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miR-663 and its putative target PP5 are two regulative elements in stress response and cell cycle control of human dermal fibroblasts

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Abstract

Nowadays non coding RNAs such as micro RNAs (miRs) are believed to play a vital role in the regulation of cellular processes. In previous experiments we found miR-663, a miR which has tumor suppressive properties and has been linked to the development of various tumor types, to be differentially expressed in senescent cells and cells suffering from cellular stress. In this work we demonstrate that miR-663 has an anti-proliferative effect in human dermal fibroblasts (HDF) and is necessary for a well-regulated response to cellular stress. We found many indications for a link between miR-663 and its putative target PP5, a phosphatase with very unique features which is highly conserved throughout the eukaryotic kingdom. Although up to now not much was known about the detailed functions of PP5, we could discover a very interesting, possibly p53 dependent mechanism in the regulation of the cellular response to oxidative stress which involves PP5 and its localization in the cell. This mechanism not only explains the unusual low basal enzymatic activity of the full length PP5 protein but also addresses the much disputed intracellular localization of PP5 and its isoforms. Theese findings describe a new miR mediated stress response pathway which controls the cell cycle of human dermal fibroblasts.

Kurzfassung

Heutzutage wird davon ausgegangen, dass nicht kodierende RNAs wie zum Beispiel micro RNAs (miRs) ein wichtige Rolle bei der Regulation von zellulären Prozessen spielen. In früheren Experimenten konnten wir zeigen, dass miR-663, eine miR welche Tumor unterdrückende Eigenschaften besitzt, unterschiedlich stark expremiert wird in seneszenten Zellen und in Zellen die zellulärem Stress ausgesetzt sind. In dieser Arbeit zeigen wir, dass miR-663 einen Wachstums hemmenden Effekt auf menschliche dermale Fibroblasten hat und notwendig ist für eine gut regulierte Antwort auf zellulärem Stress. Wir fanden viele Anzeichen für einen Zusammenhang zwischen miR-663 und seinem möglichen Ziel PP5, eine Phosphatase mit einzigartigen Merkmalen die hoch konserviert über das gesamte Reich der Eukaryonten ist. Obwohl bislang nicht viel über die genauen Funktionen von PP5 bekannt war, konnten wir einen sehr interessanten, möglicherweise p53 abhängigen Regulationsmechanismus in der zellulären Antwort auf oxidativem Stress entdecken, bei welchem PP5 und seine Lokalisation innerhalb der Zelle eine wichtige Rolle spielt. Dieser Mechanismus erklärt nicht nur die ungewöhnlich niedrige enzymatische Basalaktivität des vollständigen PP5 Proteins, sondern befasst sich auch mit der umstrittenen intrazellulären Lokalisation von PP5 und seinen Isoformen. Diese Erkenntnisse beschreiben eine neue miR mediierte Stressantwort, welche den Zellzyklus von humanen dermalen Fibroblasten kontrolliert.

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1. Introduction

1.1. Micro RNAs are a novel group of regulative molecules

One of the major research fields in cell biology is the cell cycle of human cells and its regulation. The cell cycle needs not only to be controlled to assure a correct proliferation process, but also to make an efficient repair of DNA damage possible and to be able to induce replicative or premature senescence in order to lower the risk of tumor development in old and damaged cells. Replicative senescence occurs in cell culture when cells loose their replicative potential after a certain number of population doublings and results in an irreversible growth arrest. The phenotype of replicative senescent cells includes critically shortened telomeres, a senescence associated β-galactosidase activity and the alteration of many genes including cyclin dependent kinase inhibitors. This phenotype can be artificially recreated by exposing cells to long-term subcytotoxic stress using stressing agents like H_2O_2, t-BHP, ethanol or UV-radiation and is named stress induced premature senescence (SIPS). SIPS are a valuable tool for getting a better understanding of the mechanisms behind cellular aging and the regulation of the involved genes and proteins.

Only recently a novel group of small RNA molecules, the micro RNAs (miRs), were identified and are now believed to play a vital role in the posttranscriptional regulation of proteins. The precursor molecules of miRs, called pri-miRs, are usually polymerase II transcripts of independent miR genes or portions of protein-coding introns. pri-miRs have a hairpin structure and can contain sequences for several different miRs. In mammals, an enzyme complex consisting of the RNAse III type endonuclease Drosha (also known as RN3) and the dsRNA-binding domain containing protein DGCR8 (DiGeorge syndrome critical region gene 8) processes the pri-miRs into approx. 70 nucleotides long pre-miR hairpins (Bushati et al. 2007). After being exported out of the nucleus via exportin5, the pre-miRs get cleaved into approx. 21 bp long miR duplexes by the endoribonuclease Dicer which is complexed with the TAR RNA binding protein (TRBP). This miR duplexes are similar to small interfering RNAs (siRNAs) acting in the RNA interference (RNAi). Usually only one strand in rare occasions both strands of the miR duplex are forming ribonucleoprotein complexes (miRNPs) or miR induced silencing complexes (miRISCs), the unused strand gets degraded (Rana TM. 2007). The most important proteins in these complexes are proteins of the Argonaute family (AGO1 to AGO4). AGO2 is the only one of the family which also functions in RNAi due to its ability to cleave mRNA at the center of the siRNA-mRNA duplex with its RNaseH-like P-element induced wimpy testis (PIWI) domain. Other proteins in the miRNPs function as assembly or regulatory factors of the complex or mediate the repressive miRNP functions as effectors (Peters et al. 2007).

In contrast to plants where the miRs base pair to mRNAs with almost perfect complementarity and thereby trigger an RNAi-like mechanism, the miRs in humans pair imperfectly with their targets. The most important criteria for the interaction between miR and mRNA is a continuous and perfect base pairing of the miR nucleotides 2-8 which is referred to as "seed region" (Figure 1). The binding sites for the seed region of the miR usually reside in the 3'UTR of the mRNA and are present in multiple copies. In rare occasions the binding sites lie in the 5'UTR or in coding regions of the mRNA (Doench et al. 2004).

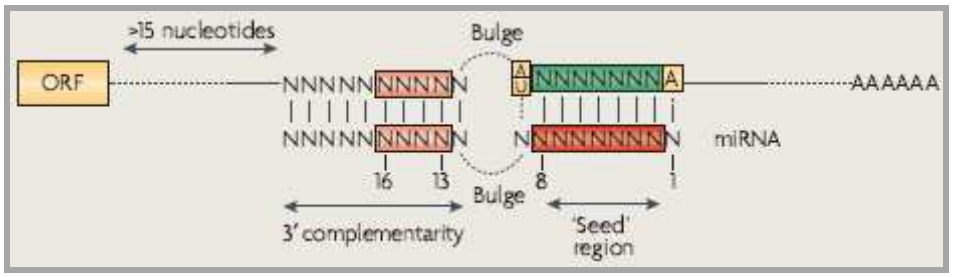


Figure 1: Principles of microRNA-mRNA interactions (Filipowicz et al. 2008)

In independent experiments it was proven that the m^7G cap is essential for the translational repression by miRs and that mRNAs containing an internal ribosome entry site (IRES) or a non-functional ApppN cap are not affected (Pillai et al. 2005). It was discovered that the central domain of AGO proteins contains limited sequence homology to the cap-binding region of eIF4E and that mutations in the amino acid sequence of AGO2 prevents it from binding to m^7GTP-Sepharose and eliminates the translational repression. The competition of AGO proteins with eIF4E for the binding sites at the m^7G cap would interfere with the eIF4E-eIF4G interaction and thereby prevent the assembly of the 40S initiation complex. The relatively low affinity of AGO for m^7G, when compared to eIF4E, is a possibly explanation for the need of multiple miRNPs for a robust repression of mRNAs (Kiriakidou et al. 2007). There are also other theories supported by various experiments that link the translational repression of miRs to the joining of the 60S subunit or to the modulation of a post-initiation step like the elongation or termination process. Among the different research groups it is still being argued about the molecular mechanisms behind the translational repression through miRs and if it occurs at the initiation or at a post-initiation step (Filipowicz et al. 2008).

In order to identify miRs that are differentially expressed in stressed, old or cells of a different cell type, microarrays are today the high-throughput method of choice. If an up- or downregulated miR is found, its putative targets are identified and given a score using specialized statistical software like TargetScan (provided by MIT), which compares the seed regions of the miRs with a databank of human mRNAs. This will deliver a list of putative targets and a respective score for each target representing its likelihood to interact with the miR. To verify the interaction of a miR and its putative target experiments such as a luciferase assay must be performed.

1.2. PUVA treatment induces reversible growth arrest

Photochemotherapy using psoralen plus UVA (PUVA) is a common treatment against the skin disorders psoriasis, T-cell lymphoma and other inflammatory skin disorders. Psoralen is usually administered orally to the patient and 1-2 h later the desired area of the skin is exposed to UVA-radiation (320-400 nm). The radiation gets absorbed by psoralen and leads to the formation of ROS (reactive oxygen species) and DNA-adducts. Hence cells which underwent PUVA treatment exhibit ICL (interstrand crosslinks) and DNA fragmentation. The damage to the DNA results in a reversible growth arrest of the cells, which is accompanied by an upregulation of $p21^{WAF1/Cip1}$ (CDKN1A), a well known cell cycle regulating protein (Ma, Wlaschek et al. 2002). The fact that PUVA treatment of human fibroblasts resulted in non permanent SIPS (stress induced premature senescence) was a very promising opportunity for us to learn more about the DNA damage repair of these cells and its regulation. One of the side effects of PUVA treatment is the excessive aging of the skin in the treated areas, linking the cellular response to this treatment directly to the aging process. Thus we decided to identify the miRs which are involved in the regulation of this process by performing a microarray with samples of young, senescent and PUVA-treated HDF (human diploid fibroblast) cells (microarray was performed by Harald Kühnel).

1.3. miR-663 is a regulative element in cancer development and cell cycle control of human cells

Among the miRs which were differentially regulated miR-663 (hsa-mir-663a) was found to be upregulated in PUVA treated cells and in senescent cells when compared to the untreated young cells. This was very promising, considering that miR-663 has already been shown to be upregulated in replicative senescence of human fibroblasts (Maes et al. 2009; Marasa et al. 2010) and the development of various tumor types. The gene of miR-663 was found to be hypermethylated and thereby inactivated in human hepatocellular carcinoma (Potapova et al. 2011) and breast cancer cells (Lehmann et al. 2008). 4-hydroxynonenal (HNE), an important second messenger with anti-proliferative and differentiative properties against various tumor cell lines, upregulates miR-663 in human leukemic HL-60 cells (Pizzimenti et al. 2009). In human gastric cancer cells miR-663 is downregulated and causes mitotic catastrophe growth arrest when transfected into the cells. It could even been demonstrated that miR-663 suppresses the growth of gastric cancer cells in vivo (Pan et al. 2010). Although in most publications miR-663 was shown to have tumor suppressive properties, two publications state that miR-663 acts as an oncogene by contributing to the proliferation of

lung cancer cells (Zhi-Yong et al. 2011) and nasopharyngeal carcinoma cells (Yi et al. 2012). Resveratrol is a natural antioxidant with cardiovascular and cancer preventive properties and decreases activator protein-1 (AP-1) activity by upregulating miR-663, which targets JunB and JunD (Tili et al. 2010). All these findings implicate that miR-663 plays an important role in the control of the cell cycle of various human cell types and make it a promising candidate for further studies.

1.4. The protein phosphatase 5 is a predicted target of miR-663

1.4.1. Structure and properties of protein phosphatase 5

By using TargetScan the protein phosphatase 5 (PP5) was predicted by Matthias Wieser as a target of miR-663 with 3 putative binding sites. PP5 is a member of the PPP family of protein serine/threonine phosphatases and is present in all eukaryotic cells examined from yeast to humans. In contrast to the other members of this phosphatase family, PP5 contains the catalytic, regulatory and subcellular targeting functions within a single polypeptide chain. PP5 can be detected in the nucleus as well as in the cytoplasm (Chinkers 2001). The C-terminus of PP5 contains a nuclear localization signal (NLS) which is essential for its translocation into the nucleus (Borthwick et al. 2001). The mechanism behind it is still unknown. Another curious fact about PP5 is its very low basal catalytic activity, considering that phophatases normally posses high turnover numbers. This low activity of PP5 arises from the auto inhibitory properties of its C-terminus and its N-terminal tetratricopeptide (TPR) region - a unique feature in the family of phophatases - which are shielding the catalytically active site of the protein. PP5 can be activated by proteolysis of the two regions or by binding of polyunsaturated fatty acids to the TPR region of PP5 (Chen et al. 1997; Sinclair et al. 1999; Kang et al. 2001).

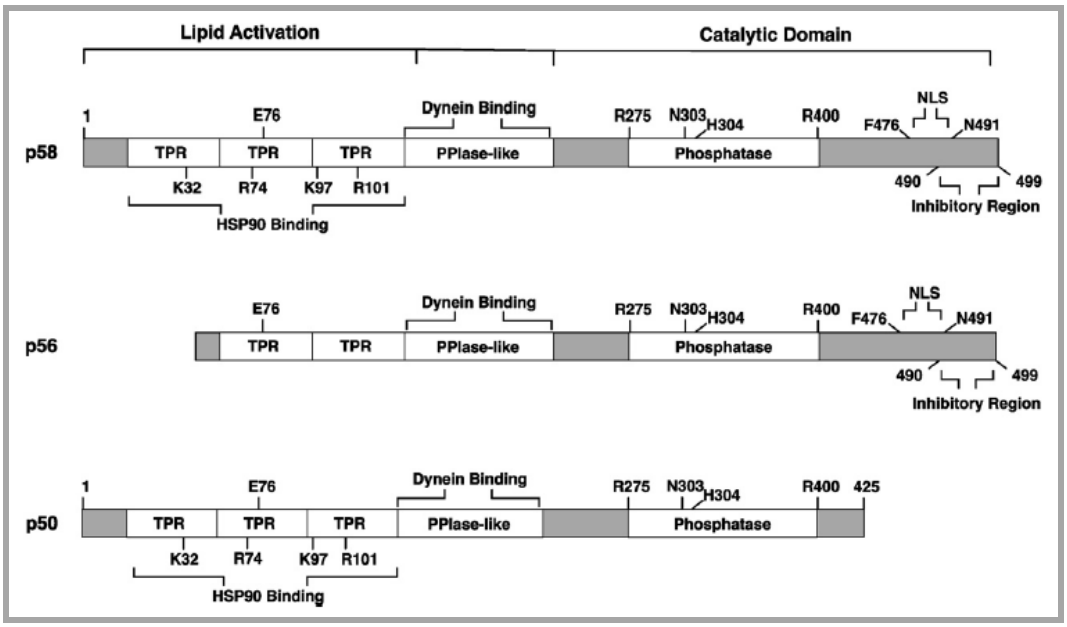


Figure 2: Structure of PP5 and isoforms of PP5 resulting from proteolysis (Hinds et al. 2007)

Various isoforms of PP5 were discovered ranging from approx. 50 kD to 130 kD, which were binding to at least one antibody specific to PP5. These isoforms were presumably resulting from proteolytic cleavage of PP5 and the subsequent formation of multimers or stable complexes with other proteins. The multimers did not occur with the full length protein and were only observed under non-reducing conditions, making it likely that the multimers are formed by the linkage between some of the Cys residues in the TPR domain (Zeke et al. 2005).

1.4.2. PP5 is a key regulator in the control of the cell cycle and DNA damage response

PP5 has already been identified as a key regulative element in cellular stress response, cell cycle control and is believed to play an important role in the development of various cancer types. The apoptosis signal-regulating kinase 1 (ASK1) which resides in the cytoplasm gets directly de-phosphorylated and thereby inactivated by PP5 *in vitro* and *in vivo*. The interaction between the two proteins was induced by H_2O_2-treatment and led to the inhibition of H_2O_2-induced sustained activation of ASK1 and ASK1-dependent apoptosis (Morita et al. 2001). PP5 is required for DNA-damage-induced ataxia telangiectasia mutated (ATM) activation by autophosphorylation of ATM on Ser 1981 and therefore for the phosphorylation of various ATM substrates like Rad17, Chk2, p53, BRCA1 and NBS1. DNA-damaged cells lacking active PP5 experience an S-phase checkpoint defect (Ali et al. 2004). PP5-deficient mice are defective in ATM-mediated cell cycle arrest at the G_2/M checkpoint in response to DNA damage caused by ionizing radiation (Yong et al. 2007). This relationship between PP5 and ATM is somewhat surprising as PP5 is normally a negative regulator of growth arrest. Suppression of PP5 expression induced the glucocorticoid receptor (GR)

transcriptional activity without the addition of hormone and increased dexamethasone-mediated induction of GR reporter activity. Dexamethasone, an inducer of GR-mediated growth arrest, leads to growth arrest by blocking the progression through the G_1/S-phase transition. This is achieved by an upregulation of $p21^{WAF1/Cip1}$ following a phosphorylation-dependent activation of p53, which implies that $p21^{WAF1/Cip1}$ and p53 may be a part of the GR-induced signaling network (Zuo et al. 1999). Furthermore it was shown that PP5-inhibition alone leads to an induction of $p21^{WAF1/Cip1}$ and subsequently to a growth arrest dependent on the phosphorylation of p53. It was also observed that recombinant PP5 can de-phosporylate p53 *in vitro* (Zuo et al. 1998). Keeping in mind that p53 is already identified as a very potent tumor suppressor and $p21^{WAF1/Cip1}$ is known to be a major cell cycle regulating protein affecting the G_1/S-phase transition, these studies prompted us to investigate the role of PP5 and its putative regulator miR-663 in the DNA damage response and cell cycle control of human cells.

