination and

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SUMOylation (Chatterjee et al., 2012). We asked whether SHP-2 might undergo beyond tyrosine phosphorylation any further post-translational modification in response to DNA damage. We observed that DNA-damaging cell treatment induced strong acetylation of SHP-2 (Fig. 5C). This effect has not been reported so far. Even more interesting was the observation that SHP-2 acetylation was an uPAR- and c-Abl-dependent process (Fig. 5D). Cell fractionation experiments revealed that only nuclear, but not cytoplasmic SHP-2 underwent acetylation upon DNA damage (Fig. 5E). To explore functional consequences of SHP-2 for cell functional behavior upon DNA damage, we relied again on DNA repair comet assay using control cells and cells with downregulated SHP-2. These experiments demonstrated that cells lacking SHP-2 were much less resistant to DNA damage and revealed a delayed DNA repair (Fig. 5F).

Discusion

The DDR network plays a cardinal role in the maintenance of genome integrity and is, as one of the key cellular mechanisms, a subject of intensive research. Most of these studies are focused on the coordinated mechanisms by which DDR proteins orchestrate at the site of DNA damage. Molecular mechanisms providing sensing and transduction of DNA-damaging signals from receptors to DDR effectors remain, however, largely unknown. Our study provides compelling evidence for the role of uPAR in regulation of signaling mechanisms underlying DDR. We show that uPAR serves as a cellular sensor for DNA-damaging signal and that loss of uPAR sensitizes cells to DNA damage and retards DNA repair. The underlying mechanism suggests uPAR-mediated regulation of the proteasomal subunit Rpn7 and the tyrosine phosphatase SHP-2 via the c-Abl tyrosine kinase (Fig.6).

The uPA/uPAR is a surprisingly multifaceted system upregulated upon numerous diseases, primarily those related to inflammation, tissue remodeling and cancer (Binder et al., 2007; Blasi and Carmeliet, 2002; Pillay et al., 2006). uPAR realizes two important cellular functions providing regulation of extracellular proteolytic cascade and serving as a signaling receptor to promote changes in cell functional behavior (Smith and Marshall, 2010). The uPAR-directed signaling can occur via uPA-uPAR binding or be uPA-independent. As a GPI-

anchored receptor lacking transmembrane and intracellular domains, uPAR associates with transmembrane proteins, such as integrins, tyrosine kinase receptors and others, to initiate signal transduction. Multiple signaling cascades induced via these co-receptor cooperations have been identified over the last decade (Blasi and Carmeliet, 2002). Though many advances have been done in the field, the mechanisms of uPAR signaling are still not completely clear and several controversies remain. At the level of cellular functions determining the cell fate in response to microenvironment, uPAR-directed signaling is believed to regulate physiological and pathophysiological conditions requiring changes in cell proliferation, migration, adhesion, and survival (Pillay et al., 2006). Due to these multifunctional properties uPAR presents many opportunities to be utilized as a target for specific therapies in diverse human diseases. However, none of the earlier studies has addressed possible involvement of uPAR in response to DNA damage that is a key event in cancer, aging, ischaemia-reperfusion injury, inflammatory, viral and other disorders.

To analyze uPAR necessity for DDR, we used a cellular model based on uPAR downregulation by means of siRNA in human primary VSMC and in human cancer cell line, as well as uPAR-deficient mouse VSMC. Our data show that uPAR deficiency in both cell types resulted in increased cell sensitization to DNA damage and impaired DNA repair. This function for uPAR has not been reported before. One recent study related to this issue has shown that transcriptional silencing of metalloproteinase 9 in combination with uPAR/cathepsin B affected DSB repair machinery in human glioma in vitro and in vivo (Ponnala et al., 2011). One further report from the same group suggested that inhibition of uPAR together with cathepsin B might be used in radiation therapy to target glioma-initiating cells. Similar to our observations, authors found changes in H2AX expression and functionality of H2AX foci after uPAR/cathepsin B downregulation (Malla et al., 2012). However, the impact of uPAR on DDR-related mechanisms independently of cathepsin B was not explored in those studies.

We show that the c-Abl non-receptor tyrosine kinase is required to mediate uPAR-related responses to DNA damage. The c-Abl tyrosine kinase is implicated in diverse cellular activities including growth factor signaling, cell adhesion, oxidative stress, and DNA damage response (Gonfloni et al., 2012). The essential role for c-Abl in molecular mechanisms and cellular responses underlying DDR has been implicated by several studies. c-Abl is known to

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control pro-apoptotic signals via interference with ATM, p73 and p53 (Yuan et al., 1999). Recent studies demonstrate that c-Abl deficiency resulted in a broad spectrum of defects in cell response to genotoxic stress, such as activation of Chk1, Chk2 and p53, nuclear foci formation, and DNA repair (Wang et al., 2011). It was proposed that multifunctional c-Abl signaling might mediate the molecular events at the interface between stress signaling, metabolic regulation, and DNA damage (Gonfloni et al., 2012). Our study adds new aspects into proposed models of c-Abl involvement in the DDR. Our findings suggest that c-Abl may orchestrate two uPAR-dependent cellular events in response to DNA damage, namely nuclear import of the Rpn7 proteasomal subunit and acetylation of the tyrosine phosphatase SHP-2. These data are along earlier observations of others showing that c-Abl acts in concert with tyrosine kinase receptors, such as VEGFR, PDGFR, FGFR and others (Anselmi et al., 2012.; Frasca et al., 2007; Li et al., 2001; Srinivasan et al., 2009). We have shown previously that PDGFR serves as a transmembrane co-receptor for uPAR to realize uPAR-directed intracellular signaling (Kiyan et al., 2005). Therefore, a scenario may be suggested that the cell-surface uPAR senses a DNA-damaging signal, associates with PDGFR and induces c-Abl activation. Alternatively, uPAR might first undergo internalization and then initiate c-Abl activation and cellular redistribution. It was shown that both mechanisms might be utilized by other membrane receptors, such as tyrosine kinase receptor to modulate DDR via multiple pathways (Squatrito and Holland, 2011).

Our findings strengthen a novel function for uPAR documented in our recent reports and classify uPAR is an important regulator of intracellular proteolysis controlling ubiquitination and proteasomal degradation of proteins determining cell functional behavior. We found that loss of uPAR resulted in deregulation of the 26S proteasome subunit Rpn7, which is an integral component of DDR. What further components of UPS might be regulated by uPAR is a question of great importance that may have an impact in the development of new therapeutic strategies aiming at uPAR targeting.

Materials and Methods

Cell culture, cell nucleofection and treatment with H_2O_2

Human primary umbilical artery VSMC were isolated from umbilical artery using explant technique in VascuLife SMC cell culture medium (CellSystems® Biotechnologie

Vertrieb GmbH, St. Katharinen, Germany). The procedure conforms to the Declaration of Helsinki and was approved by the local ethics committee. After 1st passage fibroblasts were removed from the culture by cell separation using monoclonal anti-fibroblasts antibodies (anti-CD90, Dianova) and magnetic Dynabeads® Goat anti-Mouse IgG (Invitrogen). VSMC were used between passages 2-4. Aortic VSMC were isolated from male uPAR-/- mice and uPAR+/+ (wild type) mice as controls (all on C57/BL6 background, age 10-12 weeks). All mice experiments were carried out according to the European Commission guidelines and were approved by the ethics committee of Hannover Medical School. Animals were euthanized by iv injection of 200 μl 2% avertin solution. The aortas were dissected, cut into 1–2 mm on a side pieces and subjected to enzymatic digestion as described (Fallier-Becker et al., 1990). VSMC were cultivated in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal bovine serum. MDA-MB 231 human breast cancer cell line (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (PromoCell GmbH).

DDR pathway activation was induced by cell treatment with 100 μM H_2O_2 at 37°C. For Comet assay cells were treated with 5mM H_2O_2 for 20 min on ice.

Small interfering RNAs (siRNAs) for downregulation of uPAR, SHP-2, and control silencing RNA were obtained from Santa Cruz Biotechnology and were transfected to the human UASMC using Amaxa Nucleofector™ (Lonza). Basic Primary Smooth Muscle cell nucleofector kit (Lonza) was used according to the manufacturer's instructions. Cell Line Nucleofector® Kit V (Lonza) was used for MDA-MB231 nucleofection. Efficiency of nucleofection was routinely evaluated by western blotting 24-72 hrs after cell nucleofection.

