Modulating the p53 response to DNA damage by applying different perturbations types

Dissertation

zur Erlangung des akademischen Grades
doctor rerum naturalium
(Dr. rer. nat.)

im Fach Biologie
ingereicht an der

Lebenswissenschaftlichen Fakultät
der Humboldt-Universität zu Berlin

von

Elena Cristiano, M.Sc.

Präsident der Humboldt-Universität zu Berlin
Prof. Dr. Jan-Hendrik Olbertz

Dekanin/Dekan der Lebenswissenschaftlichen Fakultät
Prof. Dr. Richard Lucius

Gutachter/innen: 1. Prof. Andreas Herrmann
                  2. Prof. Alexander Löwer
                  3. Dr. Jana Wolf

Tag der mündlichen Prüfung: 05.04.2016
TABLE OF CONTENTS

1 ABSTRACT .................................................................................................................. 4

2 INTRODUCTION ........................................................................................................... 8
  2.1 P53, THE GUARDIAN OF THE GENOME ................................................................. 8
      2.1.1 P53 response after DNA damage ................................................................. 8
  2.2 P53 DYNAMICS IN SINGLE CELLS AFTER DNA DAMAGE ................................... 11
  2.3 ENVIRONMENTAL AND CELLULAR PERTURBATIONS OF P53 ACTIVATION UPON DNA DAMAGE ................................................................. 11
      2.3.1 P53 and temperature ...................................................................................... 12
      2.3.2 P53 and cellular state ...................................................................................... 13
      2.3.3 P53 and NFκB crosstalk ................................................................................ 14
  2.4 CELLULAR MODELS ............................................................................................... 17

3 RESULTS ....................................................................................................................... 18
  3.1 TIME LAPSE MICROSCOPY AND AUTOMATED IMAGE ANALYSIS ....................... 18
      3.1.1 Feature analysis of cellular trajectories ......................................................... 20
  3.2 P53 IS NOT TEMPERATURE COMPENSATED ......................................................... 22
      3.2.1 P53 target genes respond to different temperature in distinct ways ............... 27
  3.3 P53 AND NFκB PATHWAY INTERACT UPON DNA DAMAGE .................................. 31
      3.3.1 Stimulation with TNFa prior to γ-irradiation does not affect the p53 response .... 31
      3.3.2 Inhibition of IKK2 prior γ-irradiation changes the dynamic behavior of p53 ........ 34
      3.3.3 Comparisons of different IKK2 inhibitors show that the p53 changes in dynamics are not side effects of the inhibitor ................................................................. 44
      3.3.4 Inhibition of TAK1 greatly affects p53 dynamics in single cells ....................... 47
      3.3.5 P65’s transcriptional activity is necessary to modulate p53 dynamics after γ-irradiation .............................................................................................................. 50
      3.3.6 Wip1 may contribute to linking the p53 and NFκB pathways ............................ 55
      3.3.7 Transcriptional activity of p53 is changed by inhibiting IKK2 prior γ-irradiation .... 60
  3.4 CELLULAR STATE AFFECTS P53 DYNAMIC AFTER γ-IRRADIATION ......................... 61

4 DISCUSSION .................................................................................................................. 67
  4.1 P53 DYNAMICS IN SINGLE CELLS ARE MODULATED BY A VARIETY OF PERTURBATIONS ................................................................. 67
  4.2 TEMPERATURE CHANGES AND P53 DYNAMICS .................................................. 67
  4.3 NFκB AFFECTS THE DYNAMICS OF P53 IN IRRADIATED CELLS ............................ 69
4.3.1 TNFα stimulation does not interfere with p53 activation upon irradiation in A549 and MCF10A cells.................................................................................................................................................................................. 69
4.3.2 IKK2 inhibition changes p53 dynamics in A549 and MCF10A cell lines and also affects p53 target genes expression........................................................................................................................................................................................................... 70
4.3.3 Nuclear translocation of p65 is necessary to shape p53 activation after irradiation ... 71
4.3.4 Wip1 may play a partial role in the p53 and NFkB crosstalk after irradiation .......... 72
4.3.5 TAK1 inhibition changes the characteristic of the first p53 pulse ......................... 73
4.3.6 P53 target gene transcription level are affected by IKK2 inhibition ....................... 73
4.3.7 How can we understand the p53 and p65 interaction? ........................................ 74
4.4 Cellular state affects p53 dynamics............................................................................ 75

5 CONCLUSIONS........................................................................................................... 76

6 MATERIALS AND METHODS..................................................................................... 77

6.1 Materials..................................................................................................................... 77

6.1.1 Antibodies, chemicals and kits .............................................................................. 77

6.1.2 Devices .................................................................................................................. 78

6.1.3 Solutions, buffers and cell culture media ............................................................... 79

6.1.4 qPCR Primers ........................................................................................................ 80

6.2 Methods .................................................................................................................... 81

7 REFERENCES............................................................................................................. 85

8 ACKNOWLEDGMENT................................................................................................ 90
1 Abstract

The tumor suppressor p53 plays important roles in maintaining cellular homeostasis and in preventing the formation and development of human malignancies. P53 is a main hub in the signaling response to endogenous and exogenous stress signals. After DNA damage p53 accumulates in the nucleus and regulates differential expression of target genes and cell fate decisions. Most tumor therapies, besides surgical excision, are based on the induction of DNA damage through radiation or treatment with chemotherapeutic drugs. In order to develop better treatments against cancer, it is important to understand p53 activation and function after DNA damage and to study how different perturbations may affect its activity. Previous studies on p53 activation after DNA damage have reported that it shows a series of regular discrete pulses of protein accumulation over time at the single cell level. In this work A549 and MCF10A p53 reporters cell line were used to investigate how p53 dynamics after DNA damage were affected by changes in temperature, by changes in the state of the NFκB pathway and changes in the provided growth factors. Time-lapse florescent microscopy was used to obtain single cell data with high temporal and special resolution at the single cell level. Surprisingly A549 cells treated with γ-irradiation showed higher level of p53 accumulation and increased time between p53 pulses when imaged at 30°C than cells imaged under physiological conditions. Cells imaged at 40°C showed instead higher p53 pulse frequency. P53 target gene expression was also affected by these changes in dynamics. In both A549 and MCF10A cells, p53 dynamics were changed by NFκB pathways inhibition but not activation via TNFα. Upon inhibition of the NFκB pathway the timing between p53 pulses was increased leading to changes also in p53 target genes expression. In MCF10A cells experiments done under different medium conditions proved that p53 dynamic in this cell line was shaped mainly by the presence of EGF and hydrocortisone that are usual components of the media. EGF leads to a more pulsatile p53 behavior while hydrocortisone completely abrogates the p53 response. Taken together these results showed that the p53 response after DNA damage could be greatly affected by different cellular conditions and signaling network states. These discoveries pointed out the need to study more systematically the relationship between the p53 response to a given stress and the cellular state in order to make cancer therapies more useful and effective as many factors like oncogene activation, accessibility to
nutrients or growth factors, or even pharmacological treatments could potentially affect p53 activation.
Zusammenfassung