2. Materials and Methods

2.1. Cell culture

For our experiments we used HDF5 cells, a human dermal fibroblast cell line isolated and established by Regina Grillari (Voglauer R. 2000). Additionally we used HEK-293 cells and NIH-3T3 cells for the luciferase reporter assay. HEK-293 (human embryonic kidney) cells are tumorigenic epithelial cells, which contain DNA of the Adenovirus 5. NIH-3T3 (NIH, 3-day transfer, inoculum 3 x 10^5 cells) cells are embryonic mouse fibroblasts derived from NIH-Swiss mice. All procedures involving mammalian cells were performed in a laminar flow hood except for centrifugation steps and all steps for isolating RNA or protein. The cells were cultured in an incubator at 37 °C and 5 % CO_2. HDF cells were cultured with a mixture of DMEM and HAM's F12 media at a ratio of 1:1 containing 10 % FCS and 4 mM L-glutamine. HEK-293 and NIH-3T3 cells were cultured with DMEM media containing 10 % FCS and 4 mM L-glutamine. The culture vessels for the NIH-3T3 cells were coated with 1 % gelatine in PBS for at least 10 min and washed with 1x PBS before seeding the cells. All adherent cells were passaged by washing the cells two times with 1x PBS, incubating the cells with 0.5-1 ml of 0.01 % Trypsin at 37 °C for approx. 3-5 min and resuspending them in an adequate volume of culture media. The cells were then centrifuged at 200 g for 5 min and the cell pellet was resuspended in the desired volume and the cells were seeded in the desired culture vessel. The ratio and the frequency of the passages were dependent on the cell type and the age of the cells.

2.1.1. Determination of cell concentration

Cell concentrations of cell suspensions were determined using a Bürker-Türk chamber. 500 µl of the respective cell suspension were mixed with 100 µl 0.5 % Trypan blue solution and applied onto the chamber. The cells in the marked areas of both chamber grids (a total of 10) were counted and the cell concentration was calculated using the following equation:

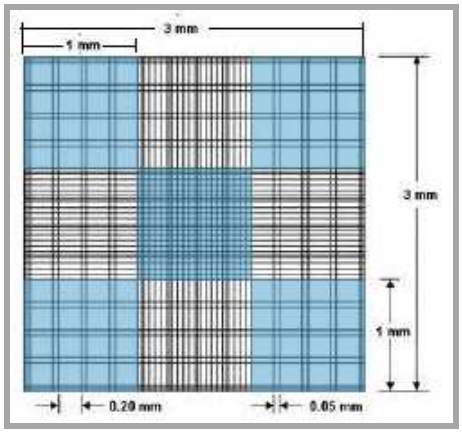


Figure 3: Bürker-Türk chamber

cell number/ml = counts x 1200

2.1.2. Transfections

2.1.2.1. Transfection with miR-663/miRctrl and miR-663 Power Inhibitor/Power Inhibitor Control

For the manipulation of miR-663 levels, miR-663/miRctrl (Ambion) and miR-663 Power Inhibitor/Power Inhibitor Control (Exiquon) together with the siPORT™ NeoFX™ Transfection Agent (Ambion) was used. The transfections were performed either in 6-well culture plates or in 96-well plates for the MTT-assay.

6-well

The cells were diluted to a concentration of 2 x 10^5 cells per 2.3 ml growth media. In the meantime, the transfection mixes were prepared as follows:

per well: 5 µl transfection agent + 95 µl Opti-MEM® (Life Technologies)

7.5 µl miR-663/miRctrl (10 µM) + 92.5 µl Opti-MEM

or 1.5 µl miR-663 Power Inhibitor/Power Inhibitor Control (50 µM) + 98.5 µl Opti-MEM

Both dilutions were incubated at room temperature (RT) for 10 min, the RNA dilutions were each transferred to a microcentrifuge tube containing an equivalent volume of transfection agent dilution and mixed carefully by pipetting the mixture up and down for 3 times. After incubation at RT for 10 min, the transfection mixes (200 µl/well) were pipetted into the 6-well culture plates and 2.3 ml of the cell suspension was added to each well.

96-well

The cells were diluted to a concentration of 3400 cells per 200 µl growth media. In the meantime, the transfection mixes were prepared as follows:

per well: 0.5 µl transfection agent + 9.5 µl Opti-MEM

1.5 µl miR-663 Power Inhibitor/Power Inhibitor Control (50 µM) + 98.5 µl Opti-MEM

Both dilutions were incubated at RT for 10 min, the RNA dilutions were each transferred to a microcentrifuge tube containing an equivalent volume of transfection agent dilution and mixed carefully by pipetting the mixture up and down for 3 times. After incubation at RT for 10 min, the transfection mixes (20 µl/well) were pipetted into the 6-well culture plates and 200 µl of the cell suspension was added to each well.

2.1.2.2. Transfection with siRNA-PP5/siRNA-ctrl

For the manipulation of PP5 levels, ON-TARGETplus siRNA-PP5/siRNA-ctrl (Dharmacon) together with the DharmaFECT® transfection agent (Thermo Scientific) were used. The cells were seeded in 6-well culture plates at a density of 2 x 10^5 cells/cm^2. On the next day, the transfection mixes were prepared as follows:

per well: 5 µl transfection agent + 195 µl Opti-MEM

2.5 µl siRNA-PP5/siRNA-ctrl (20 µM) + 197.5 µl Opti-MEM

→incubation at RT for 5 min

→RNA dilutions were each pipetted carefully into a microcentrifuge tube containing the equivalent volume of transfection agent dilution and mixed by carefully pipetting the mixture 3 times up and down

→incubation at RT for 20 min

→the growth media of the cells was substituted with 2.6 ml/well fresh growth media

→400 µl/well of the respective transfection mix was added to the cells

2.1.2.3. Plasmid transfections

Cells were transfected with plasmids using the jetPRIME® reagent (Polyplus Transfection). Transfections were performed in either 6-well or 12-well culture plates.

6-well

2 µg plasmid were diluted with 200 µl jetPRIME® buffer (Polyplus Transfection) in 1.5 ml Sarstedt tubes, vortexed and spun down. Transfection control was transfected with 200 µl jetPRIME® buffer only. 4 µl jetPRIME® reagent were added, the tubes were vortexed, spun down and incubated at RT for 10 min. The media of the cells was substituted with 2 ml fresh growth media and the transfection mixes were added drop wise to the cells. 6 h post transfection the media was substituted with 2 ml fresh growth media per well.

12-well

0.75 µg plasmid were diluted with 75 µl jetPRIME® buffer in 1.5 ml Sarstedt tubes, vortexed and spun down. 1.5 µl jetPRIME® reagent were added, the tubes were vortexed, spun down and incubated at RT for 10 min. The media of the cells was substituted with 1 ml fresh growth media and the transfection mixes were added drop wise to the cells. 6 h post transfection the media was substituted with 1 ml fresh growth media per well.

2.1.3. H_2O_2-treatment

The HDF cells were treated with 100 µM H_2O_2 for 1 h to induce the DNA damage repair mechanisms of the cells. The cells were seeded at a density of 10^4 cells/cm^2 and treated on the following day. The growth media was removed and cells were treated with media containing 100 µM H_2O_2 in parallel with cells cultured with normal media. After one hour the media of all cells was removed and substituted with normal growth media. Following the treatment, the cells were incubated at 37°C and 5 % CO2 and analyzed after indicated recovery times. For the analysis of protein or RNA, the cells were harvested and the cell pellet was resuspended in 1 ml ice-cold 1x PBS, transferred to a clean 1.5 ml Sarstedt tube and centrifuged for 10 min at 1000 g and 4 °C. The supernatant was discarded and protein or RNA was isolated from the pellet.

2.2. RNA analysis

The effect of oxidative stress on HDF5 cells on RNA level was studied by first stressing the cells with H_2O_2, then isolating the RNA and transcribing it into cDNA, which was subsequently quantified by qPCR (Real-Time quantitative PCR).

2.2.1. Isolation of the RNA

After the treatment with H_2O_2, the pellets were re-suspended in 500 µl TRIzol® (Life Technologies), mixed thoroughly, incubated at RT for 5 min and stored at –80 °C. To isolate the RNA, 100 µl of chloroform were added to each tube and after mixing them thoroughly they were incubated at RT for 5 min. By centrifuging the tubes at 12 000 g and 4 °C for 15 min the aqueous phase containing the RNA got separated from the organic phase. The aqueous phase was carefully transferred to a new 1.5 ml Sarstedt tube and the RNA was precipited by adding 500 µl isopropanol. The content of the tubes was mixed thoroughly, incubated for 10 min at RT and centrifuged at 12 000 g and 4 °C for 10 min. The RNA pellets were washed with 1 ml of 70 % ethanol and centrifuged at 7 500 g and 4 °C for 5 min. The RNA pellet was air dried and re-suspended with 20 µl NFW. The RNA was dissolved by incubating it at 55 °C for 10 min. The concentration was measured with a NanoDrop™ 1000 UV/Vis Spectrophotometer (Thermo Scientific) using the 260/280 nm ratio to measure the purity and the 260 nm absorbance to measure the DNA concentration. NFW was used as blank for the measurement. After isolation the RNA was stored at –80 °C.

2.2.2. Transcription into cDNA

For the quantification of the mRNA, the RNA samples were reverse transcribed into cDNA using the DyNAmo™ cDNA Synthesis Kit (Thermo Scientific). The reaction mix was prepared as follows:

RNA (200 ng)	7 µl
Random hexamers	1 µl
2x RT buffer	10 µl
M-MuLV RNase H^+ reverse transcriptase	2 µl
Total volume	20 µl

For each batch of cDNA synthesis, one reaction with NFW as template instead of RNA was performed as a control. The cDNA synthesis was performed using a T3 Thermocycler (Biometra) with the following parameters:

Step 1: 25 °C for 10 min

Step 2: 37 °C for 30 min

Step 3: 85 °C for 5 min

Step 4: cool to 4 °C

After the reverse transcription, each cDNA sample was diluted with 30 µl NFW.

For the quantification of miR-663 expression, RNA was reverse transcribed using the NCode™ miRNA First-Strand cDNA Synthesis and qRT-PCR Kit (Invitrogen). Prior to reverse transcription into cDNA, a poly-A-tail was added to the RNA. The reaction mixes were prepared as follows:

RNA (100 ng)	6.4 µl
5x miR Reaction buffer	2 µl
$MnCl_2$ (25 mM)	1 µl
ATP (200µM)	0.4 µl
Poly A polymerase	0.2 µl
Total volume	10 µl

→incubation at 37 °C for 15 min

Previous reaction mix	2 µl
Annealing buffer	0.5 µl
oligo dT universal RT primer (25 µM)	1.5 µl
Total volume	4 µl

→incubation at 65 °C for 5 min

→incubation on ice for 1 min

Previous reaction mix	4 µl
2x First strand reaction mix	5 µl
Superscript III RT	1 µl
Total volume	10 µl

→incubation at 50 °C for 50 min

→incubation at 85 °C for 5 min

After the transcription, each cDNA sample was diluted with 90 µl NFW. For each batch of cDNA synthesis, one reaction with NFW instead of RNA was performed as a control.

2.2.3. Quantitative real time PCR (qPCR)

For quantitation, the transcribed mRNA/miR was quantified using a Rotorgene-6000 Thermocycler (Qiagen) and the SensiMix plus SYBR (GenXpress) reaction mix. The mRNAs were quantified using a series of dilutions of standards, the miR-663 were quantified by calculating of the delta delta Ct values of miR-663 and a constitutive expressed control RNA.

For the normalization of the mRNA levels of PP5 and p21, the mRNA level of the housekeeping gene GAPDH was used. The standards were derived from a PCR reaction with the respective primers and were ranging from 10^8 to 10^3 copies. The standards were analyzed together with the samples and one control from the cDNA synthesis. The standards were analyzed in duplicates; the samples and the control were analyzed in quadruplicates. The reaction mixes were prepared as follows:

cDNA sample/standard/control	1 µl
Primer sense (PP5/p21/GAPDH)	0.25 µl
Respective primer antisense	0.25 µl
Sensi mix reaction buffer	5 µl
NFW	3.5 µl
Total volume	10 µl

For the normalization of the miR-663 level, the ribosomal S5 RNA was used. The level of the miR-663 was calculated using the delta delta Ct value of miR-663 and S5 RNA. The samples and the controls for miR-663 and S5 rRNA were prepared in quadruplicates. The reaction mixes were prepared as follows:

cDNA sample/standard/control	1 µl
Primer (miR-663/S5 rRNA)	0.25 µl
Universal qPCR Primer (10 µM)	0.25 µl
Sensi mix reaction buffer	5 µl
NFW	3.5 µl
Total volume	10 µl

The Universal qPCR Primer (10 µM) was included in the NCode™ miRNA First-Strand cDNA Synthesis and qRT-PCR Kit (Life Technologies).

2.3. Protein analysis

2.3.1. Analysis of protein levels

The effect of oxidative stress on HDF5 cells on the protein level and its mechanism was studied by preparing cell lysates and quantifying various proteins via SDS-PAGE and Western-blot. Prior to lysis the cells were stressed with H_2O_2 (see 2.1.3.) and/or their miR-663- and PP5-levels were manipulated via transfection (see 2.1.2.). Additionally to the protein levels, the localization of the proteins was investigated with immunofluorescence assays and fractionized lysis followed by SDS-PAGE and Western-blot.

2.3.1.1. Cell lysis

The cell pellets were re-suspended in 50 µl NEB buffer, transferred to a QIAshredder column (Qiagen) and centrifuged for 2 min at 4 °C and full speed. The collected flow through was applied onto the column for a second time and again centrifuged for 2 min at 4 °C and full speed. The flow through was now transferred to a clean 1.5 ml Sarstedt tube and stored at -80 °C.

For the analysis of $p53^{Ser15}$ a different lysis protocol was used:

After harvesting the cells, the cell suspensions were centrifuged for 10 min at 600 g. The pellets were re-suspended in 1 ml cold PBS, transferred to clean 1.5 ml Sarstedt tubes and centrifuged again for 10 min at 600 g and 4 °C. The supernatant was removed; the pellets were incubated in liquid nitrogen for 10 s and re-suspended in 50 µl TNE buffer. They were then incubated for 15 min on ice, shortly vortexed and centrifuged for 10 min at 6000 g and 4 °C. The supernatant was transferred to a QIAshredder column (Qiagen) and centrifuged for 2 min at 4 °C and full speed. The collected flow through was applied onto the column for a second time and again centrifuged for 2 min at 4 °C and full speed. The flow through was then transferred to a clean 1.5 ml Sarstedt tube and stored at -80 °C.

2.3.1.2. Quantification of protein content with BCA-assay

The total protein content of the protein samples was quantified using the Pierce® BCA Protein Assay Kit (Thermo Scientific). The assay was conducted according to the

manufacturers' instructions. The microplate procedure variant was performed and the protein samples were diluted 1:10 in NFW for the assay. In order to get reliable results, the standards were diluted in the corresponding buffer.

2.3.1.3. SDS-PAGE

To load similar amounts of protein, the samples were diluted in NFW according to their total protein concentration and after adding the respective amount of 4x SDS loading dye the samples were incubated for 10 min at 96 °C on a heating block. Protein samples were loaded in equal amounts for each gel, ranging from 30-50 µg of total protein per lane. The SDS-PAGE was performed with NuPAGE® 4-12 % Bis-Tris Gels (Life Technologies), NuPAGE® MOPS SDS Running Buffer (Life Technologies) and PageRuler™ Prestained Protein Ladder (Thermo Scientific) using a XCell SureLock™ Mini-Cell Electrophoresis chamber (Life Technologies). The electrophoresis was performed at 200 V for approximately 1 h.