Immunostaining

Cells grown on coverslips were fixed by addition of 10% formaldehyde to the final concentration of 2%, permeabilized in 0.1% Triton X-100 for 10 min and blocked with 3% (w/v) BSA/PBS at 4°C overnight. Cells were labeled with primary and corresponding Alexa Fluor® 488- or Alexa Fluor® 594-conjugated secondary antibody (Invitrogen) for 1 h at room temperature. Cells were then mounted with Aqua-Poly-Mount mounting medium (Polysciences) and analyzed on a Leica TCS-SP2 AOBS confocal microscope. For

immunostaining of mice Aortic VSMC, cells were incubated with 5% mouse serum in PBS followed by 1h incubation with 5% normal goat serum.

Preparation of cell lysates, Immunoprecipitation and Western Blotting

Cultured cells were lysed in RIPA buffer containing 1mM PMSF, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1mM Na3VO4, 1mM NaF and incubated for 10 min at 4°C. For whole cell lysate preparation lysates were subjected to sonication. The lysates were centrifuged at 10000 rpm for 10 min. For immunoprecipitatin 600 μg total cell lysate with 4 μg of specific antibodies was used. After 3 hours immunocomplexes were precipitated with A/G PLUS-agarose beads. Precipitates were washed 3 times in PBS buffer containing protease inhibitors and subjected to SDS-electrophoresis.

Isolation of cytosolic and nuclear fractions was performed as described (Suzuki et al., 2010).

Antibodies against P-Chk2, γH2AX, Phospho-ATM, Phospho-SHP-2, SHP-2, Ac-Lys, c-Abl, Histon H3 were from Cell Signaling. Anti-tubulin antibody was from BD Pharmingen™. Alexa Fluor 488-conjugated chicken anti-rabbit antibody and Alexa Fluor 594-conjugated donkey anti-mouse antibody were from Invitrogen.

Comet assay

Comet assay was performed using OxiSelect™ Comet Assay Kit from Cell Biolabs, Inc. according to the manufacturer's instructions.

Cell death analysis

Apoptotic cell death was analyzed by Cell Death Detection ELISA[PLUS] from Roche Applied Scince accordingly to the manufacturer's instructions.

Statistical analysis

All experiments were performed at least three times. Statistical significance analysis ($P<0.05$) was performed using a Student's t test. "*" represents statistically significant differences at P<0.*05*.

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Figure legends

Figure 1. uPAR deficiency deregulates DDR pathway activation. A. VSMC isolated from WT and uPAR-/- mice were treated with 100 μM H_2O_2 for indicated time. Phosphorylation of Chk-2 and γH2AX was assessed by western blotting. B. Kinetics of H_2O_2 -induced Chk-2 and γH2AX phosphorylation in WT and uPAR-/- mouse VSMC. Tubulin was used for normalization. C. WT and uPAR-/- mouse VSMC were treated with H_2O_2 for 1 h, then fixed and stained for P-Chk-2 (Alexa 488) and P-ATM (Alexa 594). D. Cells treated as in C were stained for γH2AX (Alexa 488) and P-ATM (Alexa 594).

Figure 2. uPAR deficiency delays DNA repair and sensitizes cells to DNA damage. A. SiCo and uPARsi -nucleofected human VSMC were treated with 5mM H_2O_2 for 20 min on ice to induce DNA damage. Right and left panels show comet tails of SiCo and uPARsi VSMC after 4 hrs of DNA repair visualizes by Vista Green DNA staining. Comet tails of nucleofected human VSMC (B) and WT and uPAT-/- mouse VSMC (C) were quantified as described in the Materials and Methods. D. SiCo and uPARsi-nucleofected human VSMC were treated with different concentrations of H_2O_2 for 20 min on ice to induce DNA damage. The number of viable cells was calculated 24 hrs after DNA damage. C. SiCo and uPARsi-nucleofected human VSMC were treated with H_2O_2 as indicated to induce DNA damage. Cell apoptosis was detected 24 hrs after DNA damage using Cell Death Elisa Kit (Roche).

Figure 3. uPAR is essential for DNA damage-induced Rpn7 nuclear import. SiCo and uPARsi -nucleofected human VSMC were treated with 100μM H_2O_2 for 1 h at 37°C. Then cells were fixed and stained for Rpn7 (Alexa 488) and P-ATM(Alexa 594). The lower panels show indicated area with higher magnification. B. WT and uPAR-/- mouse VSMC were treated with 100μM H_2O_2 for 1 h at 37°C. Then cells were fixed and stained for Rpn7 (Alexa 488) and P-ATM(Alexa 594). H_2O_2 -induced Rpn7 content in nuclear fraction was assessed

after subcellular fractionation of SiCo- and uPARsi-nucleofected human VSMC and WT and uPAR-/- mouse VSMC. Histon H3 was used as loading control.

Figure 4. c-Abl kinase is required for DNA damage-induced Rpn7 nuclear import. MDA-MB 231 cells were pretreated with 2 μM imatinib for 1 hr at 37°C prior to H_2O_2 stimulation. After stimulation with 100 μM H_2O_2 for 1 h cells were fixed and stained for Rpn7 (Alexa 488) and P-ATM(Alexa 594). B. H_2O_2 -induced association of Rpn7 and c-Abl in WT and uPAR-/- VSMC was studied using co-immunoprecipitation from whole cell lysate. C. H_2O_2 -induced c-Abl phosphorylation in WT and uPAR-/- VSMC was studied using immunoprecipitation from whole cell lysate. D. WT and uPAR-/- mouse VSMC were treated with 100μM H_2O_2 for 1 h at 37°C. Then cells were fixed and stained for c-Abl (Alexa 488) and P-ATM(Alexa 594). E. H_2O_2 -induced Rpn7 content in nuclear fraction was assessed after subcellular fractionation of WT and uPAR-/- mouse VSMC. Histon H3 was used as loading control.

Figure 5. SHP-2 is involved in uPAR- and c-Abl-related DDR. A. H_2O_2 -induced association of c-Abl and SHP-2 in WT and uPAR-/- VSMC was studied using co-immunoprecipitation from cytosolic and nuclear fractions. The lower panels show loading control and purity of both, cytosolic and nuclear fractions. B. H_2O_2 -induced SHP-2 phosphorylation in WT and uPAR-/- VSMC (upper panel) and SiCo- and uPARsi-nucleofected human VSMC (lower panel) was assessed by western blotting from whole cell lysate. Tubulin shows loading control. C. H_2O_2 -induced SHP-2 acetylation was assessed by immunoprecipitation from whole cells lysate. D. H_2O_2 -induced SHP-2 acetylation in SiCo- and uPARsi-nucleofected human VSMC and in cells pre-treated with 2 μM imatinib prior to H_2O_2 stimulation was assessed by immunoprecipitation from whole cell lysate. E. H_2O_2 -induced SHP-2 acetylation was assessed by immunoprecipitation from cytosolic and nuclear fractions. F. SiCo- and SHP-2si-nucleofected human VSMC were treated with 5 mM H_2O_2 for 5 min on ice to induce DNA damage. Comet assay was performed 4 hrs after H_2O_2 treatment. Tail moments were quantified as described in Material and Methods.