Zugang zu Nährstoffen oder Wachstumsfaktoren oder sogar pharmakologische Behandlungen die p53-Aktivierung verändern.
2 Introduction

2.1 P53, the guardian of the genome

The majority of human cancers acquire mutations that abrogate the p53 tumor suppressor network and, as a consequence, p53 is one of the most extensively studied proteins in cancer research. As a tumor suppressor it inhibits uncontrolled cell proliferation and ensures genomic integrity, while a loss of its regulatory function causes uncontrolled tumor growth (Vousden and Lu, 2002). P53 can be activated in response to DNA damage, oncogene activation, or hypoxia, and orchestrates biological responses such as apoptosis, cell-cycle arrest, senescence and DNA repair (Vousden and Lu, 2002).

Under basal conditions, p53 steady state is maintained at low level by the action of the ubiquitin ligase Mdm2 (mouse double minute 2 homolog) that targets p53 to degradation (Haupt et al., 1997). After activation by intracellular or extracellular stress stimuli, p53 response comprises three basic steps: stabilization of p53, sequence-specific DNA binding, and transcriptional activation of target genes (Yee and Vousden, 2005). P53 stabilization is primarily achieved through events that disrupt its interaction with Mdm2 and a consequent increase of nuclear p53 levels; these events involve the phosphorylation of the N-terminal transactivation domain of p53 by specific upstream signal kinases (Pei et al., 2012).

P53 gene transcription regulation is mediated by binding to specific conserved DNA regulatory sites in promoter regions (Sengupta and Harris, 2005). Post-translational modifications in the N-terminal transactivation domain and acetylation of the C-terminal domain regulate not only p53’s DNA binding affinity but also its transcriptional activity (Gu and Zhu, 2012). P53 in fact may activate or repress gene transcription by recruiting transcriptional activators such as histone acetyltransferase CBP/p300 (Iyer et al. 2004), or transcriptional repressors such as histone deacetylases Sin3 (Zilfou et al., 2001; Murphy 2003) to the promoter.

P53 release from the active state is promoted by several autoregulatory negative feedback loops, for example with Mdm2 and the phosphatase Wip1 (Harris and Levine, 2005).

2.1.1 P53 response after DNA damage

In response to γ-irradiation p53 is stabilized and activated via post-translational modification by the upstream kinases that sense the DNA damage; once activated p53 regulate the transcription of genes promoting cell cycle arrest, DNA repair apoptosis and senescence. Each type of DNA lesions triggers the activation of specific members of the phosphatidylinositol
3-kinase-related kinase (PIKK) family. Double strand breaks (DSBs) are sensed by ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK). Single stranded DNA resulting from replication errors or UV-radiation induced damage is recognized by ATM and RAD3-related (ATR) (Sengupta and Harris, 2005).

After the induction of DSBs by $\gamma$-irradiation, ATM is activated by auto-phosphorylation and recruited to the sites of DNA damage foci by the Mre11-Rad50-Nbs1 (MRN) complex that acts as a DSB sensor. Active ATM phosphorylates various downstream signaling components including Chk2, Mdm2 and p53. Phosphorylation of the N-terminal p53 region on Ser15 disrupts the binding between p53 and Mdm2 leading to p53 stabilization and accumulation. P53 accumulation is also increased by degradation of Mdm2 that is provoked by phosphorylation by ATM on Ser395 that leads to auto-ubiquitination and proteasomal degradation (Khosravi et al., 1999) (Figure 1). It has been suggested that the level of nuclear p53 is dependent on the extent of DNA damage and it correlates with cell fate. Low levels of p53 after a moderate and tolerable DNA damage result in cell cycle arrest, which enables DNA repair. High levels of DNA damage are thought to induce a tremendous increase in p53 followed by cell death (Vousden and Lu, 2002).

Figure 1: Schematic representation of ATM dependent p53 activation after DSBs. ATM (ataxia telangiectasia mutated) and Chk2 (Checkpoint kinase 2) stabilize p53 by disrupting its interaction with the E3 ubiquitin ligase Mdm2 via phosphorylation. P53 up-regulates the transcription of Mdm2 (mouse double minute 2 homolog) and Wip1 (protein phosphatase 1) that negatively feed back into the circuit. Wip1 de-phosphorylates p53 and ATM, while Mdm2 targets p53 to degradation. Green arrows indicate activation through protein interaction and phosphorylation, dashed green arrows depict transcriptional up-regulation and red arrows show inhibition.
Cell cycle arrest is mediated by p21 that inhibits cyclin dependent kinase 2 (CDK2) and cyclin dependent kinase 1 (CDK1) and prevents the G1/S and G2/M transition, respectively. GADD45 is also activated and participate in the inhibition of G2/M transition. Cell cycle arrest gives time to the cells to repair the DNA damage and prevents DNA replication or mitosis in the presence of damage (Sengupta and Harris, 2005; Vousden and Lu, 2002; Stark and Taylor, 2006).

P53 mediates apoptosis after DNA damage by activating PUMA, NOXA and BAX. Upon activation, PUMA and NOXA interact with bcl-2 anti-apoptotic proteins, freeing BAX that is then able to activate the release of cytochrome-c from the mitochondria and to initiate the cell death program via caspase activation (Shibue T. et al., 2006; Nakano K. and Vousden K.H. 2001).