2.3.1.4. Western blot

The transfer to a Roti®-PVDF membrane (Roth) was done using a XCell II™ Blot Module (Life Technologies) and transfer buffer. The transfer was performed at 30 V for approximately 75 min. The membrane was then incubated for at least 1 h in blocking solution containing milk powder. For the $p53^{Ser15}$ samples, TBS was used instead of PBS and the blocking solution contained BSA (bovine serum albumin) instead of milk powder. The antibodies were diluted in blocking solution as follows:

α-PP5 (BD Transduction Laboratories™/611021)	1:500
α-$p21^{WAF1}$ (US Biological/P1000-53)	1:1000
α-GAPDH (Santa Cruz Biotechnology/sc-25778)	1:5000
α-β-actin (Sigma-Aldrich/A5441)	1:5000
α-$p53^{Ser15}$ (Cell Signaling Technology/9284)	1:1000
α-Lamin A/C (Santa Cruz Biotechnology/sc-20681)	1:1000
goat α-mouse Alexa Fluor®680 (Life Technologies/A-21057)	1:5000
goat α-rabbit IRDye800® (Rockland/611-132-122)	1:5000

The membranes were incubated with the primary antibody solutions over night on a shaker at 4 °C in the dark. On the next day the membranes were incubated with a secondary antibody solution, according to the species of the primary antibody, for approximately 1 h at RT in a lightproof box. After each incubation step with antibody solutions the membranes were washed 3x 5 min with TPBS or TTBS. After the incubation with the secondary antibody

and the last washing step the membranes were left in PBS and analyzed on an Odyssey® infrared scanner (LI-COR). The protein bands were quantified by using the ImageJ program and normalizing the protein levels to the levels of the housekeeping proteins.

2.3.2. Analysis of the sub cellular protein localization

2.3.2.1. Fractionized cell lysis

The fractionized lysis was performed with the Qproteome® Cell Compartment Kit (Qiagen) according to the manufacturers' instructions. The assay was performed with only half of the recommended volume for the extraction buffers. For each sample 2 T25 tissue culture flasks containing HDF5 cells, which were passaged 1:4 on the day prior to the treatment, were used. The protein solutions of the different fractions (nucleus, cytoplasm, membrane, cytoskeleton) were stored at -20 °C. SDS-PAGE and Western blot were performed (see 2.3.1.3. and 2.3.1.4.), except that no BCA-assays were carried out since it was not feasible to prepare standards.

2.3.2.2. Immunofluorescence assay

For the visualization of the protein localization inside the intact cell an immunofluorescence assay was conducted. HDF5 cells were seeded at 10^4 cells/cm^2 in an 8 well µ-Slide (Ibidi) and treated with H_2O_2 (see 2.1.3.) on the following day. After the indicated recovery time in normal growth media, the cells were stained using the following steps:

→incubation with 4 % paraformaldehyde (100 µl/well) for 15 min at RT

→wash 3x for 5 min with PBS

→incubation with blocking buffer (200 µl/well) for 1 h at RT

→primary antibodies were diluted in dilution buffer and cells were incubated over night at 4 °C in the dark (100 µl/well); the ctrl with dilution buffer only

α-PP5 (BD Transduction Laboratories™/611021)	1:50
α-p53^{Ser15} (Cell Signaling Technology/9284)	1:100
α-γ-H2AX^{Ser139} (Cell Signaling Technology/9718)	1:100

→wash 3x for 5 min with PBS

→incubation with the respective secondary antibody diluted in dilution buffer at RT for 2 h in the dark

goat α-mouse Alexa Flour®594 (Life Technologies)	1:500
goat α-rabbit Dylight® 488 (Jackson Immunosearch)	1:800

→wash 3x for 5 min with PBS

→incubation with DAPI solution (0.1 µg/ml) at RT for 10 min in the dark

→wash 3x for 5 min with PBS

→store slides in PBS at 4 °C in the dark

→slides were analyzed with a Leica DMI 6000 CS fluorescence microscope

2.4. Cell growth and cell cycle analysis

2.4.1. MTT-assay

The goal of the MTT-assay was to obtain information about the impact of oxidative stress alone or coupled with the inhibition of miR-663 on the cellular proliferation of HDF5 cells. MTT is a yellow dye which gets reduced to insoluble formazan by a dehydrogenase enzyme inside the cells mitochondria. By measuring the formazan concentration with a photometer the proliferative activity of the cells can be determined.

2.4.1.1. MTT-assay with H_2O_2-treatment

Cells were seeded in 6 96-well plates (for each plate 20 wells) and treated with H_2O_2 (see 2.1.3.) on the next day in a volume of 200 µl/well. For each time point, one plate was treated with MTT after the desired recovery time was reached. Therefore 100 µl of culture media per well were removed, 10 µl/well MTT-solution were added and the cells were incubated for another 4 h. 100 µl of 10 % SDS solved in 0.01 M HCl were added to the wells and all the unused wells of the plate were filled with 200 µl PBS to compensate for evaporation effects. The plates were sealed with plastic foil and incubated for another 21 h. The absorption was measured at 570 and 690 nm with an Infinite® M200 (Tecan) microplate reader. The value for the reference wavelength of 690 nm was then subtracted from the absorption at 570 nm.

2.4.1.2. MTT-assay with H_2O_2-treatment and transfection of miR-663 Power Inhibitor/Power Inhibitor Control

In order to gain information about the influence of miR-663 on the cell growth of stressed and unstressed cells, the MTT-assay was coupled with a preceding transfection with miR-663 Power Inhibitor and Power Inhibitor Control (see 2.1.2.1.). The assay was deducted in the same way as the MTT-assay with the following changes:

Additionally to the six plates for the time points at 28, 31, 34, 37, 40 h recovery time, one plate was prepared to obtain a 0 h time point without the H_2O_2-treatment. Only half of the cells of that plate were transfected to gain additional information about the effect of the transfection on the cells. Immediately after all of the transfection mixes were distributed among the plates, the cells were seeded into the microtiter plates with a cell suspension volume of 80 µl instead of 200 µl to get a final volume of 100 µl/well. The wells of the plate

for the 0 h time point with no transfection mix were filled up with 20 µl normal growth media to adjust the volume to 100 µl/well. All unused wells of the plates were already being filled with 200 µl PBS at that early stage of the experiment to minimize the evaporation effects we still encountered in the previous assay. The cells were treated with H_2O_2 approx. 12 h post transfection (see 2.1.3.). The plate for the 0 h time point was treated with MTT 3 h after the H_2O_2-treatment of the other plates. The rest of the experiment followed exactly the protocol of the prior MTT-assay.

2.4.2. BrdU-assay

Another method we used to investigate the proliferation rate and the effects of miR-663 on the cell cycle of HDF5 cells was to first transfect cells with miR-663 and ON-TARGETplus siRNA-PP5 (Dharmacon) and their corresponding controls (see 2.1.2.). With the transfected cells a BrdU-assay was then performed and the number of BrdU-positive cells, and thereby the number of replicating cells, were determined by using flow cytometry analysis.

The cells of both transfections were passaged 1:2 24 h afterwards. 6 h after the passage, the growth media was substituted with BrdU containing (10 µM) media. After another 16 h, the cells were harvested and fixed by adding drop wise 70% ethanol, which was tempered at -20 °C, while gently vortexing the cells. Cells were incubated at 4 °C for 30 min and pelleted. 1 ml of 1 % Triton X-100 in 2 M HCl was added dropwise to the cells, while they were gently vortexed. For the denaturation of the DNA, the cells were incubated 30 min at RT and pelleted. The cells were resuspended in 1 ml of 0.1 M Na-Borat (pH 8.5) for neutralization. They were again pelleted and resuspended in primary antibody solution and incubated for 30 min at RT.

1. Antibody solution: 300 µl BTPBS + 6 µl mouse α-BrdU (BD Biosciences/347580)

Cells were washed in BTPBS, pelleted, resuspended in secondary antibody solution and incubated 30 min at RT.

2. Antibody solution: 200 µl BTPBS + 2 µl FITC-conjugated goat α-mouse (Sigma F8264)

Cells were washed in PBS, pelleted, resuspended in propidium iodide (PI) solution and transferred into FACS-tubes.

Propidium iodide solution: 200 µl PBS + 0.2 µl PI (2.5 mg/ml)

The stained cells were stored at 4 °C before analyzing them with a Calibur flow cytometer (Becton Dickinson).

2.5. Luciferase reporter assay

To verify the direct interaction of miR-663 with the predicted target sites in the 3'-UTR of the PP5-mRNA a luciferase reporter assay was performed using designed oligos containing one of the putative target sequences of the miR-663 or their mutated counterparts which were cloned into the psiCHECK^{TM}-2 vector (Promega).

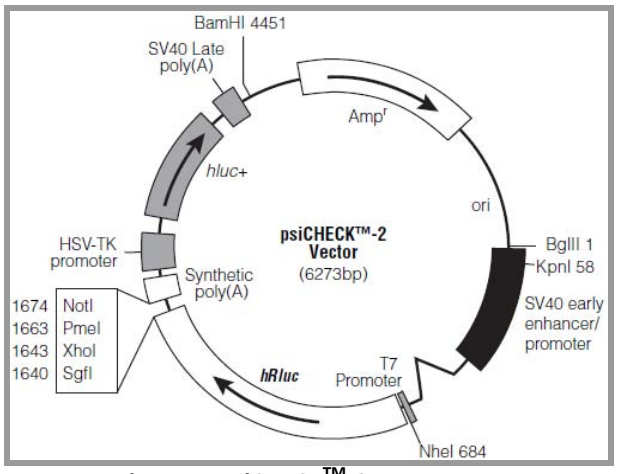


Figure 4: psiCHECK^{TM}-2 vector map

The plasmid contains a synthetic version of the *Renilla* luciferase gen (hRluc) with a multiple cloning site (MCS) in the 3'-UTR of the gen, so that the target sequences of the miR-663 could be fused to the luciferase gen, thereby putting the expression of the luciferase reporter gen under the control of miR-663. Additionally the plasmid contains another reporter gene, the firefly luciferase gen, which is not under the control of miR-663 and serves as a normalization signal between the different experiments. For the replication in bacterial cells, the plasmid has a bacterial origin and ampicillin resistance gen. The reporter constructs were transfected into HDF5 cells which were previously transfected transiently with miR-663/miRctrl or into HEK-293 cells which were expressing miR-663/miR-ctrl in a stable manner. To express miR-663 in a stable manner within HEK-293 cells, ssDNA oligos of miR-663 were designed, synthesized and cloned into a pcDNA™6.2-GW/EmGFP-miR plasmid (Life Technologies).

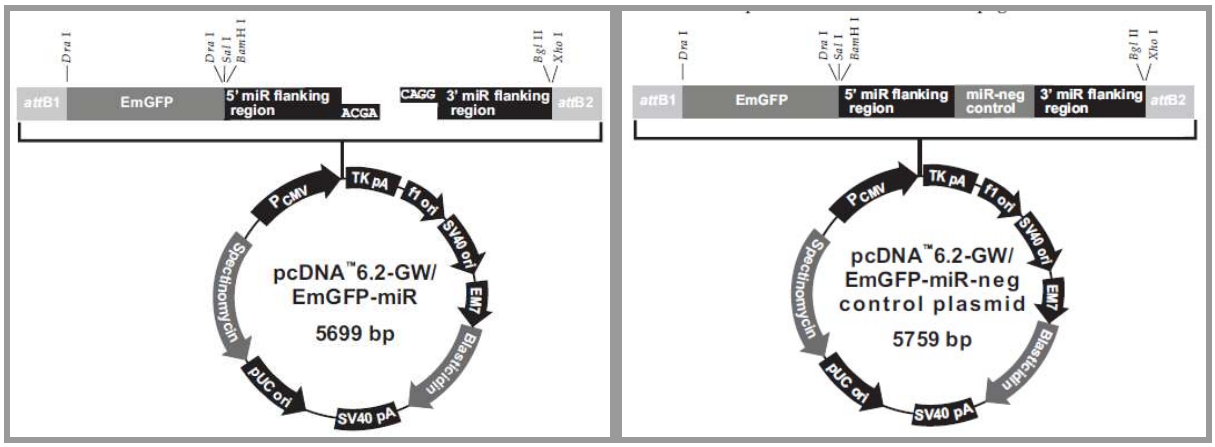


Figure 5: Vector maps of pcDNA™6.2-GW/EmGFP-miR and pcDNA™6.2-GW/EmGFP-miR-neg control

The pcDNA™6.2-GW/EmGFP-miR-neg control plasmid was used as a negative control in the luciferase experiment. Both plasmids contain a functional GFP-gen and blasticidin resistance cassette for selection and monitoring after the transfection and miR flanking regions for an optimized expression of the miR-663/miRctrl. The transfected cells were then used to conduct the reporter assay using the Dual-Glo® Luciferase Assay System (Promega).

2.5.1. Design of the PP5-mRNA 3'-UTR oligos

The oligos, each containing one of the 3'-UTR sequences of the PP5-mRNA which was identified as a putative target sequence of the miR-663 and their mutated counterparts for cloning of the reporter constructs were designed by Matthias Wieser and ordered at Integrated DNA technologies (see 2.6.2.2.). They were delivered in a lipophylized state and were solved in nuclease free water (NFW) according to their molecular mass to obtain a 200 µM solution of ssDNA and then stored at -20°C.

2.5.2. Annealing of the oligos

The annealing of the oligos was performed using the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Life Technologies) according to the manufacturers' instructions. The oligos were annealed by heating them up to 95 °C and letting them cool down for 10 min at RT.

1611

```
TOP      tcgagactagtCCCAGGCCCTGGGCTAGGGGCAGAGCAGGCCCCGCCCCAGGGCAATGTTGGAgc
BOTTOM       ctgatcaGGGTCCGGGACCCGATCCCCGTCTCGTCCGGGGCGGGGTCCCGTTACAACCTcgccgg
```

1611mut

```
TOP      tcgagactagtCCCAGGCCCTGGGCTAGGGGCAGAGCAGGCCTCTCCCCAGGGCAATGTTGGAgc
BOTTOM       ctgatcaGGGTCCGGGACCCGATCCCCGTCTCGTCCGGAGAGGGGTCCCGTTACAACCTcgccgg
```

Figure 6: Annealed PP5-mRNA 3'-UTR oligo and its mutated version; target sequence for miR-663 (green); mutated base pairs (pink); NotI overhang (blue); XhoI overhang (yellow)

2.5.3. Preparation of the vector

The psiCHECK™-2 vector was digested using the FastDigest® (Thermo Scientific) restriction enzymes XhoI and NotI and the 10x FastDigest® buffer (Thermo Scientific). The vector was diluted with NFW to 0.25 µg/µl and the reaction mix was prepared as follows:

FastDigest® XhoI	2 µl
FastDigest® NotI	2 µl
10x FastDigest® buffer	2 µl
psiCHECK™-2 (0.25 µg/µl)	2 µl
NFW	12 µl
Total volume	20 µl

→reaction mix was incubated at 37°C for 15 min on a heating block

→enzymes were inactivated by incubation at 80°C for 5 min on a heating block

The linearized vector was separated by electrophoresis on a 1 % agarose gel (2 h at 90 V) and the band containing the vector was cut out and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturers' instructions. The purified vector was eluted in 50 µl NFW and its concentration was measured (see 2.2.1.). The linearized and purified psiCHECK™-2 vector was diluted with NFW to a concentration of 30 ng/µl.