Figure 6. Schematic presentation of uPAR-mediated DDR pathway regulation. DNA damaging drugs induce activation of cytosolic c-Abl and its association with Rpn7. Rpn7 and c-Abl undergo nuclear translocation and are recruited to the DDR foci. Nuclear c-Abl is essential for DDR-induced SHP-2 phosphorylation and acetylation. Loss of uPAR interferes with c-Abl activation and nuclear import and thus compromises DNA repair.
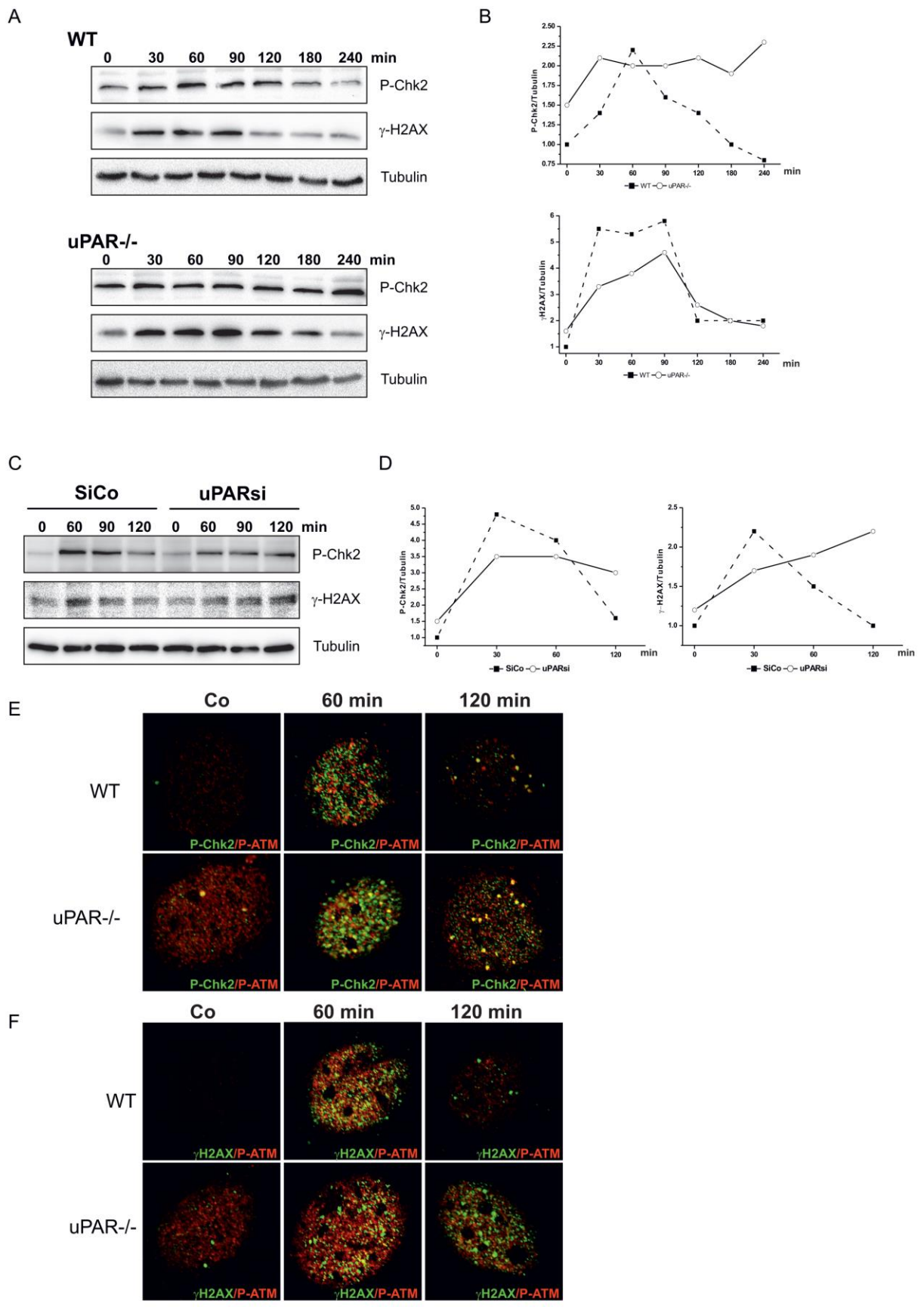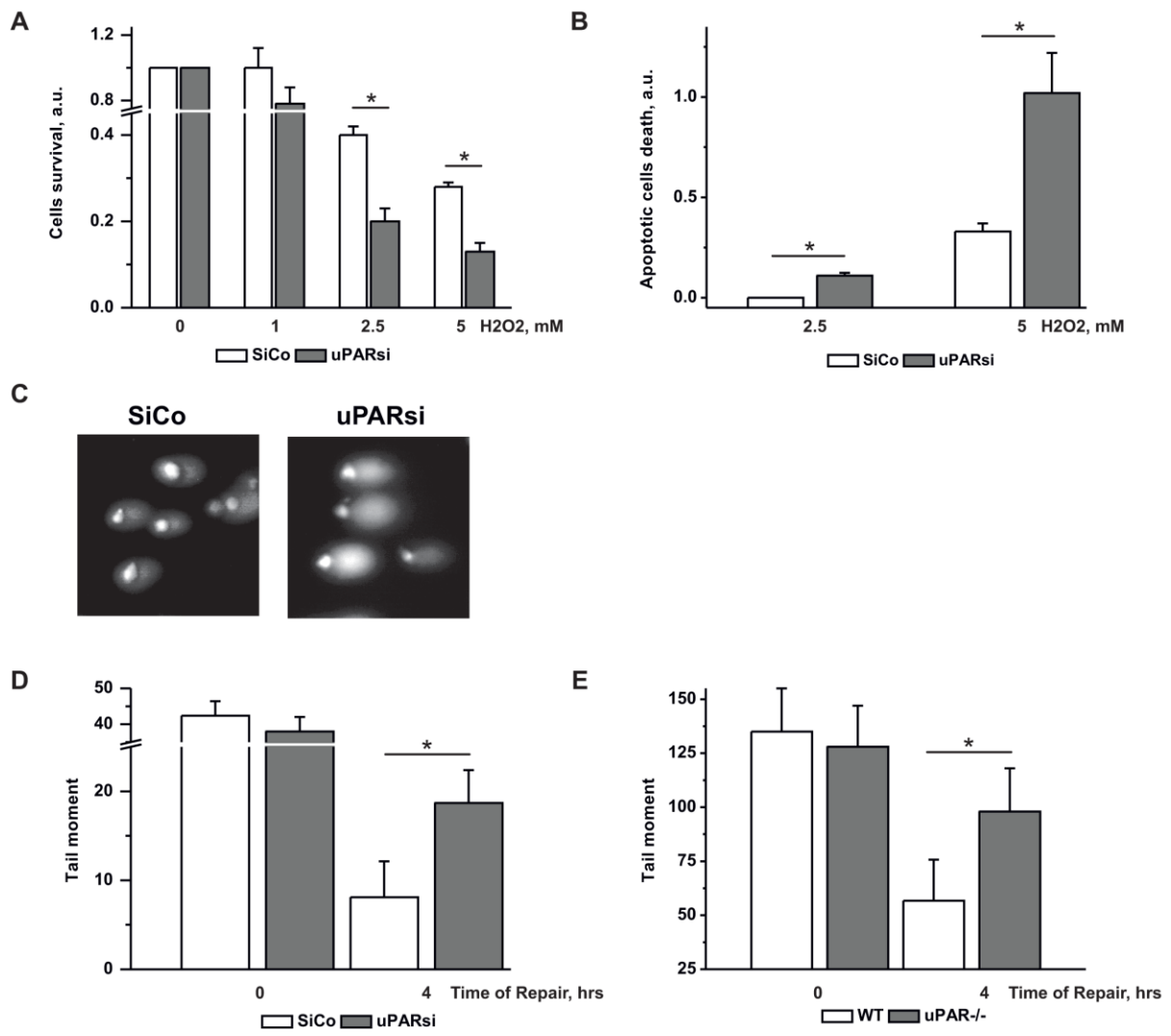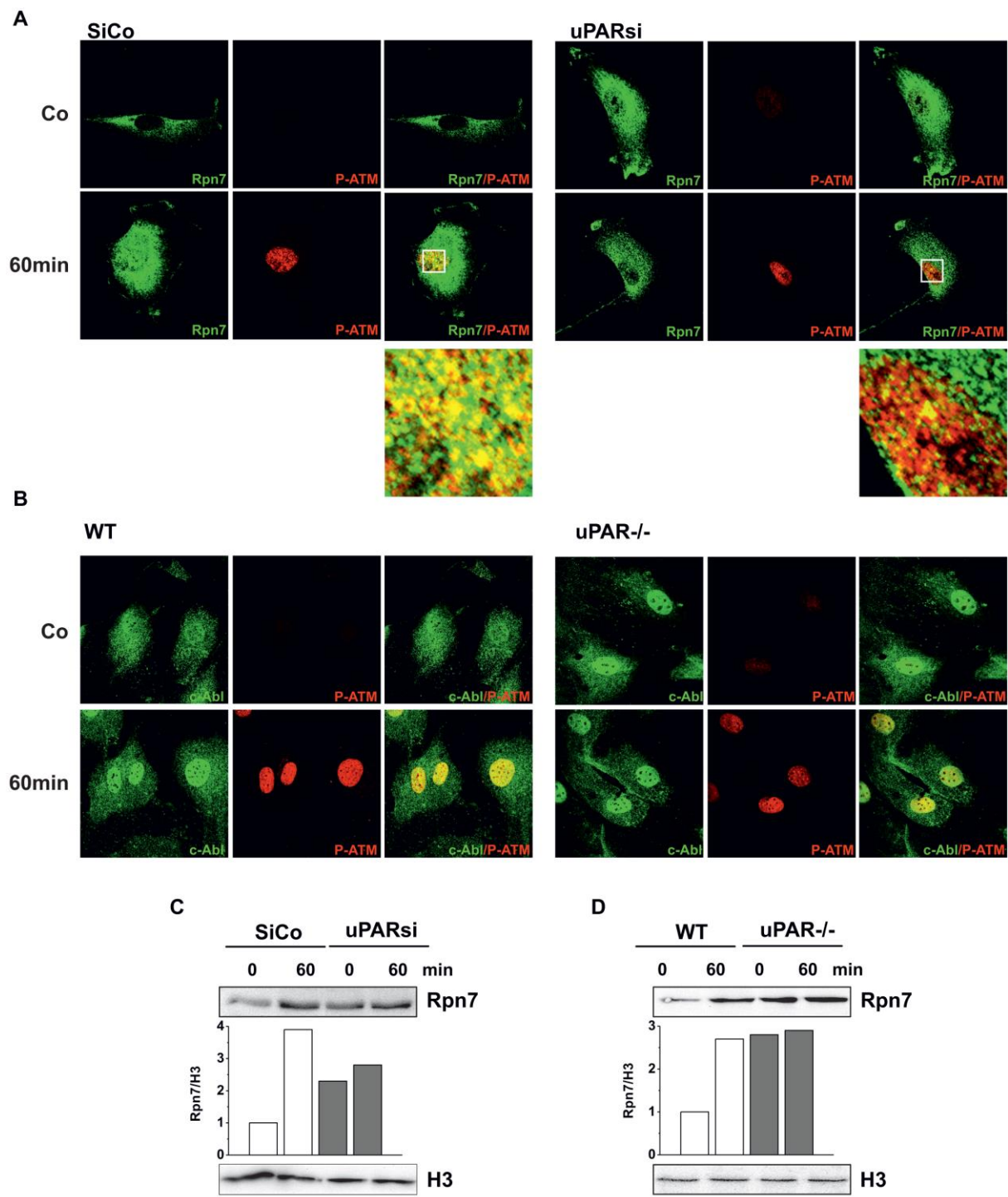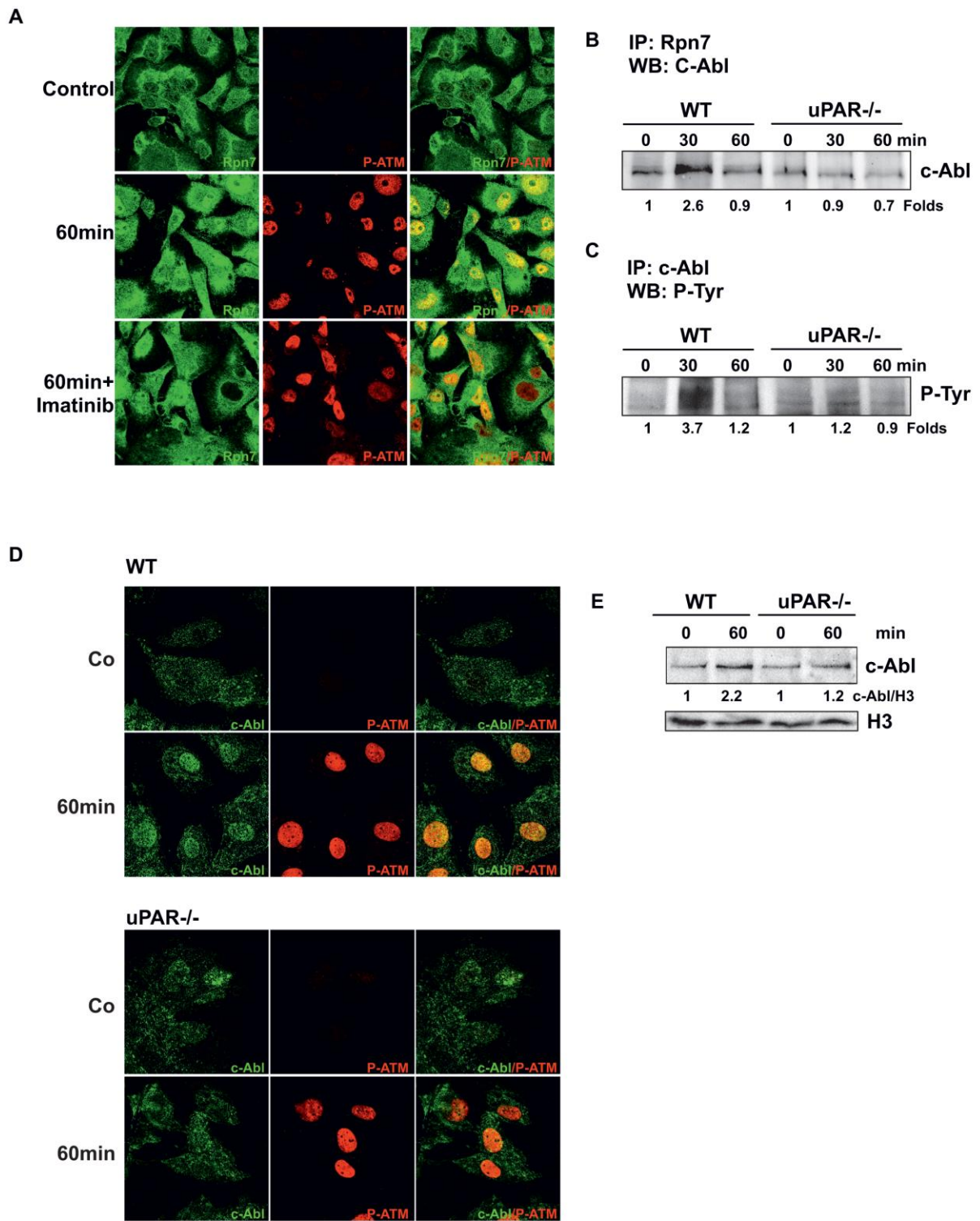
Figure 1.