P53 activation may also mediate senescence and DNA repair by activating PML and XPC respectively. The promyelocytic leukemia (PML) tumor-suppressor is the key organizer of PML Nuclear Bodies (NBs). PML acts as the scaffold of PML-NBs allowing nuclear partner proteins to shuttle in and out, a process that is regulated by SUMO-mediated modifications and interactions. NBs are implicated in multiple cellular processes including virus defense, apoptosis, and senescence (Ivanschitz L. et al., 2013).

XPC (Xeroderma pigmentosum, complementation group C) is involved in the global genome nucleotide excision repair (GG-NER) by acting as damage sensing and DNA-binding factor component of the XPC complex. XPC screens and senses DNA single strand and bind to it allowing the other XPC components to bind and initiates the nucleotide excision repair mechanism to take place (Van Der Spek P.J., 1996).

Regulatory components of the p53 network, such as Mdm2 and the phosphatase Wip1, are among the target genes. Up-regulation of Wip1 after DNA damage leads de-phosphorylation of ATM and p53 that leads to the subsequent degradation of P53 and the switching off of the DNA damage response pathway (Lu et al., 2007).

Most tumor therapies, besides surgical excision, are based on the induction of DNA damage through radiation or treatment with chemotherapeutic drugs. Cancerous tumors are characterized by cell division, which is no longer controlled as it is in normal tissue. Normal cells stop dividing when they come into contact with like cells, a mechanism known as contact inhibition while cancerous cells lose this ability. Cancer cells no longer have the normal checks and balances in place that control and limit cell division. The faster the cells
are dividing, the more likely it is that chemotherapy and DNA damage will kill the cells, causing the tumor to shrink. Cancerous cells that maintain wild type p53 can still activate apoptosis and senescence after DNA damage. For this reason wild type p53 is considered to be a radiosensitizer and DNA damage treatments are therefore generally more effective in the absence of p53 mutations.

2.2 P53 dynamics in single cells after DNA damage

In this study, dynamics of p53 is measured in single cells with high temporal resolution via time-lapse fluorescence microscopy. Many studies have already underlined the importance of measuring dynamic properties of signaling pathway molecules in order to better understand cellular response and cellular fate. The first study that connected the dynamics of a signaling molecule with differences in downstream cellular outcomes was the stimulus-specific activation of the MAP kinase pathway. Stimulation with epidermal growth factor led to a transient activation of the kinase, while nerve growth factor induced a sustained response. These differential dynamics induced proliferation and differentiation, respectively (Marshall, 1995; Nguyen et al., 1993). Concerning p53, recent studies performed via time-lapse microscopy using fluorescent reporters have revealed that differences in dynamics influence the expression rate and dynamics of p53 target genes as well. This confirms that information on a particular signaling pathway is contained not only in the state and localization of its components but also in how these components change dynamically over time (Purvis and Lahav, 2013).

In order to characterize the dynamic properties of signaling molecules it is important to observe changes in single cells. Measurements at the population level in MCF7 cells have reported that p53 after DNA damage was characterized by damped oscillation, while measurements at the single cell level demonstrated that p53 actually showed un-damped oscillation. This highlights that the underlying dynamic property of a system can be masked in population studies by factors like loss of synchronicity (Lahav et al., 2004) that may be caused by stochastic fluctuations in RNA and protein levels or differences in cellular states such as cell cycle phase (Loewer and Lahav, 2011; Spencer et al., 2009).

2.3 Environmental and cellular perturbations of P53 activation upon DNA damage

The observation that changes in p53 dynamics correlate with changes in gene transcription and consequently cell response leads to the hypothesis that even mild perturbations to the p53
pathway influencing dynamics may have an impact on cellular fate. To test this hypothesis, I focused on how p53 response after DNA damage is influenced by physical perturbation like temperature, by cellular state like proliferation state, or by the state of another pathway related to DNA damage like NFκB.

2.3.1 P53 and temperature

2.3.1.1 The effects of temperature on chemical reactions

Temperature has a major effect on chemical reactions. The Maxwell-Boltzmann distribution theory predicts that the higher the temperature is, the higher is the percentages of molecules that have enough energy to be able to react after collision. A general rule of thumb derived from Arrhenius equation (Equation 1) is that increasing the temperature by 10°C leads to a doubling of the kinetic rate of a reaction. This is usually true if the temperature range is close to room temperature. Arrhenius equation correlates the kinetic constant rate $K$ of a chemical reaction with the absolute temperature $T$. More specifically the higher is $T$, the higher $K$ gets and, vice versa, the higher the activation energy $E_a$ is, the lower $K$ gets.

$$K = A e^{-E_a/RT}$$

Equation 1: Arrhenius’ equation links the kinetic rate constant $K$ of a chemical reaction with the absolute temperature $T$ (in Kelvins), where $A$ is the pre-exponential factor that expresses the fraction of reactant molecules that possess enough kinetic energy to react, as governed by the Maxwell-Boltzmann law, $E_a$ is the activation energy and $R$ is the universal gas constant.

In biological system the majorities of reactions are catalyzed by enzymes. So while the principle of higher kinetic rates at increased temperature still holds true, in reality most enzymes and proteins start to denature at temperature approaching 40°C, leading to a decline in the reaction rates. Many enzymes in fact have an optimum catalysis rate coinciding with the body temperature of 37°C. The human core body is usually kept at constant temperature of 37 °C while the extremities like hands and feet may show 2-4 degrees lower temperatures; depending on the external weather condition the extremities temperature may be even lower (Frank S.M et al., 1994). The human body can also experience higher temperature of up to 42°C during fever.
2.3.1.2 P53 in relationship with temperature changes

Changes in temperatures may affect greatly the dynamics and activation patterns of signaling pathways. For biochemical networks, it is hard to predict the effect that these changes may have on the final outcome and on the dynamic behavior of key players. The enzymes, in fact, due to their own specific protein stability may respond differently at varying temperature changes. For this reasons some key cellular pathways, like the circadian clock, are temperature compensated. Temperature compensation ensures that the day and night rhythm is not affected by the seasonal temperature change but only by light and dark cycles (Barrett et al. 1995, Sorek and Levy, 2012). Considering the important role of p53 in the cell, we hypothesized that its activation and dynamical behavior would be temperature compensated post DNA damage as well.