2.5.4. Ligation of vector and insert

The dsDNA oligos were ligated into the linearized psiCHECK™-2 vector using T4 DNA ligase and 10x T4 ligase buffer (New England Biolabs). The amount of used vector and insert was calculated on the basis of a molar ratio between insert and vector of 5:1 and a desired amount of 60 ng of vector for the ligation. The reaction mix was prepared as follows:

T4 DNA ligase	2 µl
10x T4 ligase buffer	2 µl
ds oligo insert (10 nM)	8.4 µl
psiCHECK™-2 (30 ng/µl)	2 µl
NFW	5.6 µl
Total volume	20 µl

→reaction mix was incubated at RT for 2 h

2.5.5. Transformation of *E. coli* TOP 10 with the ligated vector

For the amplification of the reporter constructs One Shot® TOP10 Chemically Competent *E. coli* cells (Life Technologies) were transformed with the ligated vectors. As negative controls, one ligation without insert and one transformation without plasmid were performed. 5 µl of each ligation was pipetted to 50 µl of bacterial cell suspension and incubated on ice for 5 min. The cells were incubated in a water bath at 42°C for 30-40 s and immediately cooled on ice for 5 min. 250 µl of SOC-media (RT) were added to each reaction and the cells were incubated on a heating block at 37°C and 1200 rpm for 1 h. The cell suspensions were platted on LB-plates containing 100 µg/ml ampicillin and incubated over night at 37°C. On the following day the plates were checked for colonies and if possible 10 colonies of each plate were chosen for further cultivation. A pipette tip was used to inoculate a culture vessel filled with 3 ml liquid autoclaved LB-media containing 100 µg/ml ampicillin. The liquid cultures were incubated over night on a shaker at 37°C.

2.5.6. PCR colony screening

To verify the successful incorporation of the insert into the vector, a PCR screening of the colonies, chosen for the cultivation in liquid media, was conducted in parallel. The pipette tips used for the inoculation of the liquid media were each dipped into a PCR-tube containing PCR-screening mix before being dumped into the liquid media. The PCR-screening mix contained two primers (see 2.6.2.3.), binding left and right of the vectors multiple cloning site, leading to a PCR product approx. 50 bp larger if the ligation was successful. The PCR-screening mix was prepared as follows:

DNTPs (10 mM)	0.4 µl
Primer psiCHECKseq sense (10 µM)	0.4 µl
Primer psiCHECKseq antisense (10 µM)	0.4 µl
10x Polymerase buffer (Biotherm)	2 µl
DNA Polymerase (Biotherm)	0.1 µl
NFW	16.7 µl
Total volume	20 µl

A PCR was performed using a T3 Thermocycler (Biometra) and the following program specifications:

Step 1: 93°C for 10 min

Step 2: 93°C for 30 s

Step 3: 60°C for 30 s

Step 4: 72°C for 45 s

Step 5: repeat step 2 to step 4 for 34 times

Step 6: 72°C for 5 min

Step 7: cool to 4°C

The PCR products were separated by electrophoresis with on a 2 % agarose gel (130 V for 2.5 h).

2.5.7. Mini-/Maxi-prep of the reporter constructs

From the liquid cultures which were identified as positives by the PCR-screening, a cryo stock was prepared by mixing 200 µl of cell suspension with 100 µl of sterile glycerol and storing the mixture at -80°C. The rest of the cell suspension was centrifuged for 5 min at full speed and the pellet was used to perform a mini prep using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturers' instructions. The reporter constructs were eluted in 30 µl NFW and their concentration was measured (see 2.2.1.) before they were stored at -20°C. The reporter constructs were sequenced by a commercial provider to assure their identity.

After verification by sequencing, the cryo stocks of the reporter constructs were used to inoculate each a 500 ml baffled culture flask filled with 100 ml liquid LB-media containing 100 µg/ml ampicillin. The cultures were incubated over night on a shaker at 37°C. On the following day a maxi prep of each culture was performed using the EndoFree Plasmid Maxi Kit® (Qiagen) according to the manufacturers' instructions. The last two centrifugation steps of the protocol were performed at 4700 g for 1 h instead of 15000 g for 30 min. After measuring the concentration of the purified plasmid DNA (see 2.2.1.) the reporter constructs were diluted with NFW to a final concentration of 1 µg/µl and stored at -20°C.

2.5.8. Transient transfection of HDF5 cells with miR-663/miRctrl

For the Dual-Glo® Luciferase Assay (Promega) HDF5 cells were transfected transiently in a 6-well culture plate with miR-663 Power Inhibitor and Power Inhibitor Control (see 2.1.2.1.). On the following day the cells were transfected with the psiCHECK^{TM}-2 reporter constructs and the dualglow luciferase assay was performed as described in 2.5.11. without the use of blasticidin in the growth media.

2.5.9. Cloning of the miR-663 insert into the pcDNA™6.2-GW/EmGFP-miR vector

To perform the Dual-Glo® Luciferase Assay (Promega) with cells which are expressing miR-663 in a stable manner, HEK-293 and NIH-3T3 cells were transfected with a pcDNA™6.2-GW/EmGFP-miR-663 plasmid and its respective control plasmid pcDNA™6.2-GW/EmGFP-miR-neg. The plasmid was generated in the same manner as the reporter constructs. The ss oligos of the miR-663 gen (see 2.6.2.4.) were designed by Matthias Wieser and ordered at Integrated DNA technologies. Before the miR-663 insert was ligated into the vector, the pcDNA™6.2-GW/EmGFP-miR-663 plasmid was modified at its multiple cloning site by introducing a small insert containing the restriction sites for NotI and PstI.

The modified pcDNA™6.2-GW/EmGFP-miR plasmid was digested using the FastDigest® (Thermo Scientific) restriction enzymes PstI and NotI and the 10x FastDigest® buffer. The reaction mix was prepared as follows:

FastDigest® PstI	2 µl
FastDigest® NotI	2 µl
10x FastDigest® buffer	2 µl
pcDNA™6.2-GW/EmGFP-miR (0.25 µg/µl)	2 µl
NFW	12 µl
Total volume	20 µl

→reaction mix was incubated at 37 °C for 30 min on a heating block

→enzymes were inactivated by incubation at 80 °C for 5 min on a heating block

The linearized vector was separated by electrophoresis on a 1 % agarose gel (2 h at 90 V) and the band containing the vector was cut out and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturers' instructions. The purified vector was eluted in 50 µl NFW and its concentration was measured (see 2.2.1.). The linearized and purified pcDNA™6.2-GW/EmGFP-miR vector was diluted with NFW to a concentration of 10 ng/µl.
The ligation mix was prepared as follows to achieve a molar ratio of 1 : 5 between plasmid and vector:

T4 DNA ligase	2 µl
10x T4 ligase buffer	2 µl
ds oligo insert (10 nM)	8.5 µl
pcDNA™6.2-GW/EmGFP-miR (10 ng/µl)	6 µl
NFW	1.5 µl
Total volume	20 µl

2.5.10. Stable transfection with pcDNA™6.2-GW/EmGFP-miR-663 and pcDNA™6.2-GW/EmGFP-miR-neg

Prior to transfection, we determined the appropriate blasticidin concentration for the HEK-293 and the NIH-3T3 cells. Cells were passaged 1:4 into a 12-well culture plate and blasticidin was added to the growth media on the next day in concentrations ranging from 0-25 µg/ml. After 5 days the blasticidin containing growth media was renewed and another 4 days later the lowest concentration which killed all cells was determined as 8 µg/ml.

On the day before the transfection, Hek-293 and NIH-3T3 cells were passaged 1:3. The transfection was performed in a 6-well culture plate (see 2.1.2.3.). 24 h post transfection selection pressure was applied by substituting the media with blasticidin containing (8 µg/ml) growth media. The cells were cultured with blasticidin containing growth media until the cells resistant to blasticidin were selected. The GFP fluorescence was checked under the microscope.

2.5.11. Transfection of the cells with the reporter constructs and performing of the Dual-Glo® Luciferase Assay

Hek-293 cells expressing miR-663/miR-ctrl or HDF5 cells transiently transfected with miR-663/miR-ctrl were seeded into 12-well culture plates at a density of $3x10^5$ cells/well. For each cell line 8 wells were seeded, one for each reporter construct, one for the empty vector and one for transfection agent only. 48 h later the cells were transfected with the psiCHECK^{TM}-2 reporter constructs (see 2.1.2.3). For the HEK-293 cells blasticidin containing (8 µg/ml) growth media was used instead of normal growth media to maintain selection pressure.

48 h after transfection the cells were harvested, washed once with cold PBS (4°C) and pelleted. 240 µl of Glo Lysis Buffer (Promega) were added to the cell pellets and the cells were dissolved by pipetting up and down. Both substrates were prepared according to the manufacturers' instructions and stored light protected at RT. The measurements were performed in triplicates. For each measurement 75 µl/well cell lysate were transferred to a black 96-well plate. 75 µl of the first substrate were added to each well and the activity for the firefly luciferase was measured. 75 µl of the second substrate were added to each well, the activity for the *Renilla* luciferase was measured and the ratio of the two activities was calculated.

2.6. Reagents and sequences

2.6.1. Reagents

NEB (nuclear extract buffer)

50 mM Tris HCl, pH=7.5
0.5 M NaCl
1 % NP-40 substitute
1 % Sodium deoxycholate
0.1 % SDS
2 mM EDTA
cOmplete EDTA-free Protease Inhibitor Cocktail (Roche)

TNE buffer

100 mM Tris HCl, pH=8
300 mM NaCl
1 mM EDTA
2 % Triton X-100
Add freshly cOmplete EDTA-free Protease Inhibitor Cocktail (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche)

Transfer buffer

25 mM Bicine
25 mM Bis-Tris
20 mM EDTA

PBS

0.24 g KH_2PO_4
1.8 g Na_2HPO_4 x 2 H_2O
0.2 g KCl
8 g NaCl
Dissolve in 1000 ml and adjust pH to 7.4 with HCl

TPBS

0.1 % TWEEN 20 (Merck) in PBS

Blocking solution (milk powder)

3 % milk powder in TPBS

TBS

8 g NaCl
0.2 g KCl
8 g Tris
Dissolve in 1000 ml and adjust pH to 7.4 with HCl

TTBS

0.1 % TWEEN 20 (Merck) in TBS

Blocking solution (BSA)

5 % BSA in TTBS

Blocking buffer

0.3 % Triton X-100 (Sigma)
5 % FCS (fetal cow serum)
in PBS

Dilution buffer

0.3 % Triton X-100 (Sigma)
1 % BSA
in PBS

BTPBS

1 % BSA in TPBS

2.6.2. Sequences

2.6.2.1. qPCR primers

GAPDH

Sense	5′	TGTGAGGAGGGGAGATTCAG	3′
Antisense	5′	CGACCACTTTGTCAAGCTCA	3′

p21

Sense	5′	GGCGGCAGACCAGCATGACAGATT	3′
Antisense	5′	GCAGGGGGCGGCCAGGGTAT	3′

PP5

Sense	5′	ACGGAGCGCTTGTGCTCGTC	3′
Antisense	5′	GGCTGCCAGCAACATGGCAC	3′

miR-663

	5′	AGGCGGGGCGCCGCGGGAC	3′

S5 rRNA

	5′	CAGGGTCGGGCCTGGTTAGTACTTG	3′

2.6.2.2. psiCHECKseq primers

Sense	5′	TACCTTCGGGCCAGCGACGA	3′
Antisense	5′	GCCAGTTACCACCCGCCGAC	3′

2.6.2.3. miR-663 oligos

miR-663

```
TOP     ggccgcCCTTCCGGCGTCCCAGGCGGGGCGCCGCGGGACCGCCCTCGTGTCTGTGGCGGTGGGATCCCGCGGCCGT
BOTTOM      cgGGAAGGCCGCAGGGTCCGCCCCGCGGCGCCCTGGCGGGAGCACAGACACCGCCACCCTAGGGCGCCGGCA

        GTTTTCCTGGTGGCCCGGCCATGctgca
        CAAAAGGACCACCGGGCCGGTACg
```

miR-663 seed sequence (green); NotI overhang (blue); PstI overhang (grey)

2.6.2.4. PP5-mRNA 3'-UTR oligos

1611

TOP tcgagactagtCCCAGGCCCTGGGCTAGGGGCAGAGCAGGCCCCGCCCCAGGGCAATGTTGGAgc
BOTTOM ctgatcaGGGTCCGGGACCCGATCCCCGTCTCGTCCGGGGCGGGGTCCCGTTACAACCTcgccgg

1611mut

TOP tcgagactagtCCCAGGCCCTGGGCTAGGGGCAGAGCAGGCCTCTCCCCAGGGCAATGTTGGAgc
BOTTOM ctgatcaGGGTCCGGGACCCGATCCCCGTCTCGTCCGGAGAGGGGTCCCGTTACAACCTcgccgg

1875

TOP tcgagactagtATGGCTCCTCCCCCACTCAAGCAATAGGGCCCCGCCATAGGAAGACCCCCAGgc
BOTTOM ctgatcaTACCGAGGAGGGGGTGAGTTCGTTATCCCGGGGCGGTATCCTTCTGGGGGTCcgccgg

1875mut

TOP tcgagactagtATGGCTCCTCCCCCACTCAAGCAATAGGGCCATTCCATAGGAAGACCCCCAGgc
BOTTOM ctgatcaTACCGAGGAGGGGGTGAGTTCGTTATCCCGGTAAGGTATCCTTCTGGGGGTCcgccgg

1916

TOP tcgagactagtAAGACCCCCAGAGAGAGGGTCAGCAGGGGGGCCCCGCCTGCGCCTCCCCTCCTAgc
BOTTOM ctgatcaTTCTGGGGGTCTCTCTCCCAGTCGTCCCCCCGGGGCGGACGCGGAGGGGAGGATcgccgg

1916

TOP tcgagactagtAAGACCCCCAGAGAGAGGGTCAGCAGGGGGGCCTTTCCTGCGCCTCCCCTCCTAgc
BOTTOM ctgatcaTTCTGGGGGTCTCTCTCCCAGTCGTCCCCCCGGAAAGGACGCGGAGGGGAGGATcgccgg

target sequence for miR-663 (green); mutated base pairs (pink); NotI overhang (blue); XhoI overhang (yellow)

3. Results

3.1. Treatment with 100 µM H_2O_2 for 1 h induces non-lethal DNA damage and activates DNA damage repair

Since we hypothesized a role of miR-663 in stress response and DNA damage repair, we wanted to apply a sub-lethal treatment with a DNA damage inducing reagent. We treated the HDF5 cells with H_2O_2 and determined the optimal concentration and duration for the treatment (data not shown). 1 h of treatment with culture media containing 100 µM H_2O_2 and subsequent cultivation with normal media produced the desired result. The treatment with 100 µM H_2O_2 resulted in the phosphorylation of the histone variant H2AX leading to γ-H2AX (Figure 7). γ-H2AX is an indicator for DNA double-strand breaks (DSBs) and DNA damage repair by activation of the DNA damage response (DDR) (Podhorecka et al. 2010).

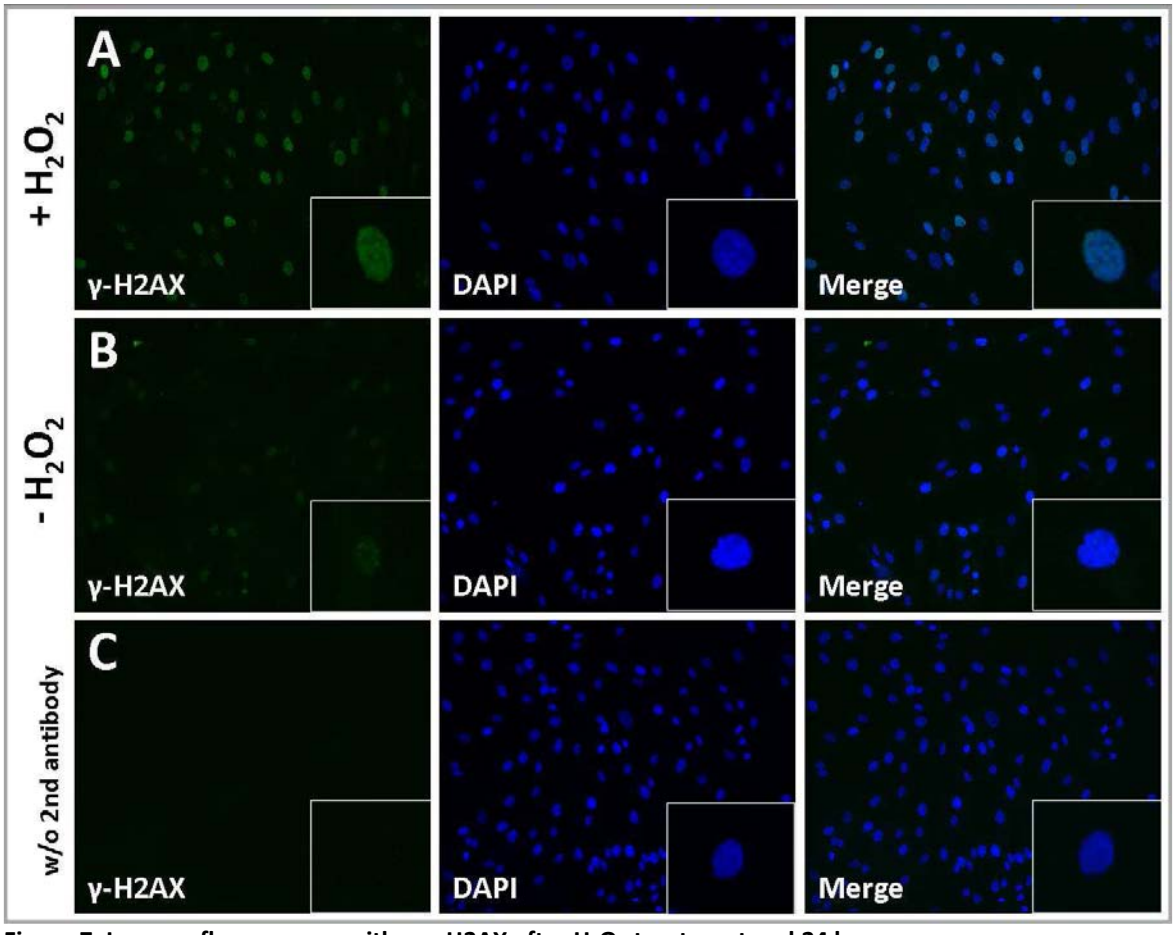


Figure 7: Immunofluorescence with α-γ-H2AX after H_2O_2-treatment and 24 h recovery

(A) Cells were treated with 100 µM H_2O_2 for 1 h and recovered for 24 h in normal culture media. **(B)** Cells were not treated with H_2O_2. **(C)** Cells were not treated with H_2O_2 and primary antibody.