Figure 2.

A

SiCo   uPARsi

Co

Rpn7   P-ATM   Rpn7/P-ATM   Rpn7   P-ATM   Rpn7/P-ATM

60min

Rpn7   P-ATM   Rpn7/P-ATM   Rpn7   P-ATM   Rpn7/P-ATM

B

WT   uPAR-/-

Co

c-Abl   P-ATM   c-Abl/P-ATM   c-Abl   P-ATM   c-Abl/P-ATM

60min

c-Abl   P-ATM   c-Abl/P-ATM   c-Abl   P-ATM   c-Abl/P-ATM

C

SiCo   uPARsi

0   60   0   60   min

Rpn7

Rpn7/H3

H3

D

WT   uPAR-/-

0   60   0   60   min

Rpn7

Rpn7/H3

H3

Figure 3.

60

A

Control

Rpn7   P-ATM   Rpn7/P-ATM

60min

Rpn7   P-ATM   Rpn7/P-ATM

60min+
Imatinib

Rpn7   P-ATM   Rpn7/P-ATM

B IP: Rpn7
WB: C-Abl

	WT			uPAR-/-		
	0	30	60	0	30	60 min
	1	2.6	0.9	1	0.9	0.7 Folds

c-Abl

C IP: c-Abl
WB: P-Tyr

	WT			uPAR-/-		
	0	30	60	0	30	60 min
	1	3.7	1.2	1	1.2	0.9 Folds

P-Tyr

D WT

Co

c-Abl   P-ATM   c-Abl/P-ATM

60min

c-Abl   P-ATM   c-Abl/P-ATM

uPAR-/-

Co

c-Abl   P-ATM   c-Abl/P-ATM

60min

c-Abl   P-ATM   c-Abl/P-ATM

E

	WT		uPAR-/-	
	0	60	0	60 min
	1	2.2	1	1.2 c-Abl/H3

c-Abl

H3

Figure 4.