Previous studies of p53 in relation to temperature have mainly explored the relationship between temperatures and binding affinity with DNA in WT p53 and p53 mutant.

In 50% of Human cancer p53 is mutated and 95% of the mutations occur in the DNA binding domain (Nigro J.M., et al. 1989, Hainaut P. and Hollstein M. 2000). As a result from these studies, it emerged that some p53 mutant can recover p53-binding activity by lowering the temperature (Zhang W. and Guo X.Y., 1994).

2.3.2 P53 and cellular state

The reduced blood supply to tumor tissue influences its microenvironmental conditions, such as the supply of oxygen, nutrients and growth factors. Chronic starvation seems to be important factors, which may determine proliferation, metabolism, and the response of tumor cells to various treatments. The effects of serum starvation on cells have important implication on the overall cell fate and also cell signaling pathway activation. In a previous study it has been shown that serum starvation prevents senescence in the presence of DNA damaging agents without abrogating p53 activation (Leontieva et al., 2010). Another study has shown that in normal cells serum starvation induces cell cycle arrest via ATM dependent p53/p21 activation and protects from cisplatin induced toxicity. In contrast, proliferation of cancer cells is only moderately reduced by serum starvation whereas cisplatin toxicity is enhanced (Shi et al. 2012).

Growth factors and mitogenic signals present in the serum are activating many signaling pathways such as MAPK and AKT/PI3K, that boost cellular proliferation and also shape activation of other signaling pathways that cross-talk with them such as p53. This cross-talk ultimately determines cellular outcomes. In this context, cell culture environment is an
artificial setting where cells are pushed towards a fast pace of proliferation that is not observed in intact tissues. Understanding when it is opportune to perform an experiment under starving condition is very important in order to obtain results that are reliable and closer to the in-vivo condition. In this study I explored the effect of media starvation on p53 dynamics after DNA damage in MCF10A cells.

2.3.3 P53 and NFκB crosstalk

The NFκB transcription factor family is involved in many pathways and can be activated by several stimuli such as TNFα, growth factors, LPS and genotoxic stress. NFκB belongs to a category of transcription factors that is rapidly activated upon stimulation. Sequestered in the cytoplasm under basal conditions by IκBα, NFκB is rapidly transported to the nucleus upon activation due to phosphorylation and degradation of IκBα. The most abundant form of the transcription factor, a dimer of the subunits p65 (RelA) and NFκB1 (p50), responds to most stimuli and activates cellular responses ranging from proliferation to inflammation and apoptosis. TNFα together with IL-1 are the two major pro-inflammatory cytokines that activate the canonical NFκB pathway. Upon binding of the cytokines to the TNFα receptor (TNFR) the signaling cascade is activated leading to a series of events that trigger the activation of the IKK complex. The IKK complex consists of catalytic kinase subunits (IKK1 and IKK2) and the regulatory non-enzymatic scaffold protein NEMO. The activated IKK complex phosphorylates and targets IκBα to proteasomal degradation allowing p65 to enter the nucleus. P65 activation leads also to the expression of the IκBα gene which function as a negative feedback loop terminating the NFκB response (Figure 2) (Scheidereit C., 2006; Gilmore 2006; Hoffmann et al., 2006).

P65 activity is also regulated by several post-translational modifications like phosphorylation and acetylation, and recently methylation and ubiquitination also have been proposed (Natoli G., 2008; Yang X.D., et al., 2009). Phosphorylation can result in increase and decrease of p65 activity; the most studied and known phosphorylation sites are: Ser 276, Ser 536 and Ser 468. Phosphorylation of Ser 536 represents an active mark for canonical NFκB activation and is mediated by IKKs (more specifically IKK2), by ribosomal subunit kinase-1 (RSK1), and TANK binding kinase (TBK1).
Figure 2: Schematic representation of p65 activation mediated by TNFα stimulation. RIP (Receptor-Interacting Protein) and TAK1 (Transforming growth factor (TGF)-β-activated kinase 1) phosphorylate and activate the IKK (I-KappaB-Alpha kinase complex). The IKK complex phosphorylates IκBα (I-KappaB-Alpha) that leads to ubiquitination and then leads to the degradation of IκBα by the proteasome, resulting in the translocation of p65-p50 to the nucleus. The IKK complex is formed by IKK1, IKK2 and NEMO, IKK2 and IKK1 are the catalytic subunits while NEMO is the regulatory one. Once in the nucleus the p65-p50 dimer binds to its consensus sequence (5-GGGACTTTC-3) and positively regulates the transcription of genes involved in immune and inflammatory responses, cell growth control, and apoptosis. Green arrows indicate activation through protein interaction and phosphorylation, dashed green arrows depict transcriptional up-regulation, and red arrows show inhibition.

Recent studies have shown that similar to p53, the NFκB pathway is also activated upon γ-irradiation (Janssens and Tschopp, 2006; Wu and Miyamoto, 2007). Both p53 and p65 share ATM as the main activator of the pathway (Li et al., 2001; Piret et al., 2009). ATM activates NEMO and by shuttling into the cytoplasm triggers the activation of TAK1 that leads eventually to activation of the IKK complex and consequently release of p65 in the nucleus (Figure 3) (Hinz et al., 2010).
Figure 3: Schematic representation of ATM dependent P65-p50 and p53 activation after DSBs. ATM is activated upon DNA damage, phosphorylates and stabilize p53 and at the same time phosphorylates and activates NEMO and TAK1. Activation of NEMO and TAK1 leads to activation of the IKK complex that in turn phosphorylates IkBα and targets it to degradation in order for the p65-p50 to be released into the nucleus. IKK2 can phosphorylate p53 and target it to degradation while Wip1 can de-phosphorylate p65 thereby inhibiting high affinity binding with DNA promoters.