3.2. miR-663 regulates the cellular stress response and the cell cycle of HDF5 cells

To test our hypothesis that miR-663 plays an important role in the cellular response to DNA damage and thereby in the regulation of the cell cycle, we conducted MTT-assays with and without a preceding transient transfection of the HDF5 cells with miR-663 Inhibitor and its respective Inhibitor Control. The transfection was performed directly in a 96-well plate with 6 independent replicates. For every MTT-assay half the cells were treated with H_2O_2 for 1 h. The time frame for our measurements ranged from 28 to 40 hours post H_2O_2-treatment, which according to previous assays (data not shown) is the time when the cells were resuming their growth.

3.2.1. H_2O_2-treatment induces a temporary growth arrest

When treating HDF5 cells without prior transfection with miR-663 Inhibitor, cells treated with H_2O_2 had fewer cells which were dividing and were suffering a temporary growth arrest. The cells overcame the growth arrest about 37 h after the treatment (Figure 8). Considering that HDF cells need 48-72 h for one population doubling, the differences seen in the formazan level after the treatment are quite substantial. The formazan levels at the respective time points are displayed in the left panel; the right panel shows the increase in formazan per hour for the indicated time period. The reduced increase in proliferative activity, seen with the untreated cells 40 h after the treatment, was presumably a result of nutrient depletion.

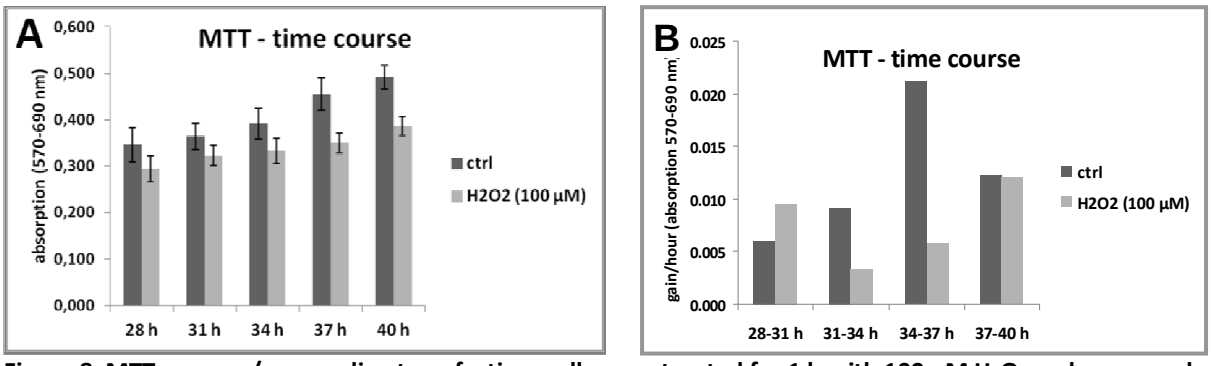


Figure 8: MTT-assay w/o preceding transfection; cells were treated for 1 h with 100 µM H_2O_2 and recovered in normal media for the indicated time

(A) Formazan level at the respective time point. **(B)** Average gain of formazan per hour over the last 3 hours, counted from the respective time point.

3.2.2. miR-663 Inhibition increases the proliferative capacity of HDF5 cells

Inhibition of endogenous miR-663 using the miR-663 Inhibitor resulted in an increase in proliferative activity even when the cells were not being treated with H_2O_2 (Figure 9). The inhibition of miR-663 could not been verified by measuring the levels of miR-663 as the inhibition is a result of the formation of highly stable heteroduplexes with the inhibitor and does not involve the degradation of miR-663.

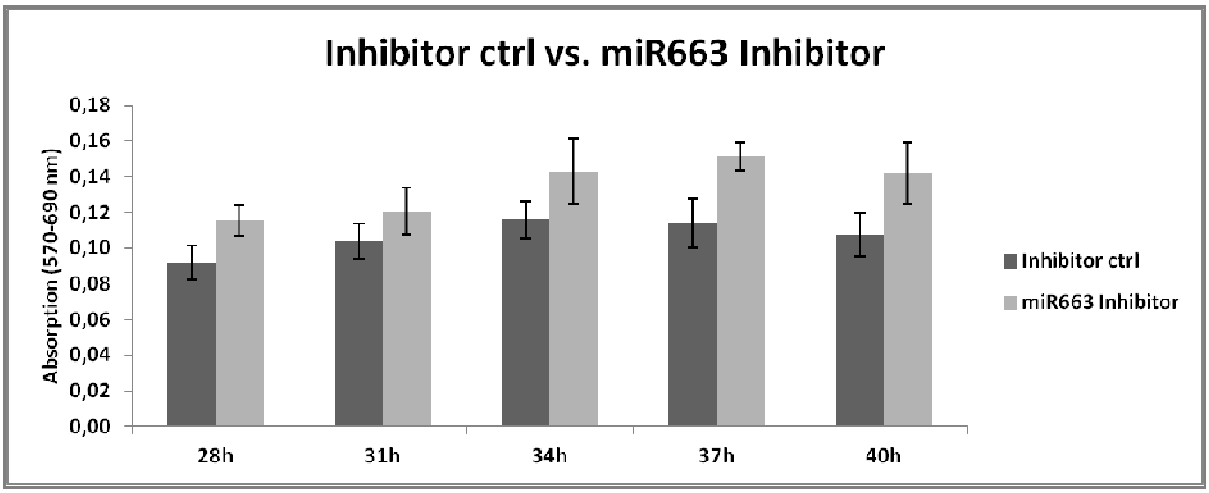


Figure 9: MTT-assay with preceding transfection of miR-663 Inhibitor/Inhibitor Control

This led us to the conclusion that miR-663 plays also under normal conditions without any oxidative stress treatment a part in the regulation of the cell cycle. Cells where endogenous miR-663 is targeted have a higher proliferative capacity compared to native cells. Considering that miR-663 is reported in several studies (see 1.3.) to have tumor suppressive properties the results seem to be in line with the assumptions made about the regulative functions of miR-663. After 37 hours the concentration of formazan didn't increase anymore and occurs equally with the cells transfected with miR-663 Inhibitor and Inhibitor Control. Thus we concluded that this might be due to limitation of nutrients.

3.2.3. miR-663 is necessary for an effective stress response

To investigate the role of miR-663 in stress response we analyzed the effect of H_2O_2-treatment combined with the miR-663 Inhibitor transfection. The data originates from the same experiment as shown in Figure 9. The formazan levels of the stressed cells were normalized to the levels of their unstressed counterparts of the respective time point. The decrease in proliferative capacity of the cells transfected with the miR-663 Inhibitor was more substantial than of the cells transfected with the Inhibitor Control (Figure 10). So it

seemed that in contrast to the unstressed cells, miR-663 was increasing the proliferative capacity of the cells which underwent oxidative stress treatment. The stronger decrease of the proliferative capacity seen with the cells containing less miR-663 is a sign of the importance of miR-663 for a well-regulated response to oxidative stress. Without this regulation the effect of the stress may be fatal for some of the cells leading to apoptosis and thereby to a decreased proliferative capacity of the cell population.

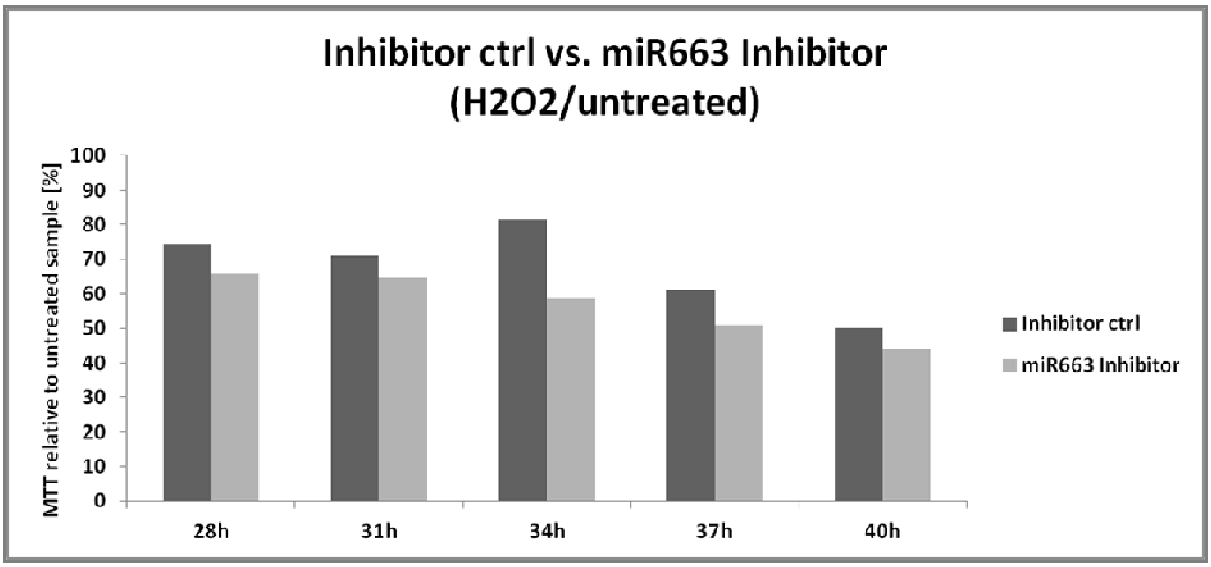


Figure 10: MTT-assay with preceding transfection of miR-663 Inhibitor/Inhibitor Control and H_2O_2-treatment; values are relative to respective untreated sample

3.2.4. miR-663 acts via PP5 and p21

One possible explanation for the observed phenotype is that miR-663 acts via PP5 on p21 to regulate the cell cycle of HDF5 cells in stress response. PP5, which was one of the predicted targets of miR-663 with three high-scoring target sites in its 3'-UTR, has been reported in various publications to act on p21 via de-phosphorylation of p53 at Ser15. If the interaction between PP5 and p53 is direct or indirect has not been clarified so far (see 1.4.2.).

3.2.4.1. Luciferase reporter assay could not prove direct interaction between miR-663 and PP5

The method of choice to verify the direct interaction between miR-663 and PP5 was a luciferase reporter assay. HDF5 cells were transfected transiently with miR-663 and its control before they were transfected with the reporter constructs and the luciferase assay was performed. To express miR-663 in a stable manner, HEK-293 and NIH-3T3 cells were transfected with pcDNA™6.2-GW/EmGFP-miR-663. When the selection of the miR-663

positive cells was complete, they were transfected with the reporter constructs and the luciferase assay was performed Despite all the variations and retries which were performed in the course of the experiment, no direct interaction between the putative target sites of the PP5 3'-UTR and miR-663 could be proven with the luciferase assay (Figure 11).

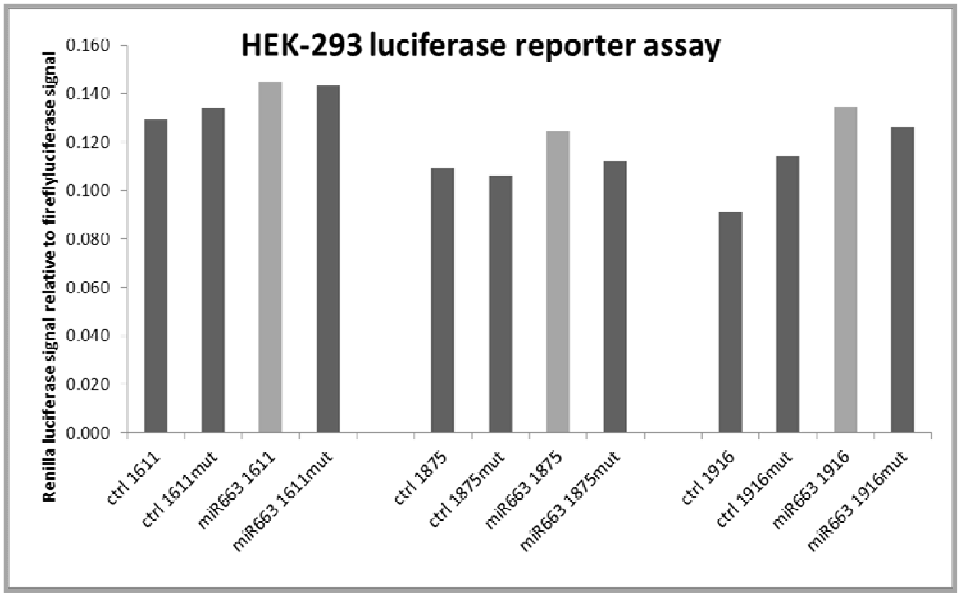


Figure 11: Luciferase reporter assay using HEK-293 transfected with pcDNA™6.2-GW/EmGFP-miR-663

3.2.4.2. miR-663 acts at least indirectly on PP5

Still the interaction between miR-663 and PP5, direct or indirect, could be proven by transfecting HDF5 cells with miR-663 and its respective miRctrl and subsequent analysis of the protein levels by performing a SDS-PAGE and a western blot with the cell extracts (Figure 12). The transfection with miR-663 resulted in a reduction of the predominant form of PP5 at 58 kD by approx. 9 % after 48 h and 21 % after 72 h when compared to the cells transfected with miRctrl. We also detected another form of PP5 at approx. 90 kD which was reduced by approx. 50 % after 48 h and 60 % after 72 h.