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A

IP:c-Abl
WB: SHP-2

	WT		uPAR-/-		
	0	60	0	60	min

Cyt _____ SHP-2

Nucl _____ SHP-2

    1   2.5   1   0.6   Folds

	Cytosolic				Nuclear				
	WT		uPAR-/-		WT		uPAR-/-		
	0	60	0	60	0	60	0	60	min

_____ Tubulin

_____ NP 62

B

WT			uPAR-/-			
0	60	120	0	60	120	min

_____ P-SHP-2

    1   1.5   1.9   1   0.6   0.7 P-SHP-2/Tubulin

_____ Tubulin

SiCo			uPARsi			
0	60	120	0	60	120	min

_____ P-SHP-2

    1   1.6   1.7   1   1.3   1.2 P-SHP-2/Tubulin

_____ Tubulin

C

IP:Ac-Lys
WB: SHP-2

IgG	0	30	60	min

_____ SHP-2

        1   1.5   2.7   Folds

D

IP:Ac-Lys
WB: SHP-2

SiCo		uPARsi		
0	60	0	60	min

_____ SHP-2

    1   3.5   1   0.9   Folds

Control		Imatinib		
0	60	0	60	min

_____ SHP-2

    1   2.7   1   0.7   Folds

E

IP:Ac-Lys
WB: SHP-2

Cytosolic fraction		Nuclear fraction		
0	60	0	60	min

_____ SHP-2

            1   2.8 Folds

F

Figure 5.

Figure 6.

4. Discussion

Maintenance of genome integrity is the critical mission of all living cells that constantly exposed to different endogenous and exogenous DNA damaging agents. For this aim a complex well coordinated network of mechanisms are evolved that prevents genomic instability towards such inevitable and constant threats. DNA damage response is a signal transduction process that has the ability to sense, transduce and repair DNA damage at the site of injury. In case of impairment or inability of repair mechanism, DDR may trigger mechanisms based on type and location of DNA lesion that shift the cell fate towards senescence and apoptosis. These complex processes require crucial function of many regulators orchestrating different signaling pathways.

uPAR is a multifunctional receptor that besides its well known role in pericellular proteolysis is involved in a wide range of intracellular signaling processes during cell migration, invasion and proliferation. So far many studies have been performed to elucidate uPAR diverse roles in cell physiology and still a lot remains to be discovered about the underlying mechanisms.

In the present doctoral project we provided novel evidences for uPAR-mediated regulation of DDR during DNA repair mechanism and cell senescence as an important consequence of DNA damage. Our study focused mainly on UPS and its regulatory role in DDR-directed cellular events. As an experimental approach to down- and up-regulation of uPAR expression, we used small interfering RNA combined with the lentiviral cell infection or with cell nucleofection. In addition we used cells isolated from the uPAR-deficient mice.

Manuscript 1:

Telomeres, the ends part of chromosomes, have a specialized chromatin structure to protect chromosome ends from being recognized as double-stranded DNA breaks. It is reported that damage to telomere structure might be irreparable and contribute to telomere dysfunction that induces DNA damage response towards senescence or apoptosis [85]. The irreparability of telomeric tracts may be the consequence of their functions in preventing chromosomal fusions. Though many efforts have been made to elucidate the mechanism of DNA damage response, still not much is known about the DDR related telomeric regions of genome.

TRF1 and TRF2 are the critical components of functional telomere that interact directly with double stranded telomeric DNA. Specifically, TRF2 is responsible for the formation and maintenance of shelterin structure. Numerous studies demonstrated the result of telomere

uncapping by interference with TRF2 expression that contributes to telomere dysfunction and cell senescence [105, 107, 146]. In our study we showed that low doses of the anti-cancer drug Doxorubicin (DOX) induced senescence in VSMC that was accompanied by decrease in TRF2 protein level. We showed further that TRF2 regulation was a hallmark of senescence response in DOX treated cell. TRF2 downregulation by means of siRNA induced cell senescence in normal and DOX treated cells, that was abrogated in our replenishing experiments. Our data confirm the impact of TRF2 regulation on activation of DDR-related pathways towards senescence.

In this thesis we reported for the first time the role for multifunctional receptor uPAR in controlling telomere function during senescence response. The involvement of uPA system in cellular senescence has been previously discussed. A more than 50-fold increase in uPA activity was reported in senescent endothelial and fibroblast cells [147, 148]. PAI-1, the inhibitory member of uPA/uPAR system, was shown to be an essential mediator of replicative senescence leading to down-regulation of PI(3)K–PKB signalling and nuclear exclusion of cyclin D1 [149]. Also the increased uPAR expression was associated with senescence-associated secretory phenotype in different cell lines [150].

Our data based on in vivo and in vitro targeting of uPAR suggested that uPAR is an active regulator of cellular senescence in VSMC.

DNA-damaging drug, Doxorubicin, is one of the most effective antitumor agents, though DOX application is limited due to its cardiotoxic effect. Our data suggested that in addition to cardiomyocytes, DOX also affect VSMC via induction of senescence that contributes to vascular dysfunction. Our present finding provides novel evidence for the vascular toxicity side effect of DOX and the underlying mechanisms.

We reported that genotoxic drug DOX enhances TRF2 degradation via affecting two main processes of the UPS mechanisms, namely TRF2 ubiquitination and proteasomal degradation. These data are in agreement with the previously known role of DOX in activation of UPS in other cell types that results in degradation of many transcriptional and survival proteins [151]. Importantly we showed that uPAR is a key regulatory factor required in both processes of ubiquitination and proteasomal degradation.

Ubiquitination is a multi-step process, accomplished by the concerted, well organized action of different enzymes. E3 ubiquitin ligases are a large family of proteins that are engaged in transfer of ubiquitin to a substrate protein. SIAH1 is an E3 ubiquitin ligase whose role in direct ubiquitination of TRF2 via p53 pathway during replicative senescence has been recently discussed. In this thesis project we observed the activation of P53/ Siha1 pathway in DOX treated cells. Our results further evidenced that uPAR was required for TRF2 ubiquitination. Whether or not uPAR is involved in regulation of other enzymes of ubiquitination apparatus remains to be elucidated. Clearly, further research is required to identify the mechanisms by which uPAR can affect TRF2 ubiquitination. A role for deubiqutinating enzymes on TRF2 degradation is still unclear.

26S proteasome is the chief site of regulatory protein turnover in cell. Its regulatory role in DDR in both protein turnover and protein recruitment has been well defined. In our present project we showed that TRF2 serves as a substrate for proteasomal degradation in DDR-related senescence. We found that uPAR silencing abrogated DOX-induced proteasomal activity in human cells and in VSMC from uPAR-deficient mice. The data suggest a novel regulatory role for uPAR in proteasome activity that is essential for control of telomere function during senescence. In order to elucidate how uPAR may affect proteasome activity, we performed further experiments (the data are presented and discussed below in supplementary parts).

Overall, our findings suggest that uPAR might have multiple functions in regulation of not only cell surface related but also intracellular proteolysis.

Manuscript 2:

Precise regulation of DNA damage response is crucial for cellular survival after DNA damage, and its abrogation often results in genomic instability contributing to cell apoptosis or cancer. Over last decades large efforts have been performed to improve knowledge in DDR area that have introduced several key molecules and regulatory mechanisms of signaling initiation and transduction that was unknown before.

Among all regulatory molecules, the components of ubiquitin proteasomal system known to play cardinal roles in many aspects of DNA damage repair mechanism in formation of DNA foci, resolution and related subsequent signaling [152, 153]. 26S Proteasomal, a highly

conserved member of UPS has recently attracted many research interests. It involves not only in protein turnover but has also non-proteolytic functions in protein recruitment of DNA repair factors [152]. Many ubiquitinated proteins are known to bind to the 26S but escape proteolysis, only to be deubiquitinated and released [154]. It is reported that individual subunits of the 19S have non-proteolytic roles inside the nucleus and can be recruited to specific gene promoters in various eukaryotes [155]. Elucidating the regulatory mechanisms of 26S proteasomal components is of particular interest and still not much known about it.