ATM is not the only player that is shared between the p53 and NFκB pathways. Previous studies have reported that IKK2 upon DNA damage can phosphorylate p53 on Ser 362 and 366 promoting p53 degradation via the SCF complex and independently from Mdm2 (Xia Y., et al. 2009). Moreover other studies have shown that IKK2 inhibition can stabilize p53 accumulation and acetylation with subsequent activation of p21 and apoptosis (Yang et al., 2010). It has also been reported that Wip1 (p53 negative regulator) acts as phosphatase of Ser 536 of p65. Dephosphorylation at S536 reduces p65’s interaction with p300 and hence transcription of NFκB target genes (Chew et al., 2009).

Taken together these evidences pointed out that there might be a crosstalk between p53 and NFκB pathway and that the state of one pathway may affect the induction of the other. In this work we investigated how p53 dynamics are affected by the status of p65 upon γ-irradiation.
2.4 Cellular models

To study the p53 response to DNA damage under a variety of experimental conditions on an individual cell level we used a MCF10A and an A549 reporter cell line. MCF10A is an immortalized and non-transformed breast epithelial cell line that expresses wild type p53. It is dependent on epidermal growth factor (EGF) and insulin to proliferate, it is negative for estrogen receptor and it has a stable near-diploid karyotype. Genomic deletions of p16, p14ARF as well as MYC amplifications, arose after immortalization and allow continuous cell culture (Debnath et al., 2003).

A549 is a type II alveolar epithelium lung adenocarcinoma cell line (Foster et al., 1998). This cell line has a K-Ras mutation and although it expresses wild type p53, it was demonstrated to form p53-heat shock 70 protein complexes (Lehman et al., 1991).

Transgenic fluorescent p53 reporters have been previously shown to mimic the dynamics of endogenous p53 (Batchelor et al., 2008; Lahav et al., 2004). MCF10A cells were infected with lentiviruses containing UbC-p53-Venus and EF1α-H2B-CFP (Histone 2 B) transgenes, where p53-Venus is constitutively expressed by the human Ubiquitin C promoter (UbC) and H2B-CFP is constitutively expressed through the human elongation factor 1α promoter (EF1α). The H2B-CFP construct has the sole purpose of marking cellular nuclei so that automated tracking can be done.

The A549 p53-Venus reporter cell line has been described before (Chen at al. 2013). The cells were further transfected via lentivirus infection in our lab with EF1α-H2B-CFP construct to allow automated tracking.
3 Results

3.1 Time Lapse microscopy and automated image analysis

Time-lapse fluorescent microscopy was used to study cellular behavior at the single cell level. Cells were imaged using a Nikon Ti inverted light microscope with a perfect focus system and incubator to maintain temperature at 37°C and CO₂ concentration at 5%. Cells were imaged after γ-irradiation for 24h in 15 minutes intervals in the phase contrast, YFP and CFP channels. After each experiment, flat field images were acquired for each fluorescence channel at increasing level of exposure time to create a calibration mask. This mask was applied to raw images to remove background noise coming from the camera and even out differences of fluorescence illumination in the field of view.

We used a custom written MATLAB software to segment each nucleus according to H2B-CFP fluorescence (Figure 4a, b, c) and to track the cells through all time points of an experiment. Touching nuclei were separated by a watershed algorithm. For each nucleus that was identified during segmentation, a doughnut shape like area of a given width was placed around it. This area was used to estimate fluorescence in the cytoplasm. The algorithm ensured that the estimated cytoplasmic area did not overlap with cytoplasm or nuclei from neighboring cells. (Figure 4 c, d, e, and f). In this manner, we were able to measure the absolute fluorescence intensity of proteins fused with fluorophores localized both in the nucleus and in the cytoplasm at each time point. The coordinate of the center of mass of each nucleus was calculated and the cells were tracked from one time point to the next using a custom algorithm. This algorithm, based on “greedy matches”, connects cells based on distance, area and fluorescence intensity of the nuclear marker. Initial assignments of cells were used to further refine segmentation and tracking. Since p53 localizes in the nucleus, we used only the nuclear absolute fluorescence intensity for the analysis. Integrated p53 nuclear fluorescence intensity, that is the total measured level of p53 in the nucleus, was normalized by the nuclear area for every cell at each time point. Normalization by the nuclear area results in the mean intensity of nuclear p53 that is a measure of p53 nuclear concentration; furthermore normalization by the area avoids changes in the p53 intensity that are due only to aberration in the segmented nuclear area. P53 mean trajectories extracted from each tracked cell are synchronized in-silico to cell division. Cell division is detected in the nuclear marker channel as the fluorescent intensity of the nuclear marker doubling during the S phase of mitosis and dropping back to initial level after cellular division has taken place.
Despite the normalization by the area, p53 mean trajectory over time for each tracked cells presents a great variability after irradiation (Figure 5A). Stochastic events together with different cellular states may influence the actual protein amounts and the response behavior. Previous studies have already reported single cell heterogeneity (Loewer and Lahav, 2011; Spencer et al., 2009). In order to have information on the average behavior from each observed cell population we calculated and plotted the median (Figure 5a solid blue line). To represent the variability of the p53 response after irradiation we plot the interquartile range from the 25th to 75th percentile of all data points (Figure 5B).
A549 cells treated with 10 Gy γ-irradiation A) P53 trajectories of single cells in light blue (100 cells) and median of the p53 response (dark blue line). B) Median of p53 and interquartile range from 25th to 75th percentile of all data points (shaded area).

3.1.1 Feature analysis of cellular trajectories

After obtaining time-resolved p53-YFP trajectories for hundreds of cells for each condition, we proceed to further analyze characteristic features of these measurements. A custom MATLAB algorithm based on a watershed algorithm was used to identify pulses within a trajectory (Loewer et al. 2010). Upon identification of peaks and valleys of maximum and minimum mean intensity, we extracted and statistically analyzed properties like: amplitude of the peak, width of the peak at half maximum, time of peak maximum and slope (Figure 6A).