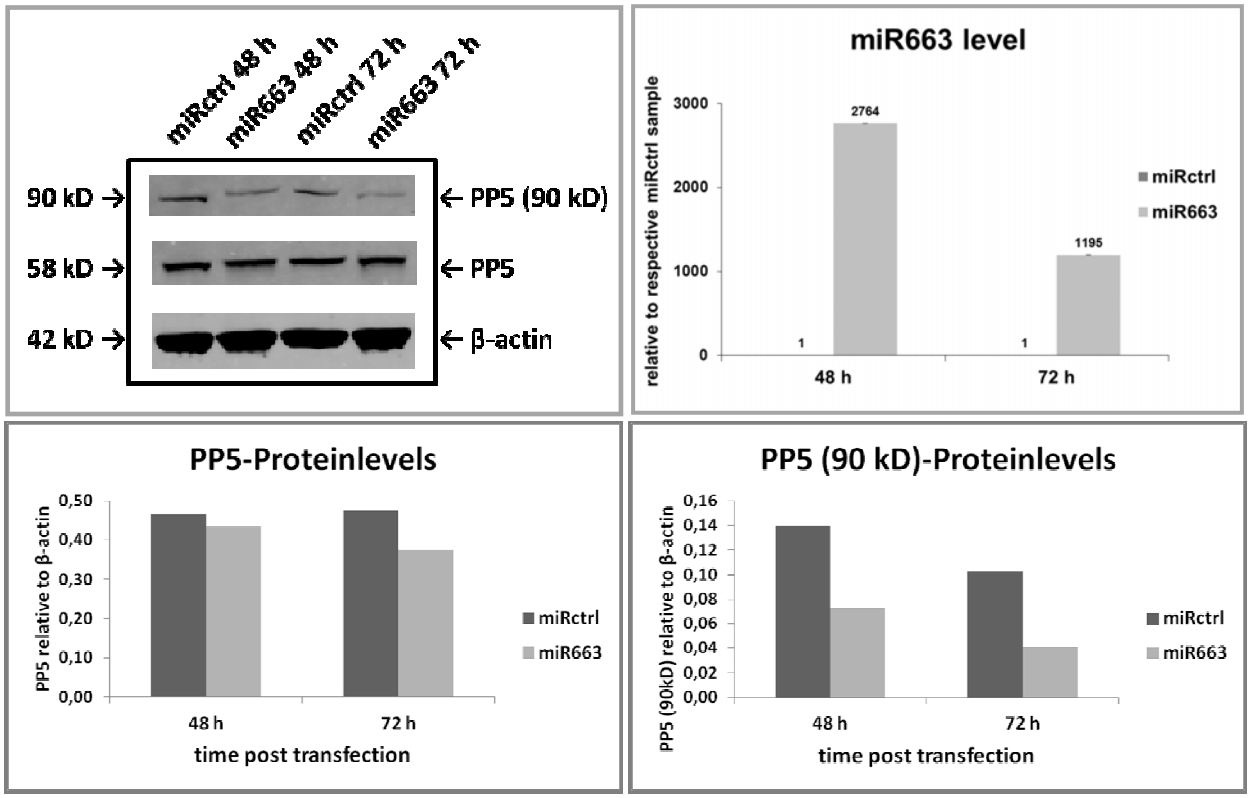


Figure 12: HDF5 cells were transfected with miR-663/miRctrl, cell lysates were prepared and RNA was isolated 48 h and 72 h post transfection

On the other hand, transfection of HDF5 cells with miR-663 Inhibitor reduced the downregulation of the predominant PP5 form at 58 kD and the 90 kD form of PP5 after oxidative stress treatment. Additionally the upregulation of p21 and the 45 kD form of PP5 was reduced (Figure 13). The predominant PP5 form at 58 kD got downregulated after H_2O_2-treatment by 59 % in cells transfected with Inhibitor Control and by 76 % in cells transfected with miR-663 Inhibitor. The 90 kD form of PP5 got downregulated by 42 % and 70 % respectively. p21 got upregulated by 226 % and 212 % respectively. In this blot yet another form of PP5 at 45 kD was being detected which was upregulated by 42 % and 70 % respectively.

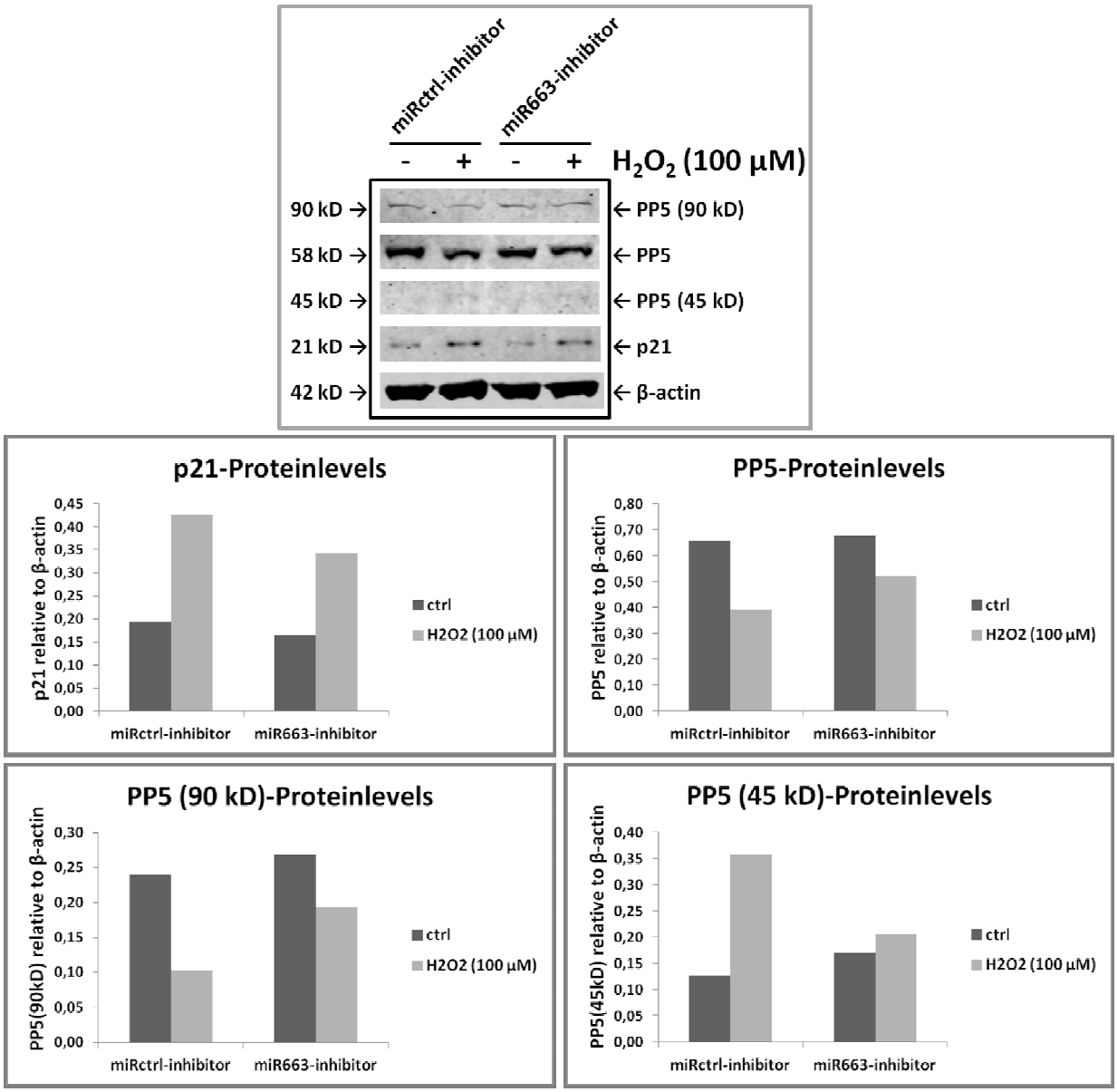


Figure 13: HDF5 cells were transfected with miR-663 Inhibitor/Inhibitor Control and subsequently treated with 100 µM H_2O_2 for 1 h and recovered 24 h in normal media before cell lysates were prepared

Even if the direct interaction between miR-663 and PP5 could not be proven, an at least indirect effect on p21 and the various forms of PP5 with or without oxidative stress treatment was clearly demonstrated by the experiments. To obtain a deeper understanding of the involved mechanisms behind the observable effects, the role of PP5 in stress response and the regulation of the cell cycle were yet to be clarified.

3.3. PP5 is promoting cell growth and gets downregulated by cellular stress

3.3.1. PP5 is downregulated in senescent cells and in cells suffering from cellular stress

By analyzing cell extracts from previous experiments we discovered that PP5 gets also downregulated in senescent cells and cells treated with tBHP (tert-Butyl hydroperoxide) (treatment was performed by Harald Kühnel) similar to the effect observed with H_2O_2-treatment (Figure 14). Hence the mechanism PP5 is involved in seems not to be specific to one type of cellular stress, but more to be a general mechanism of cell cycle control and stress response.

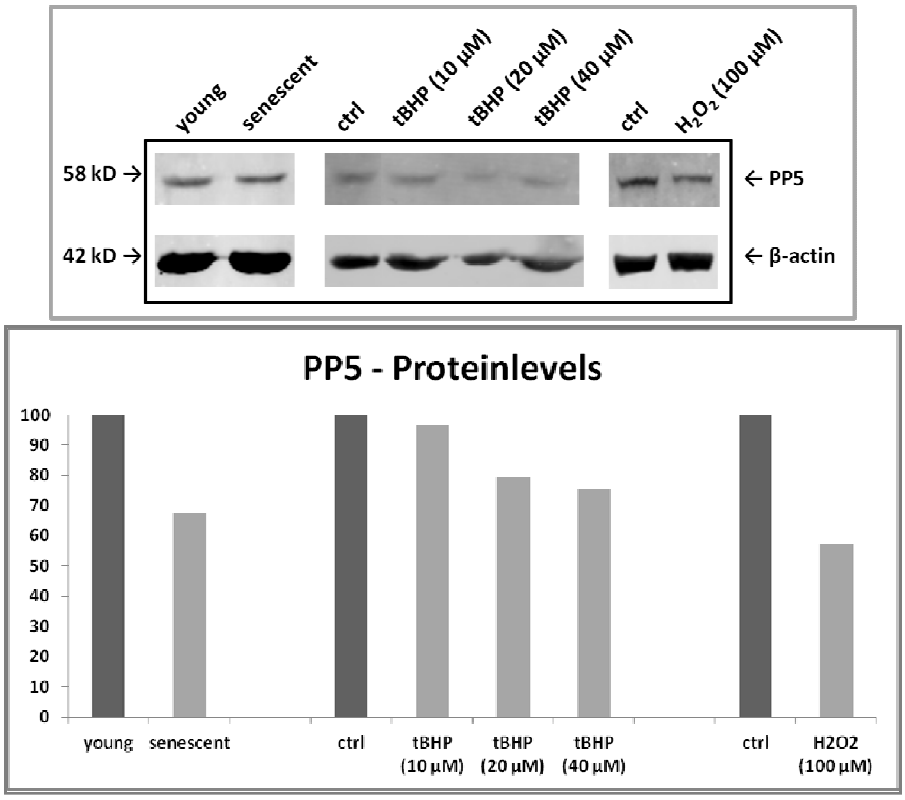
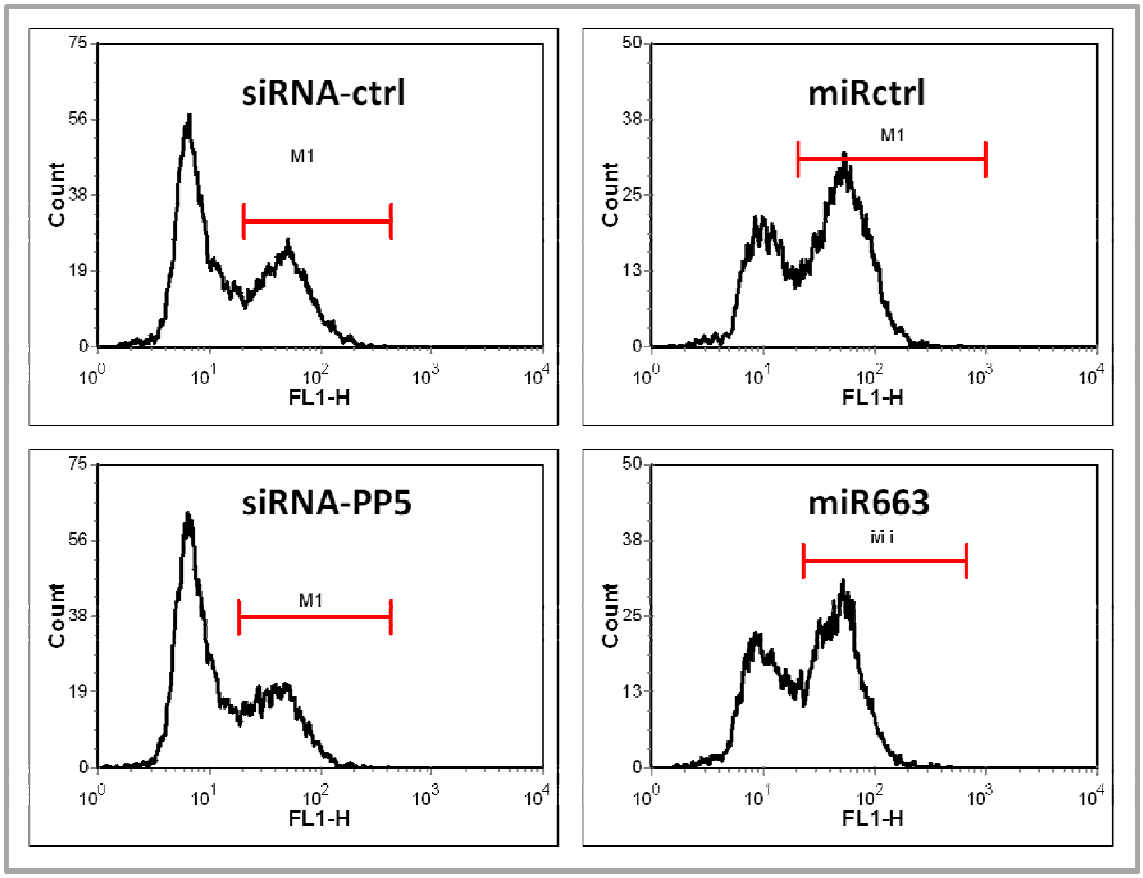


Figure 14: PP5 protein levels normalized on the respective control. Cells from young and old donor; cells treated for 1 h with different concentrations of tBHP and recovered for 24 h; cells treated for 1 h with 100 µM H_2O_2 and recovered in normal media for 24 h

3.3.2. Knockdown of PP5 leads to reduction of the proliferative capacity

To emphasize our evidence on the regulative effect of PP5 on the cell cycle of HDF5 cells and to see if a direct repression of PP5 with siRNA (small interfering RNA) can mimic the reduction of proliferative capacity caused by miR-663 (Figure 9), we conducted a BrdU-assay after transfecting the cells with miR-663 or siRNA-PP5 and their respective controls (Figure 15). The reduction of BrdU positive cells compared to the respective controls was for both, miR-663 and siRNA-PP5 transfected cells, at around 13 %. Thus, the effect of PP5 on the proliferative capacity of HDF5 cells could be proven and siRNA-PP5 did mimic the effect of miR-663 perfectly. The reduction of the proliferative capacity caused by the downregulation of PP5 can be explained with the upregulation of p21, whereas the process by which PP5 accomplishes that is still unclear.



sample	BrdU positive cells [%]	relative to ctrl [%]
miRctrl	64.0	100.0
miR663	55.8	87.2
siRNA-ctrl	39.0	100.0
siRNA-PP5	33.9	86.9

Figure 15: Flow cytometry analysis of HDF5 cells treated with BrdU 30-46 h post transfection with siRNA-PP5/siRNA-ctrl or miR-663/miRctrl

3.4. miR-663, PP5, p53 and p21 are part of a mechanism controlling cellular stress response

The tumor repressor protein p53, which is located in the nucleus and acts in its phosphorylated state $p53^{Ser15}$ as a transcriptional activator for p21, could be a possible link between p21 and PP5 (see 1.4.2.). By de-phosphorylation of $p53^{Ser15}$, PP5 would under normal conditions repress the transcription of p21. Under stress conditions like oxidative stress treatment however, PP5 is downregulated and $p53^{Ser15}$ could remain in its active phosphorylated form, p21 levels would be elevated and as a result the cells would suffer from a temporary growth arrest (Figure 8). Keeping this in mind, PP5 should under normal conditions be located in the nucleus as an active nuclear form and be deactivated or shuttled out of the nucleus under cellular stress conditions. To test our hypothesis we treated HDF5 cells with H_2O_2 and measured the protein levels of $p53^{Ser15}$ and p21 (Figure 16).