In the first part of this doctoral project we revealed one novel function for uPAR in DDR mechanisms that mediates specific ubiquitination and proteasomal degradation of proteins determining cell functional behavior towards senescence. Accordingly, in the second part of our research we asked whether uPAR could also be involved in DNA repair mechanisms based on its regulatory role on UPS. We applied a model to induce a repairable DNA damage response in different cell type. DDR foci are intracellular structures where DDR signalling originates. It includes a high local concentration of DDR factors at damage sites providing a specific set of markers for the detection of an activated DDR. The foci detection in our experimental model revealed position of the DNA lesions, which was resolved after repair mechanisms.

Our data showed that during DNA damage the proteasome subunit Rpn7 translocates from cytosol to the nucleus and be recruited to DNA damage foci.

High mobility of 26S proteasome is a critical property of the proteasome. Mutant cells, whose proteasomes do not efficiently accumulate in the nucleus, are defective in DNA damage repair [156]. Furthermore, *cut8* mutants are inefficient in degrading cyclin-B during anaphase [157], which occurs in the nucleus. These observations support the idea that proteasomes must move and accumulate in the nucleus in order to regulate DNA damage repair that is in accordance with our data.

The mechanism of active translocation of proteasomal core complex and the regulatory particles in mammalian cells has been recently discussed. Using the advantage of the in vitro assembly system, it has been shown that proteasome passed the nuclear border in the form of an intact preassembled proteasome, the 20S+ particle, which is imported through nuclear pore complex. However it is proposed the remainder of the 19S RP subunits may be independently

targeted to the nuclear compartment [158]. Many shuttling transport receptors have been detected that might be involved in translocation of proteasome complex [158].

We showed here that nuclear translocation of Rpn7 a component of 26S requires Rpn7 association with the tyrosine kinase c-Abl. Indeed c-Abl tyrosine kinase has been widely associated with various aspects of the DDR (reviewed in [159]). Long before it was found that nuclear localization signal (NLS) containing c-Abl shuttles between cytoplasm and nucleus and is involved in carrying molecules in both compartments [160, 161]. These data suggest that the nuclear-cytoplasmic shuttling of this tyrosine kinase may play a critical role in the regulation of c-Abl biological function upon DDR. In agreement with these studies, our data revealed that c-Abl indeed plays a critical role in Rpn7 translocation during DDR.

We reported that in the absent of uPAR all mobility activities of Rpn7 and c-Abl become impaired that leads to the cell inability to repair DNA damages and cells prone to apoptosis.

We next addressed a possible mechanism of direct involvement of uPAR as a cell surface receptor in c-Abl localization and function. C-Abl is a member of the Src family contains three domains: SH3, SH2 and Src homology (SH1) domains. These features enable c-Abl to interact with many intracellular signalling molecules such as phosphatase SHP-2.

SHP-2, a tyrosine phosphatase implicated in diverse signaling pathways induced by growth factors and cytokines, is also involved in DNA damage triggered signalling and cellular responses [162]. It has demonstrated previously that SHP-2 plays an important role in controlling uPAR-dependent signalling and functions in human VSMC [43]. Taken together all these notions, we supposed that SHP-2 might be a direct mediator between c-Abl and uPAR. Our data revealed strong activation of SHP-2 after DNA damage treatment. Also in our experiment SHP-2-depleted cells, showed impairment of DNA repair upon induction of DNA damage that support the current findings on cellular significance of SHP-2 involvement in DDR pathways and chromatin stability[162, 163]. We found that c-Abl was associated in nuclear fractions with SHP-2 in uPAR- and DNA damage-dependent fashion. Additionally, we observed that DNA-damaging cell treatment induced strong acetylation of SHP-2 in uPAR- and c-Abl dependent fashion. This effect that has not been reported so far suggests a role for c-Abl on SHP-2 activation. Whether or not SHP-2 mediates uPAR dependent nuclear signalling remains an open question.

Finding the precise mechanism of uPAR-directed c-Abl nuclear translocation and significance of its interaction with SHP-2 upon DDR requires further intensive studies.

Discussion on supplementary data

The 26S proteasome is a protein complex consisting of two parts, the catalytic 20S core particle and the 19S regulatory particle composed of ATPase (Rpt) and non-ATPase (Rpn) subunits. So far many mechanisms have been proposed for regulation of 26S proteasomal activity.

It is known that assembly and disassembly of the 26S proteasome is a conserved mechanism for regulation of proteasomal activity in both yeast and mammalian cell. Posttranslational modification of different subunit could also affect proteasomal function. In our project we decided to elucidate the possible mechanism of uPAR-mediated proteasomal activity through analyzing proteasomal assembly. Our experiments based on proteasome immunoprecipitation followed by mass spectrometry revealed that the assemblies of some components of 19S regulatory particles to the core proteasome are significantly impaired in DOX treated uPARsi cells (Fig S1). The MS data were further verified by immunoblotting (Fig S2). Our data showed that the deregulation of proteasome assembly was mostly pronounced among 19S non ATPase particles, particularly Rpn7 and Rpn8. 19S regulatory particles are known to be involved in recognizing polyubiquitin-linked proteins, and translocation of substrates into the catalytic chamber of the 20S core [164, 165]. Each individual components of the 19S particle has its own regulatory role. Rpn7 is known to be required for the integrity of the 26S complex by establishing a correct lid structure [166]. Cells containing the mutant form of Rpn7 showed a defect in the assembly/ maintenance of the 26S proteasome. Rpn7 is also necessary for the incorporation/ anchoring of Rpn3 and Rpn12 to the lid that suggests the key role of Rpn7 in integrity of the 26S complex [166].

Our findings showed that in the absence of uPAR the proteasome undergoes changes in molecular assembly that might cause for change in its activities. These data suggest that uPAR regulate proteasomal activity via interfering with incorporation of specific regulatory particle to the proteasomal holoensyme and consequently proteasomal assembly.

Although the pivotal role of the proteasome is well established in eukaryotic cells, the mechanism of proteasome activity and the assembly mechanism of the 19S regulatory particle and 26S holoenzyme are still not fully elucidated.

Our studies based on cell fractionation assay, showed increased proteasomal activity in nucleus after DOX treatment that is inline with resulted degradation of nuclear telomeric TRF2 (Fig S4). Increased nuclear proteasome activity requires translocation of proteasome complex to the nucleus. Therefore impaired translocation of 26S proteasomal from cytoplasm to the nucleus resulted in deregulation of proteasomal assembly. In support for this view, our preliminary experiments showed DOX induces accumulation of Rpn7 in the nucleus that is impaired in uPARsi cells accompanied by reduced proteasomal activity (Fig S3). These preliminary data might raise the hypothesis that DOX induced proteasomal nuclear activity is the result of translocation and assembly of proteasomal components to the nucleus mediated by uPAR.