The period was calculated using autocorrelation and defined as the time of the first maximum of the corresponding function. For calculating integrated intensity (the area under the curve), the area of a rectangle with the height of the first time point was subtracted (Figure 6b, Figure 6c). Wilcoxon rank-sum test was used to compare population properties among different experimental conditions. In order to counteract the problem of multiple comparisons among conditions we further test the p-values with the Holm-Bonferroni method.
Figure 6: A) Features extracted from each trajectory: peaks and valley of maximum and minimum p53 levels (red and green dots), amplitude, width, slope and period; B) The integrated intensity of the induced p53 is obtained by subtracting the basal p53 integrated intensity (rectangular area created by multiplying the first p53 data point by the time); C) Total p53 integrated intensity.
3.2 P53 is not temperature compensated

P53 is a key transcription factor that gets activated upon a variety of stresses. Its function is required for the maintenance of cellular homeostasis. Considering the important role of p53 in the cell, we wanted to assess if p53 activation dynamic after DSBs was temperature compensated. P53 response was measured at 30°C, 37°C and 40°C in A549 untreated or treated with 10 Gy γ-irradiation (Figure 7).

![Figure 7: Median of p53-YFP trajectories (solid line) and interquartile range (shaded area) under normal conditions and upon 10 Gy irradiation at 30°C (left panel), 37°C (middle panel) and 40°C (right panel). Number of cells tracked from each condition range from 100 to 400.](image)

By analyzing features of the individual trajectories, I observed that irradiated cells at 30°C show stronger p53 response compared to cells at 37°C: pulses have a longer width (Figure 8b), the peak of the pulses occurs later (Figure 8C), amplitudes and periods are increased (Figure 8D and E) and the average number of pulses is decreased (Figure 8F). The slope of the increase is not affected (Figure 8A).

P53 dynamics of cells irradiated at 40°C (if compared to 37°C) show the opposite behavior such as: decreased period (Figure 8E, 3.5h for 40°C against 4.5h of 37°C), increased slope and amplitude (Figure 8A, D). The overall p53 dynamic behavior is affected more strongly at 30°C than at 40°C because there is a 7°C temperature gap (against a 3°C) from the physiological temperature condition (37°C). In confirmation of this, p53 induction in cells irradiated at 34°C follows the trend observed in cells treated at 30°C; the alteration, however, is milder (Figure 9).
Figure 8: Box-plot representation of the feature analysis showing the median (red line), the 25th-75th percentile (colored area), the maximum and minimum with 1.5x the interquartile range (whiskers) and the outliers (red cross). A) Slope of increase of the first pulse B) Width of the first pulse C) Timing of the first pulse D) Amplitude of the first pulse E) Period and F) Average number of pulse plotted as bar graph with respective standard deviation.
Surprisingly, control trajectories for 30°C and 40°C conditions show p53 activation as well (Figure 7). The median trajectory of control cells imaged at 30°C show clear p53 pulses with a broad interquartile range, while control cells imaged at 40°C show a conserved first peak and a smaller interquartile range and therefore overall less variability. By examining examples of single cell trajectory we can appreciate that many of the control cells imaged at 40°C show in fact activation of p53 and regular pulses (Figure 10c, right panel). This finding is consistent with studies revealing that hyperthermia could sensitize cells by activating ATM (Guan et al, 2002; Hunt et al, 2007). Examples of single cells trajectories of control cells imaged at 30°C show also p53 activation that resembles p53 activation in the respective irradiated cells (Figure 10a right and left panel).

The median trajectory of control cells imaged at 37°C (Figure 7) show no activation of p53 and a small interquartile range. By examining single cells trajectories we appreciate that indeed the majorities of cells do not show p53 activation while few show spontaneous p53
pulse within the trajectories or full p53 activation with the regular pulsatile p53 behavior (Figure 10B, right panel).

Figure 10: Example trajectories of p53 fold induction in single A549 control cells (right panel) and cells irradiated with 10 Gy (left panel) at 30°C, 37°C and 40°C (A, B, C).

Greater differences in cell dynamics are observed between control cells and irradiated cells under physiological temperature than in irradiated and untreated cells imaged under hypo- and hyperthermia. This difference is reflected also in the feature analysis for control and irradiated cells under 37°C that shows extensive changes (Figure 8). Consistent with the observation made on examples of individual trajectories (Figure 10), the percentage of non-reacting cells is higher in control cells imaged under physiological condition than in control cells under...
hypo- and hyperthermia (Figure 11A), confirming that both hypothermia and hyperthermia can induce p53 activation.

The period calculated for the overall control cell population (Figure 8E) shows great variability for each temperature condition. Moreover the median value of control cells imaged under 37°C and 40°C differs from the one of the respective irradiated cells. This difference could be due to the high heterogeneity in p53 dynamic pattern found in the control group. In fact, by analyzing the period distribution of only the fraction of oscillating cells we can observe no difference between control and irradiated cells imaged at the same temperature (Figure 11C); we can also confirm that changes in temperature affect the timing between peaks of p53 trajectories as the majority of the cells show a timing of 8.5h under 30°C, 4h for cells under 37°C and 3h in cells under 40°C.
3.2.1 P53 target genes respond to different temperature in distinct ways

Changes in p53 dynamics have been shown to affect also p53 target genes transcription (Purvis et al. 2012, Chen et al. 2013, Borcherds et al. 2014). More in detail, these studies revealed that pulsatile p53 behavior is a cellular control mechanism that suppresses high activation of genes for cellular apoptosis, senescence and cell cycle arrest. Pulsing p53 allows the activation of target gene to a degree that the cell can stop, repair the DNA and re-enter into the normal cell cycle. Contrarily, sustained p53 accumulation would lead to high activation of p53 target genes with a consequent cellular outcome leading towards apoptosis or senescence. Changes in p53 dynamics were achieved either by changing the DNA damage type (DSBs lead to a pulsatile p53 behavior while UV treatment leads to a p53 accumulation) (Purvis et al. 2012) or by increasing the dose of the damage (Chen et al. 2013).