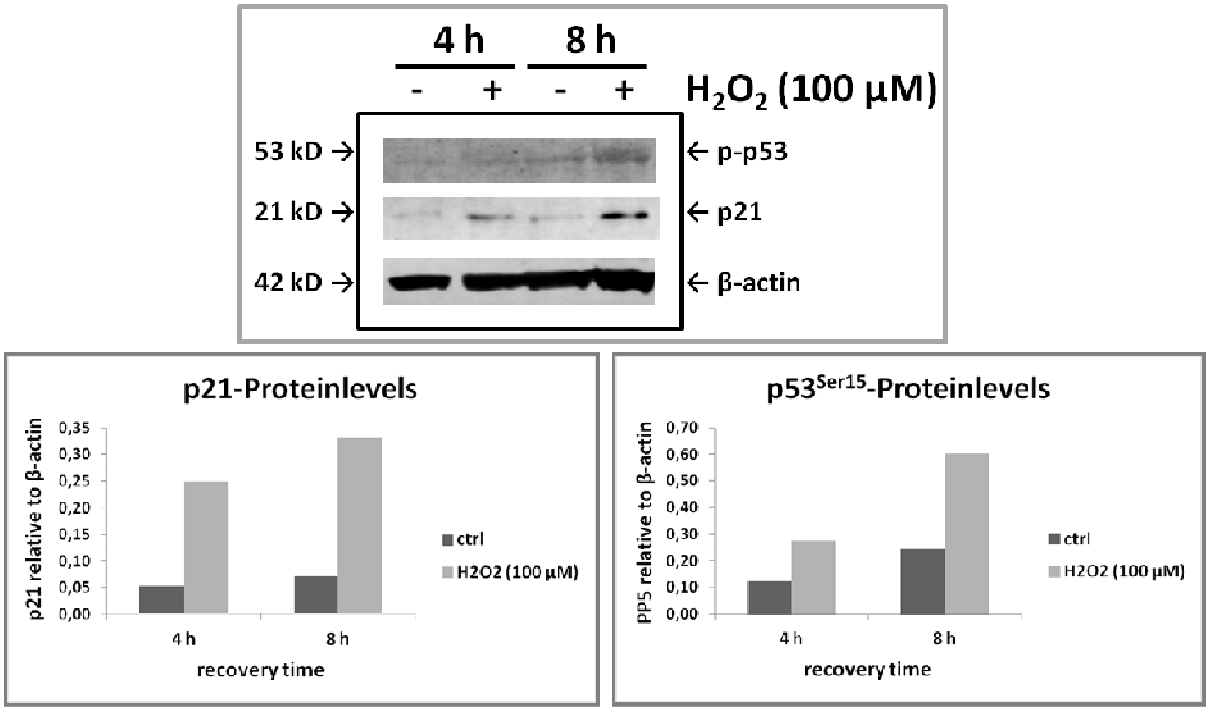


Figure 16: HDF5 cells were treated for 1 h with 100 µM H_2O_2 and recovered in normal media for the indicated time; cell lysates were prepared according to the TNE buffer protocol

The protein levels of $p53^{Ser15}$ and p21 reacted to the oxidative stress treatment as hypothesized by us.

3.4.1. Early cellular stress response by shuttling of nuclear PP5

We conducted immunoflourescence assays with antibodies for PP5 and $p53^{Ser15}$ under normal conditions and with H_2O_2 treated cells to see if our assumptions about the localization of PP5 were right. The images showed that 5 h after the stress treatment PP5 levels were decreased and $p53^{Ser15}$ levels were increased in the nucleus (Figure 17). These observations can be explained by the presence of a fast mechanism which relocates the active nuclear form of PP5 to the cytoplasm in response to cellular stress.

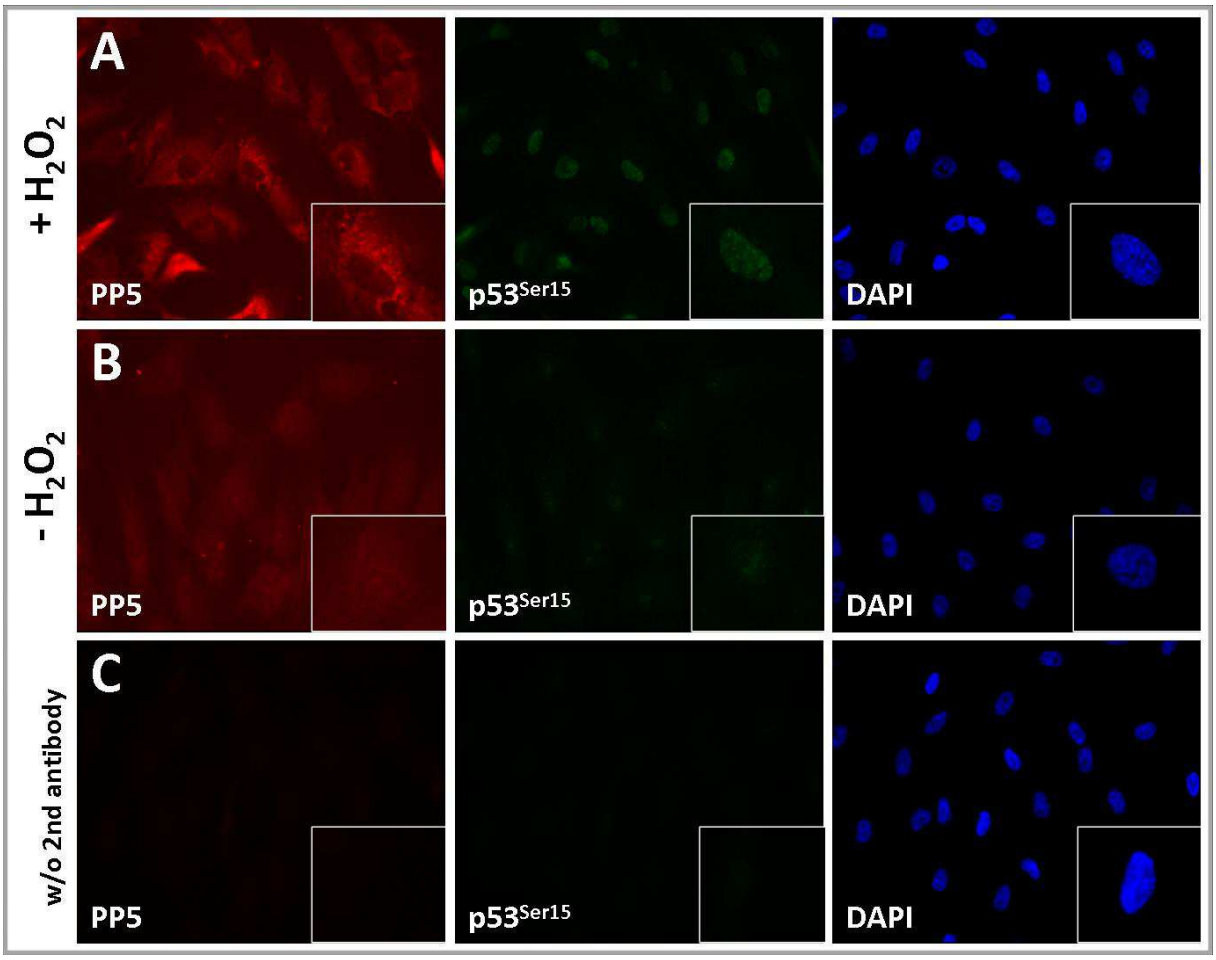


Figure 17: Immunofluorescence with α-PP5 and α-$p53^{Ser15}$ after H_2O_2-treatment and 5 h recovery

(A) Cells were treated with 100 µM H_2O_2 for 1 h and recovered for 5 h in normal culture media. **(B)** Cells were not treated with H_2O_2. **(C)** Cells were not treated with H_2O_2 and primary antibody.

24 h after the treatment the level of PP5 in the nucleus was returning to normal which implies that the damage done by the oxidative stress treatment might already have been repaired and the growth arrest was about to be reversed. The $p53^{Ser15}$ level was still up, which might be due to a delayed reaction. The PP5 level in the nucleus of some of the untreated cells also seemed to be decreased. This was observed when the cells grew too dense in the culture vessel and might be a sign that PP5 is also involved in the mechanism of contact inhibition (Figure 18).

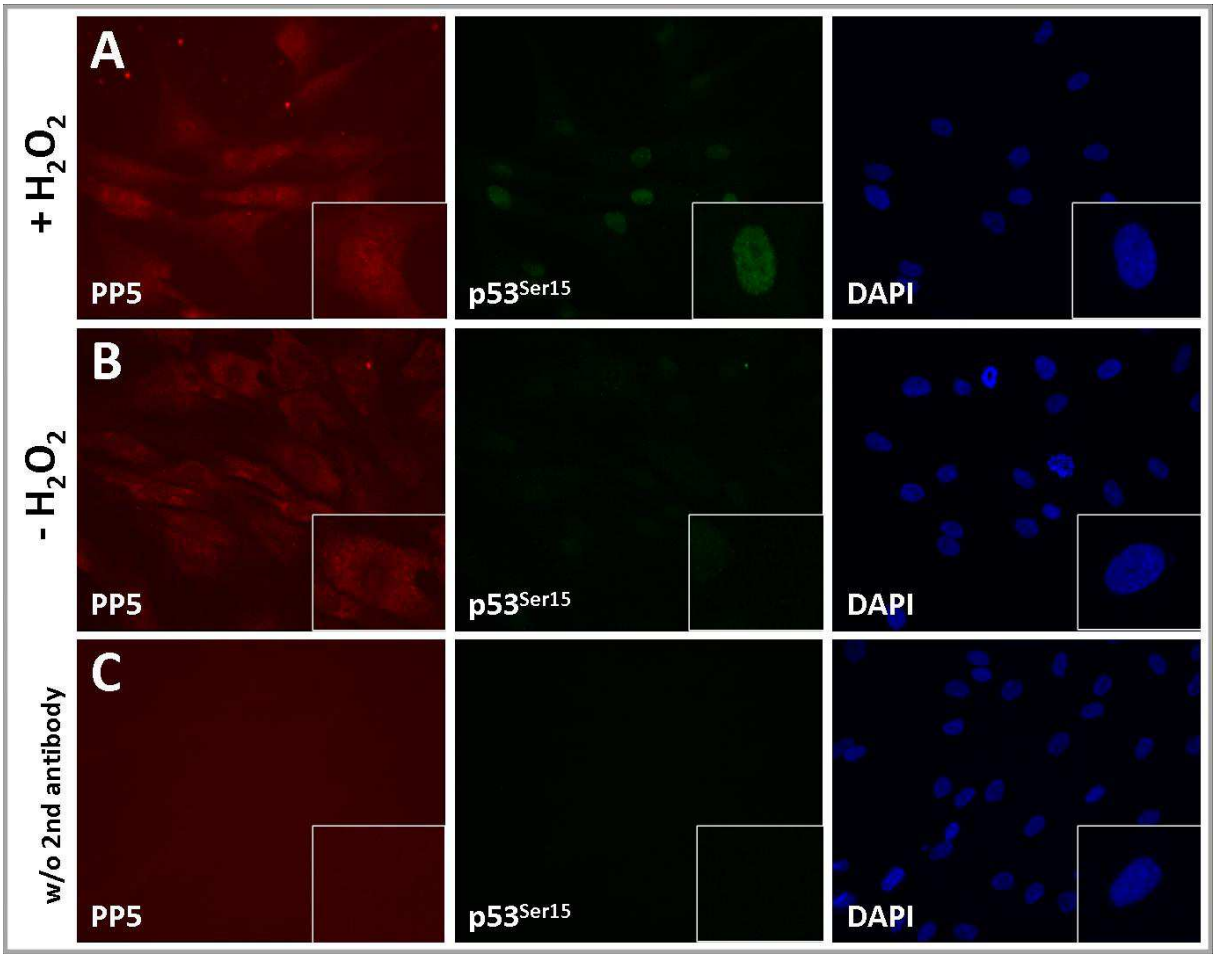


Figure 18: Immunofluorescence with α-PP5 and α-$p53^{Ser15}$ after H_2O_2-treatment and 24 h recovery

(A) Cells were treated with 100 µM H_2O_2 for 1 h and recovered for 24 h in normal culture media. **(B)** Cells were not treated with H_2O_2. **(C)** Cells were not treated with H_2O_2 and primary antibody.

3.4.2. Nuclear PP5 forms a stable complex with a molecular weight of 90 kD

As already mentioned in the introduction, PP5 is known to feature a putative NLS (nuclear localization signal) and exists in various forms resulting from proteolytic cleavage and possibly other processes. In previous protein analysis we already detected two more forms of PP5 at approx. 90 kD and 45 kD (Figure 12 and 13), so we were very keen on finding out which of the them is located in the nucleus and thereby de-phosphorylating $p53^{Ser15}$. We performed a fractionized lysis after treating the HDF5 cells with H_2O_2 (Figure 19) and analyzed the protein levels via SDS-PAGE and subsequent western blot. GAPDH was used as the marker for the cytoplasm fraction and Lamin A and C for the nuclear fraction. The markers showed the expected pattern, thus we can be sure that the fractionation worked properly. The only form of PP5 present in the nucleus was the 90 kD form. On the other hand, in the cytoplasm we could only detect the predominant form at 58 kD and the form at 45 kD. The amounts of the predominant PP5 form at 58 kD which were present in the membrane fraction are presumably carryovers from the cytoplasm fraction and can also be seen with GAPDH. The PP5 form at 90 kD however is not present in the cytoplasm fraction and thereby can't be the result of a carryover.

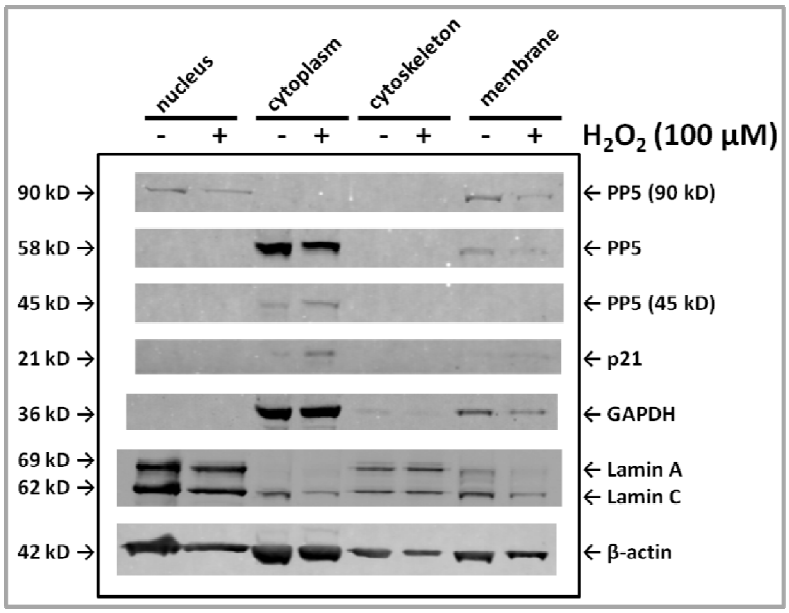


Figure 19: Fractionized lysis of HDF5 cells treated for 1 h with 100 µM H_2O_2 and recovered in normal media for 24 h

Because of these interesting findings we decided to analyze how the protein levels of the different forms of PP5 react to oxidative stress treatment over the course of time. We treated the cells with H_2O_2, took protein samples at the indicated time points and performed SDS-PAGE and western blot. The predominant form of PP5 at 58 kD and the form at 90 kD showed the same pattern and were decreased after the treatment whereas p21 levels were increased. The strongest effect was visible 24 h after treatment and 48 h after the treatment the levels were beginning to go back to normal. This further supported the hypothesis that

the treatment with H_2O_2 leads to a temporary growth arrest which gets reversed after the cells repaired the damage. The PP5 form at 45 kD however showed a strong increase in the beginning which got reduced to normal levels over time (Figure 20).