5. References

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6. Supplementary data

a
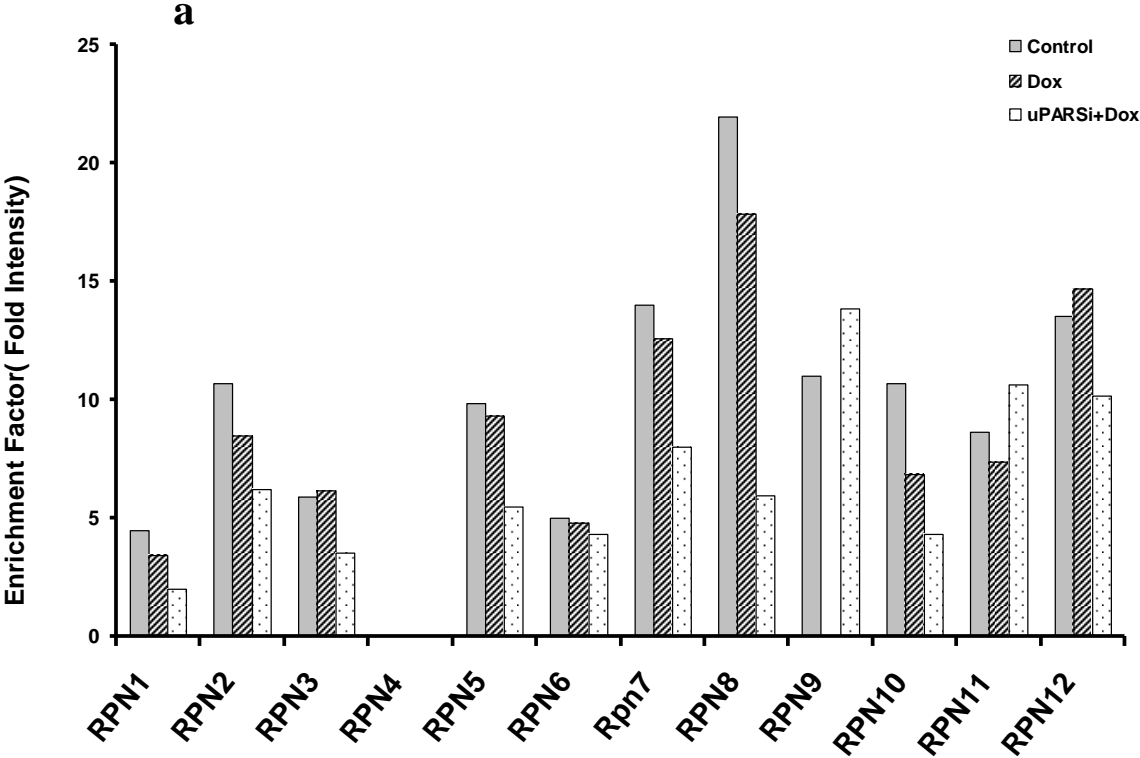
26S Proteasome non-ATPase regulatory particles

b
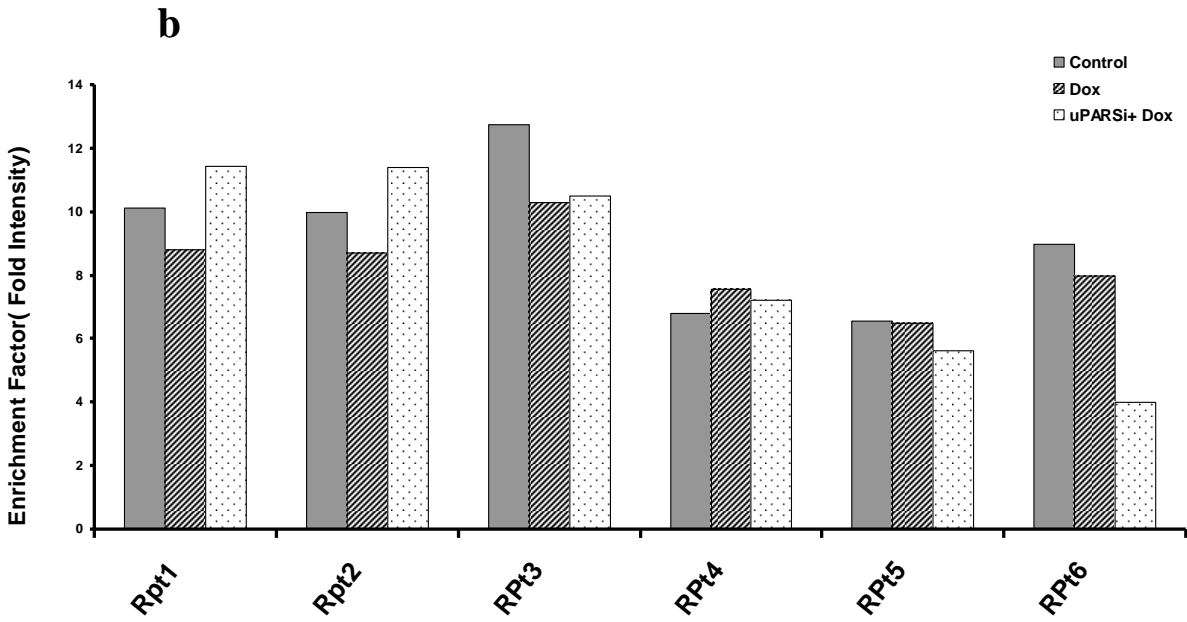
26S Proteasome ATPase regulatory particles
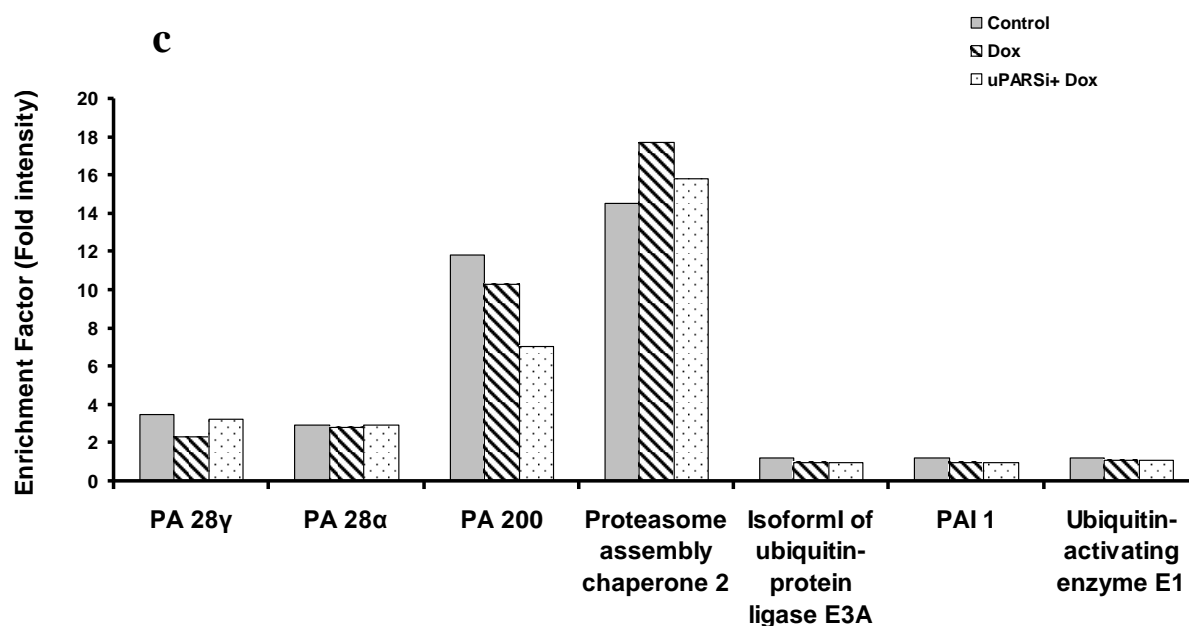
Fig S1. Quantitative mass spectrometry of purified 20S proteasome.
Proteasome and its interacting proteins were co-immunoprecipitated from Sico and uPARsi cells treated with doxorubicin (DOX) using antibody against α subunit of 20S core particle. Isolated proteins were subjected to ESI-LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific) for quantitative analysis. Enrichment factor were calculated for each individual peptides as the ratio of normalized intensity of peptide peak to the total input cell extract. a. Interaction of non-ATPase subunits of 19S regulatory particles to the proteasome core particle. b. Interaction of ATPase subunits of 19S regulatory particles to the proteasome core particle. Interaction of regulatory proteins to the proteasome core particle. Interaction of Rpn7 and Rpn8, the non-ATPase regulatory subunits with proteasom were markedly impaired in uPARsi cells after DOX treatment. Proteasome activator 28α (PA28α), proteasome activator 200(PA200), Plasminogen activator inhibitor (PAI1).
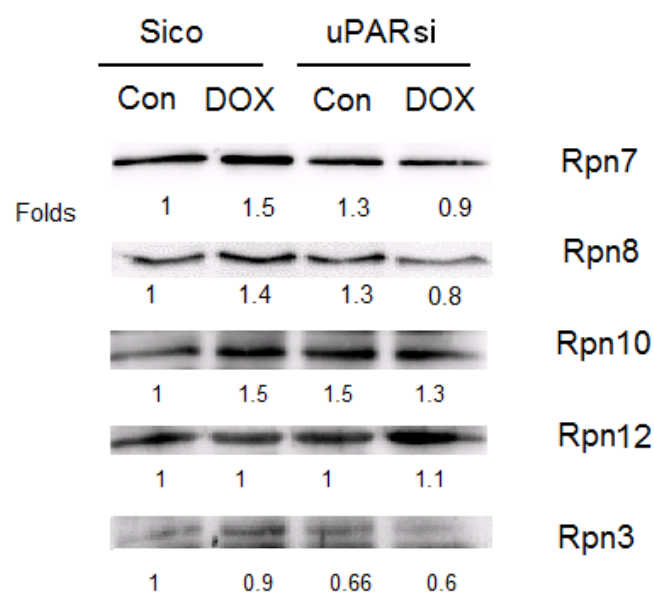
Fig S 2.Intreraction of 19S regulatory particles to the 20S core proteasome in control silencing (Sico) and uPAR silencing (uPARsi) cells treated with doxorubicin.
Proteasome purification performed by imunoprecipitation followed by western blotting. **7**
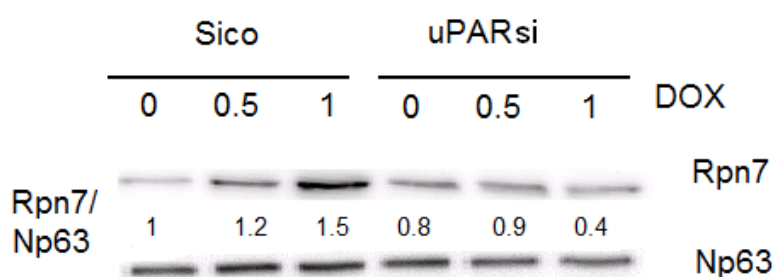
Fig S3. Rpn7 nuclear content in control and uPARsi cells treated with doxorubicin
DOX -induced Rpn7 content in nuclear fraction was assessed after subcellular fractionation of SiCo- and uPARsi-nucleofected human VSMC. Np63 was used as loading control. The cells were lysed 24h after DOX treatment in SiCo and uPARsi-nucleofected human VSMC.
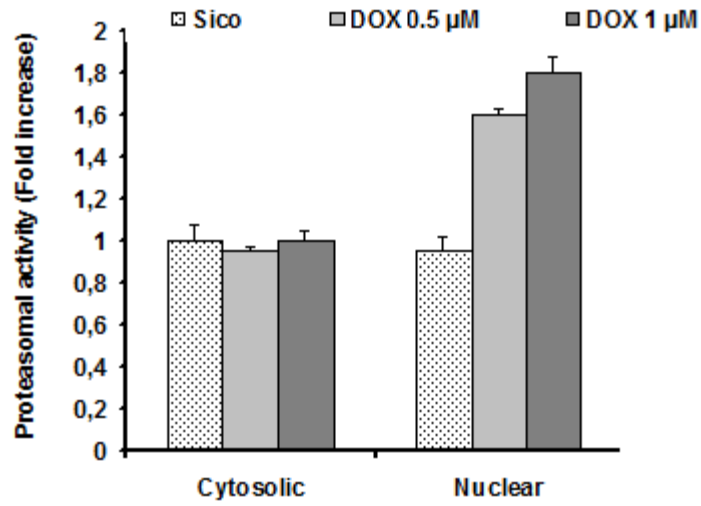
Fig S4. Increased proteasomal activity in nucleus after Doxorubicin (DOX)