In order to study if changes in p53 dynamics observed at different temperature would affect also the expression of p53 target genes and in which manner, A549 cells were harvested after 5h and 24h upon 10 Gy irradiation and under normal conditions at 30°C, 37°C and 40°C. QPCR was performed and the expression of P21, XPC, BAX, PML and Gadd45a was studied (Figure 12). Interestingly, I observed that gene transcription responded with great variability to differences in temperature. Focusing on the fold of induction normalized by the respective control (Figure 12, right panel), it could be detected that PML showed higher level of transcription at 40°C after 5h if compared to 37°C induction and at the same time showed no change at 30°C. Genes like XPC and Gadd45a showed instead higher level of induction after 5h upon irradiation at 30°C if compared to 37°C fold of induction and no induction at 40°C. All these three genes at 24h showed the same level of induction independently of the temperature. P21 and BAX did not show differences in gene induction for all the three temperatures.

When we focus on the non-normalized data (Figure 12, left panel), the gene expression profile observed is generally consistent with the normalized data, but it also bears more variability in gene expression. PML and Gadd45a show higher level of induction at 24h at 40°C and 30°C respectively; and BAX shows higher level of induction after 5h at 40°C while P21 shows higher level of induction at 24h for 30°C and 40°C.
Figure 12: In left panel, bar plots with the average fold of gene induction and standard deviation for PML, XPC, GADD45a, BAX and p21 genes are shown. A549 cells were cultured under 30°C, 37°C and 40 °C and harvested after 5h and 24h after 10 Gy irradiation. In the right panel the fold of gene induction in irradiated cells is normalized by the fold of induction in the respective control.

Difference in gene expression after irradiation at different temperature may point out that there are genes that are more sensitive to changes in p53 frequency, genes that are more sensitive to the total p53 level or genes that responds to both parameters. For this reason the average p53-YFP integrated intensity for each condition was calculated (as described in the previous paragraph 3.1.1). The average integrated intensity at 30°C shows higher values and variability if compared with the one at 37°C (Figure 13). At 40°C the average integrated intensity is almost double of the one at 37°C and shows higher variability.

Figure 13: Bar graphs representing the normalized (upper row) and raw (lower row) average integrated intensity of p53-YFP trajectories.
Taken together these data show that PML seems to be more sensitive to p53 level variations (the rate at which p53 fluctuate in the nucleus), while Gadd45a and XPC are more sensitive to the total p53 concentration in the nucleus. BAX and P21 seem to be not greatly affected by temperature.

As already mentioned in this chapter, previous studies showed that a pulsatile p53 behavior correlates to a more transient p53 gene transcription activity while sustained high p53 levels correlate with strong induction of p53 target gene transcription and terminal cell fates (Purvis et al. 2012; Chen et al. 2013). As p53 level are increased at 30°C I expected also an increase in target gene transcription. This behavior was eventually not observed; it may be important to remark that although p53 levels are higher at 30°C, dynamically it is still mainly oscillating and this may explain why the increase in p53 target genes transcription was not observed.
3.3 P53 and NFκB pathway interact upon DNA damage

We have shown in the previous chapter that p53 dynamics after γ-irradiation are affected by temperature. In this chapter, we analyzed if p53 dynamics could be affected by the state of other signaling pathways as well.

3.3.1 Stimulation with TNFα prior to γ-irradiation does not affect the p53 response

In order to study if p53 dynamics are affected by activation of the canonical NFκB pathway, A549 cells were stimulated with 10ng/ml TNFα 1h prior to 10 Gy γ-irradiation or 2h post irradiation and imaged for 24h. P53 dynamics of the TNFα stimulated cells were compared with cells being only irradiated (control), (Figure 14). However, no differences were observed between control and TNFα treated cells.

Figure 14: Median of p53-YFP trajectories (solid line) and interquartile range (shaded area) of untreated cells upon 10 Gy irradiation (left graph); cells treated with TNFα 1h before irradiation (middle graph); cells treated with TNFα 2h after irradiation.

The effect of TNFα prior 10 Gy irradiation was tested also in MCF10A cells (Figure 15). No differences was observed in cells treated with TNFα 1h, 3h and 5h prior to damage induction.

To validate activation of the NFκB pathway by TNFα, immunofluorescence was performed in untreated and treated A549 and MCF10A cells. As expected both cell lines responded with p65 nuclear translocation 30 minutes after stimulation.
Figure 15: Median of p53-YFP trajectories (solid line) and interquartile range (shaded area) of MCF10A untreated cells upon 10 Gy irradiation (left graph); cells treated with 10ng/ml TNFα 1h, 3h and 5h before 10 Gy irradiation.
Figure 16: Immunofluorescence performed on MCF10A cell (upper panel) and A549 cells (lower panel) treated with 10ng/ml TNFα and harvested after 30 minutes (right column) and the respective control cells (left column).
3.3.2 Inhibition of IKK2 prior γ-irradiation changes the dynamic behavior of p53

In order to test if p53 dynamics were affected by inhibition of the NFκB pathway, A549 cells were treated 1h prior 10 Gy irradiation with 15 µM IKK2 inhibitor (Calbiochem IV, IKK2i) and imaged for 24h.

Surprisingly by preventing p65 activation we observed changes in p53 dynamics upon irradiation (Figure 17) with the time between peaks being larger than in untreated cells.

In Figure 18 we show examples of p53 trajectories observed in the cell populations of untreated cells and IKK2i treated prior irradiation. In the cells treated with IKK2i prior irradiation new phenotypes were present such as cells not reacting, or showing just the first pulse, or showing increasing level of p53, while untreated cells were less variability, as the majority showed a pulsatile behavior;

Figure 17: Median of p53-YFP intensity (solid line) and the interquartile range represented as shaded area of cells untreated before irradiation (violet) and cells treated with 15 µM IKK2i prior irradiation (orange).
In order to study more in detail the difference in p53 dynamic between the two conditions, dynamic features of the trajectories were extracted and plotted (Figure 19). The percentage of non-reacting cells (cells not responding or responding with only one peak) was doubled upon IKK2 inhibition (Figure 19E), consistent with what was observed in example trajectories (Figure 18). The period was increased by 2h for the IKK2i treated cells (6h for IKK2i treated cells and 4h for the untreated one); consequently all pulses peaked at later time points (Figure 19A, B). In order to determine if the observed increase in the period is due to a real shift at the level of single trajectory or is due to the higher variability in treated cells, the distribution of the period was plotted over only the pulsing fraction of cells (Figure 19F). The difference in period was still observed in cells treated with the IKK2i, confirming that indeed a shift to longer timing between peaks occurred.