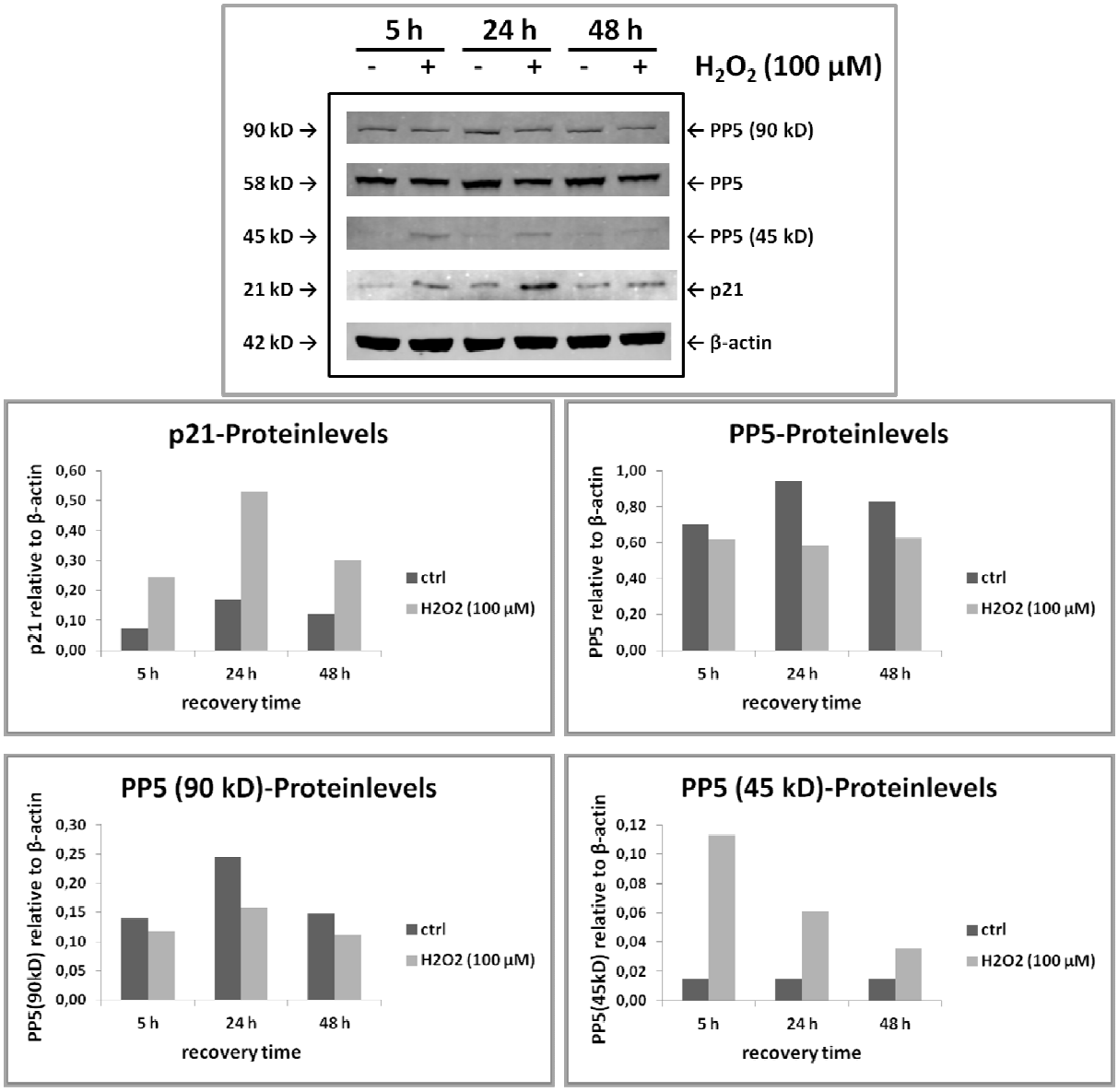


Figure 20: HDF5 cells were treated for 1 h with 100 µM H_2O_2 and recovered in normal media for the indicated time

Being the only form of PP5 which is present in the nucleus and considering how its protein levels react to oxidative stress treatment, the identity of the 90 kD form needed to be investigated. Our assumption was that the 90 kD form is either a dimer of proteolytical cleaved PP5 or a stable complex of PP5 with other proteins. To test our thesis we performed a SDS-PAGE with low and high concentrations of β-mercaptoethanol and with DTT as alternative reducing agent (Figure 21).

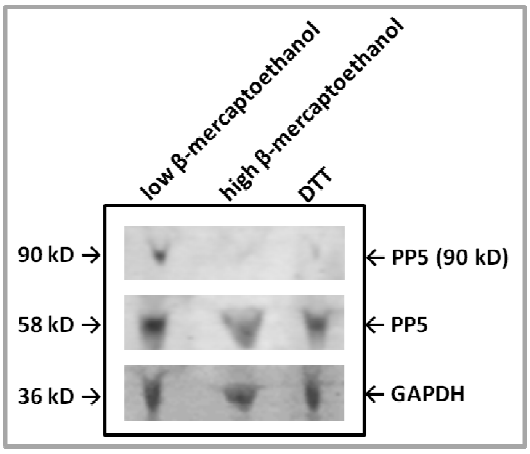


Figure 21: HDF5 cell lysates in loading dyes with different reducing capacity

Under high reducing conditions, as well as with DTT as reducing agent, the band for the 90 kD form disappeared. Thus the complex or dimer PP5 forms in the nucleus seems to be stabilized by disulphide bonds.

3.4.3. Late cellular stress response by post-transcriptional regulation of PP5 through miR-663

Besides the early DNA damage response via shuttling of PP5, exists the late response via post-transcriptional regulation of PP5 through miR-663. This presumably has the function to cut off the fresh supply of PP5 from the cytoplasm. In order to get a better understanding of this late stress response, we analyzed the mRNA/miR-663 and the protein levels of HDF5 cells after H_2O_2-treatment over the course of time. On the mRNA level a clear correlation between the levels of miR-663 and PP5-mRNA was visible, another piece of proof for an at least indirect interaction (Figure 22).

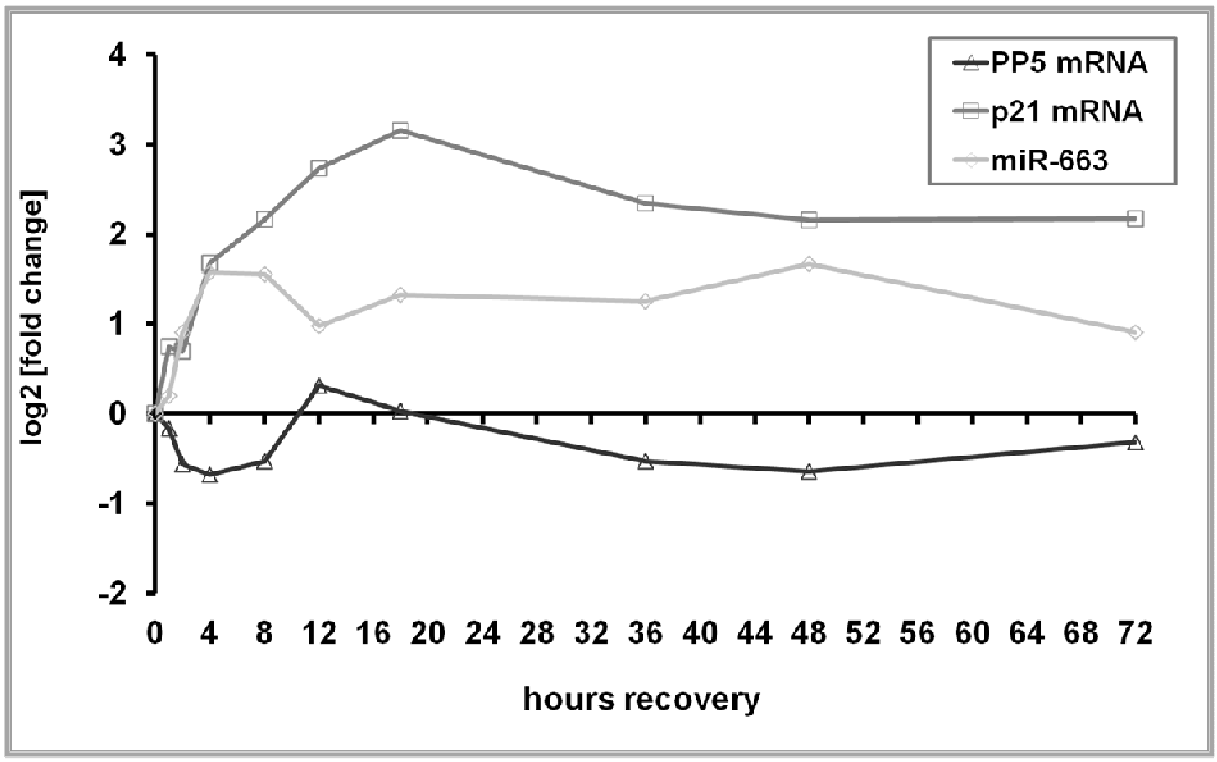


Figure 22: Time course of miR-663 and mRNA of PP5 and p21 after HDF5 cells were treated for 1 h with 100 µM H_2O_2 and recovered in normal media for the indicated time

Also the increased levels of p21-mRNA upon oxidative stress treatment matched the results from the analysis of the protein levels and supported our assumption that p21 was the cause of the temporal growth arrest seen with the MTT-assays. The protein analysis confirmed the results from previous experiments regarding the behavior of p21 and the different forms of PP5 (Figure 20). Both analyses indicated that the peak of the stress reaction was around 24 h post treatment and that the levels of mRNA and proteins were returning to normal afterwards. This correlates with the results from the MTT-assay (Figure 8) and demonstrates that the stress was at a sub-lethal level and the cells were resuming their growth after the damage was repaired.

4. Discussion

Considering the results of our experiments and the findings made by other research groups, miR-663 is undoubtedly of great importance for the regulation of the cell cycle and seems to play a vital role in cancer cell development and cellular stress response. In various publications it was demonstrated that miR-663 is differentially regulated in tumor cells and that it possesses tumor suppressive properties in most cases. Yet the mechanism how miR-663 acts on the cells is still not clear. Some targets of miR-663 have already been identified like JunB and JunD (Tili et al. 2010), TGFB1 (Zhi-Yong et al. 2011).

With our experiments, we demonstrated that the treatment of HDF5 cells with a sub-lethal dose of H_2O_2 results in a temporal growth arrest. In regard to the levels of miR-663 and PP5 the oxidative stress treatment did mimic the effect of replicative senescence and stress treatment with PUVA and tBHP. Further it was shown that the inhibition of miR-663 has a pro-proliferative effect and the transfection of cells with miR-663 has an anti-proliferative effect. This is in line with the results of various publications that found evidence for a tumor suppressive effect of miR-663. Under oxidative stress conditions however the inhibition of miR-663 resulted in an anti-proliferative effect. Thus it seems that miR-663 is necessary for an effective response to cellular stress.

To link miR-663 to its putative target PP5 we conducted several luciferase reporter assays which were all inconclusive. First HDF5 cells were used for the experiments which were not ideal because of their limited growth and their low stress tolerance to repeated transfections. HEK-293 cells seemed to be a good alternative because of their high growth rates and their high stress tolerance. Another possible problem of the assay was the depletion of the artificially introduced miR-663 before the cells were transfected with the reporter constructs. Thus we created HEK-293 cells which were expressing miR-663 in a stable manner by transfecting them with pcDNA™6.2-GW/EmGFP-miR-663. This way we could assure that at the time we transfected the cells with the reporter constructs miR-663 was still present in the cells and thereby could act on the mRNAs transcribed from the plasmid. Still the results were not conclusive and later experiments showed that HEK-293 cells posses a very high basal level of PP5 compared to HDF5 cells. The endogenous PP5 mRNA could be acting as a sponge for any miR-663 introduced to the cells and thereby leaving no or too few miR-663 to act on the mRNAs derived from the Dual-glow plasmid. The usage of cells which are not expressing miR-663 in a native way and posses a relatively low level of PP5 such as NIH-3T3 cells would pose a solution for these problems. Disappointingly the NIH-3T3 cells silenced or got rid of the transfected plasmids after showing high transfection efficiencies in the first days post transfection. The usage of CHO cells would have been one more favorable alternative, but as time and material was running out we had not the resources to perform such an experiment. A different explanation for the problems we experienced could be that our reporter constructs each contained only one putative

binding site for miR-663 and that for an effective inhibition more or all of the three binding sites are necessary. Yet we must admit that the direct interaction between miR-663 and PP5 and therefore the suggested mechanism for the late DNA damage response remains to be proven.

For an at least indirect interaction between miR-663 and PP5 we found very convincing proof in all of our experiments. The anti-proliferative effect of miR-663 could be mimicked perfectly by knocking PP5 down via siRNA. Transfection with miR-663 decreased the protein level of PP5 and inhibition of miR-663 resulted in less reduction of PP5 after oxidative stress treatment. When analyzing the mRNA level of stressed cells there is a clear correlation between PP5 and miR-663 over the course of time. PP5 and miR-663 are definitely linked together the only question is if this interaction is direct or mediated by another molecule. JunB and JunD which are both parts of the AP-1 (activator protein 1) transcription factor are possible candidates for linking miR-663 to PP5 indirectly.

For the mechanism behind the temporal growth arrest which was induced by the oxidative stress treatment we came up with the following theory:

After its synthesis in the cytoplasm PP5 gets translocated into the nucleus. In the nucleus PP5 transforms into an active nuclear form of approx. 90 kD by forming a dimer or a stable complex with another protein after proteolytic cleavage of the C-terminus and/or the N-terminus. By removing the autoinhibitory region of the C-terminus and/or a part of the N-terminal TPR-regions PP5 would increase its phosphatase activity substantially. The PP5 dimer or complex could be stabilized by disulphide bonds between the remaining TPR-regions which is supported by our experiments under high reducing conditions. Another research group also came to the conclusion that induced by stress treatment PP5 may form enzymatically active multimers stabilized by disulphide bonds between the TPR-regions after being proteolytically cleaved (Zeke et al. 2005). This active form then represses the phosphorylation of p53 at Ser15 directly or indirectly and thereby prevents the activation of the transcription of p21. Although it has already been published that recombinant PP5 can de-phosphorylate $p53^{Ser15}$ *in vitro* it needs to be demonstrated that the active nuclear form of PP5 at 90kD can do the same by conducting a phosphatase assay with $p53^{Ser15}$.

Under stress conditions the 90 kD form of PP5 gets shuttled out of the nucleus, p53 gets phosphorylated and activates the transcription of p21. This leads to a growth arrest by blocking the G_1/S-phase transition. In order to maintain the low level of PP5 in the nucleus, miR-663 cuts off the fresh supply from the cytoplasm by inhibiting the translation of PP5. The PP5 form at 45 kD may be a result of proteolytic cleavage of the predominant form at 58 kD in order to prevent the translocation into the nucleus or it can result from the active nuclear form which got shuttled out of the nucleus after being dismantled. This theory is supported by our experiments which show that the protein levels of the 90 kD form and the predominant form at 58 kD decrease after oxidative stress treatment or transfection with miR-663. Inhibition of miR-663 leads to less reduction of the protein levels. Another interesting fact is that the protein level of the 45 kD form gets increased by oxidative stress and that this effect can be reduced by inhibiting miR-663. The immunofluorescence assays, which are illustrating the mechanism of the early stress response via shuttling of nuclear

PP5, and the analysis of the mRNA levels over the course of time further support our theory and the results from the protein analyses.

There exists also evidence that PP5 inactivates ASK1 when cells are treated with H_2O_2 (Morita et al. 2001). Thereby PP5 inhibits ASK1-dependent apoptosis, so the downregulation of PP5 which resides in the cytoplasm through miR-663 would lead to apoptosis instead of a temporary growth arrest if it would be severe enough. In that way the level of miR-663 could be determining if the cell is trying to repair the DNA damage or becomes apoptotic.

Still the identity of the active nuclear form of PP5 must be determined and the interaction between miR-663 and PP5 needs to be clarified. A suitable way to identify the form at 90 kD might be by isolating the protein via immunoprecipitation and performing a MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry) with the purified protein.

But even after proving the direct interaction between PP5 (90kD) and $p53^{Ser15}$ and discovering the true identity of the active nuclear form of PP5, the mechanisms behind the proteolytic cleavage of PP5 and the shuttling of PP5 still remain to be investigated. By analyzing the amino acid sequence of PP5 with the NetNES 1.1 Server provided by the Technical University of Denmark, we found a putative NES (Nuclear Export Signal) between amino acid number 165 and 172 of PP5, which is necessary for the nuclear export, mediated by XPO1 and can be blocked by leptomycin b (Calbiochem). The access to the NES may be blocked as long as PP5 forms a dimer or a stable complex with another protein and remains in its active nuclear form. The cellular response to DNA damage then somehow breaks up the dimer or complex and thereby leads to its translocation to the cytoplasm. A colleague (Lucy Terlecki) has already performed the first immunofluorescence assays coupled with leptomycin b treatment and despite of having still some issues, the first results are looking very promising. Another possibility for getting a better insight into these mechanisms could be the creation of PP5 with mutations in the NLS, the NES, the serine residues in the TPR-regions or the binding sites for the proteases which are cleaving PP5. The identity of the proteases which are cleaving PP5 and their regulation would also be interesting targets for following experiments.

Recently a paper was published (Yi et al. 2012) in which the authors stated that miR-663 has pro-proliferative properties and acts as an oncogene in nasopharyngeal carcinoma (NPC) cells and that they identified p21 as a direct target of miR-663. This contradicts our theories and results in many ways. The fact that they found miR-663 upregulated in NPC cells compared to human immortalized nasopharyngeal epithelium cells does not necessarily conflict with our findings as miR-663 was already identified to act as an oncogene by contributing to the proliferation of lung cancer cells (Zhi-Yong et al. 2011). The cell cycle control of tumor cells is highly altered and cannot be compared to native cells. To verify p21 as a direct target of miR-663 they conducted a luciferase reporter assay using the same plasmid as we did. However the way they performed the assay is not conclusive. We found

compelling evidence for our theories by performing various independent experiments using a wide range of techniques. Nevertheless the way they conducted the luciferase assay must be further investigated and revised.

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