7. Appendix

Appendix

Table of figures:

Appendix

Acknowledgements

Appendix

I wish to express my love and gratitude to my beloved families; my mom Floria, my father Ahmad and my sister Mahzad for their understanding, endless love and their supports during my studies. Their encouragement and belief, has led me to be where I am today.

Last but the greatest thank to the omnipresent God, for giving me the strength and the courage to deal with the challenges and to complete another stage of my life.

Curriculum vitae

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Academic Profile

Oct 2009 – Present	**PhD candidate** Hannover Medical school, Germany.
2005 - 2009	**Assistant researcher** Department of Reproductive Endocrinology & Embryology, Avicenna Research Institute
2002 – 2005	**Master of Science** Biochemistry, Alzahra university, Tehran, Iran
1996 – 2001	**Bachelor of Science** Cell and Molecular Biology, Azad university, Tehran Iran

Awards & honors

2009- March 2013	DAAD (Deutsche Akademische Austauschdienst) Scholarship
2005	Winner of the Prize for best poster presentation (11th Iranian Congress on Fertility and Sterility)

Research Experience

Oct 2009 – Present	Urokinase receptor in regulation of cellular senescence and DNA damage response: role for the ubiquitin-proteasomal system. PhD candidate, Hannover Medical School
2007- 2009	Study the expression profile of sperm specific genes in non obstructive azoospermic patients Assistant researcher, Avicenna Research Institute, Tehran, Iran
2006- 2008	Effect of folic acid and zinc sulfate supplementation on sperm function tests in infertile male Assistant researcher, Avicenna Research Institute, Tehran, Iran
2005- 2007	Study the relationship between sperm function test and outcome of Assisted reproductive techniques Assistant researcher, Avicenna Research Institute, Tehran, Iran
2003 –2005	Purification of polyclonal Anti-Ubiquitin Antibodies for detection of defective Sperm in Infertile Male *(Design an*

Imunocytochemistry assay for detection of defective sperm)
Masters thesis

Conferences and Workshops:

Nov 2011	Life in Motion: Dynamics of Molecules and Systems, Barcelona, PhD Student Symposium
Mar 2011	XIIIth International workshop on molecular & cellular biology of plasminogen activation, Cambridge
Aug 2011	Functional Genomics in Hematology,Hannover Medical school
Sep 2010	Gene regulation, cell differentiation and tolerance Hanover Biomedical research school, Germany
May 2007	Mutations in phospholipid hydroperoxide glutathione peroxidase (PHGPX) gene in Iranian infertile men. *2th international Student award of Reproduction Medicine*

Laboratory skills:

• Magnetic Sorting and FACS analysis
• Immunostaining, Chromatin IP, ELISA and Western Blotting
• PCR, Real time PCR, RNA & DNA isolation, transformation of E.*coli*, cell
  transformatin
• Confocal and Fluorescence Microscopy
• Protein purification
• Animal work

Publications

.Urokinase receptor mediates doxorubicin vascular smooth muscle cells senescence via proteasomal degradation of TRF2. Journal of vascular biology, 2013;50:109-123. *Mahshid Hodjat, Herman Haller, Inna Dumler, Yulia Kiyan*

.Relationship between sperm chromatin status and ICSI outcome in men with obstructive azoospermia and unexplained infertile normozoospermia. Rom J Morphol .Embryol. 2011;52(2):645-51 *Sadeghi MR, Lakpour N, Heidari-Vala H, Hodjat M, Amirjannati N, Hossaini Jadda H, Binaafar S, Akhondi M*

.GSTM1 and GSTP1 polymorphisms and glutathione S-transferase activity:Iranian infertile men. Tehran University Medical Journal. 2009; 66(12): 878-887, *Mirfeizollahi A, Farivar Sh, Akhondi M.M, Modarresi M.H, Hodjat M, Sadeghi M.R.*

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.Sperm ubiquitination correlates with human semen quality. Iranian Journal of public Health.2008; 37(2):32-40. *Hodjat M., Talebian A., Akhondi M.M., Zeraai H., Shabani A. and Sadeghi M.R.*

Extra Curriculum

. Executive secretary of 1st and 2nd sperm Functional test workshop, Avicenna Research Institute, 23 Feb 2006/ 2007, Tehran, Iran.

.Training technical staff and students. 2005-2009. *Assistant researcher, Avicenna Research Institute*

Appendix

Declaration for dissertation / Erklärung zur Dissertation

I hereby declare that I have made my dissertation entitled:

"Urokinase receptor in regulation of cellular senescence and DNA damage response: role for the ubiquitin-proteasomal system "

independently, and used only the specified tools and resources. For all used information the source was indicated. This work has not been in the same or similar form submitted as master thesis, diploma or any other degree in any other institution.

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel

"Urokinase receptor in regulation of cellular senescence and DNA damage response: role for the ubiquitin-proteasomal system"

selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die

zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere

Prüfungsarbeit verwendet.