Differences between the two conditions in the shape of the first pulse were also found: in the IKK2i treated cells the slope of increase was slower for the first and second peak (Figure 19C). This trend was also observed in later peaks but it’s not always conserved throughout all the experiments. On the contrary the slope of decrease of the first pulse was not changed for both conditions and this characteristic is conserved through the experiments (Figure 19C). The width of the first peak is longer in IKK2i treated cells (Figure 19D). It seems that in
IKK2i treated cells p53 accumulation is slower while degradation is at the same rate as in the untreated cells (Figure 19C); this leads to pulses that are not symmetric.

Figure 19: Box plot representation of dynamic features for each experimental condition (untreated cells in light blue and IKK2i treated cells in orange). * p ≤ 0.05 ** p < 0.01 *** p < 0.001, Wilcoxon rank-sum test comparing IKK2i treated population cells to the control cells; null hypothesis: the two populations are the same. F) Representation of the period distribution over the fraction of oscillating cells for untreated cells and IKK2i treated cells.
Taken together these results showed that p53 dynamics are modulated by the state of the NFκB pathway: while activation of the canonical NFκB pathway prior or post irradiation does not affect p53 dynamics, inhibition of the NFκB pathway and more precisely of IKK2 affects greatly the period of p53 trajectories and the shape of the pulses.

The IKK2i inhibitor was tested via Western blot in MCF10A cells. Untreated and cells pre-treated with 15 µM IKK2i were stimulated with 5ng/ml TNFα. In IKK2i treated cells, IκBα is not degraded upon TNFα stimulation indicating a strong IKK2 inhibition (Figure 20).

![Western blot showing IκBα level in MCF10A cells stimulated with 5ng/ml TNFα in the presence or absence of 15 µM IKK2i (Calbiochem IV). Cells were incubated 1h with IKK2i and harvested 15 minutes after TNFα stimulation.](image)

**3.3.2.1 P53 dynamics are modulated by IKK inhibition at different time points**

Next, we tested whether inhibiting IKK at different time points after irradiation would lead to the same modulation of p53 as observed in cells pre-treated with IKK2i prior to irradiation. I added the inhibitor 1.5h, 2.5h and 5h after 10 Gy irradiation and compared the measured p53 response to untreated cells and pre-treated cells (Figure 21).
Figure 21: Median of p53 induction and interquartile range of all data are shown for each condition. Pretreated cells with IKK2i 1h before 10 Gy irradiation (upper-left); cells treated with IKK2i 1h post irradiation (upper right), cells treated 2.5h after irradiation (lower left) and cells treated 5h after irradiation (lower right). Untreated cells are shown in blue as comparison.

Surprisingly, the effect on p53 accumulation is rapidly observed upon treatment, independent of the timing. I estimated that IKK2i acts within 1h from treatment. The timing distribution of the first 5 pulses is shown for each condition (Figure 22). The slope of increase of the first 5 pulses is shown for each condition (Figure 23).

The effect of IKK2i treatment can be seen already in the timing of the first pulse that its shifted to a higher value in cells treated 1.5h after irradiation (Figure 22, T1 green box) and in the decreased slope of increase (accumulation rate) of the second pulse (Figure 23B).

In cells treated 2.5h after irradiation the effect of the inhibitor on the period and slope of increase is detectable from the second pulse on (Figure 22, T2 yellow box, Figure 23B), while treatment 5h after irradiation only affects dynamics and pulse shape from the third pulse on (Figure 22, T3 pink box, Figure 23C).
Figure 22: The distribution of the timing of the first 5 pulses (T1-T5) is shown in box plot for each condition.

Taken together these results show that inhibition of p65 activation upon treatment with IKK2i has the same effect on p53 dynamics (longer period and slower p53 accumulation) independently whether the inhibitor is added prior or post irradiation.
Figure 23: The slope increase of the first 5 pulses is shown for each condition. * $p \leq 0.05$ ** $p < 0.01$ *** $p < 0.001$, Wilcoxon rank-sum test comparing IKK2i treated population cells to the untreated cells (light blue), null hypothesis the two population are the same.
3.3.2.2 IKK2 inhibitor activity proved to be concentration dependent and to increase the percentage of oscillating and regular cell in MCF10A cells

To validate the observed modulation of p53 dynamics in another cell line, MCF10A cells were used. The cells were stimulated with increasing concentration of IKK2i prior to 10 Gy irradiation (Figure 24). The p53 dynamic behavior after irradiation of MCF10A cells showed higher heterogeneity than in A549 cells. In irradiated MCF10A cells only the first pulse is synchronous and therefore observable in median trajectories. After the first pulse, cells lose synchronicity and show a great variety of dynamical patterns ranging from pulsing to accumulation (D. Friedrich, 2014 Master thesis).

Figure 24: Median of p53 induction after 10 Gy irradiation in untreated MCF10A cells (dark blue line) and the interquartile range of all cell population data (shaded area), and for cells treated with 15 µM IKK2i (light blue line and shaded area). Medians of p53 induction in treated cells with lower IKK2i doses are represented in dashed lines.

This heterogeneity was reflected in the wide interquartile range. Surprisingly, cells treated with increasing concentration of IKK2i showed a shift towards a more pulsatile and less heterogeneous behavior. The average number of pulses per trajectory was almost the same in treated and untreated cells, however, the percent of oscillating cells is doubled in IKK2i treated cells. Moreover the interquartile range became smaller at increasing concentration of the IKK2i, indicating also that the trajectories tend to acquire a more homogeneous behavior (Figure 25).

41
In untreated MCF10A cells the period and its standard deviation were very high and they decreased greatly upon treatment with the IKK2i (from 1 µM onward) (Figure 25).

This result indicates that the longer period and higher standard deviation is due to the high heterogeneity of the p53 induction of this cell line. In fact by plotting the distribution of the period over only the fraction of pulsating cells we can observe that untreated cells showed a period distribution on lower values (Figure 26). Cells treated with high doses of IKK2i
showed a shift in the distribution towards higher values consistently with what we observed in A549 cells.

![Distribution of the period for oscillating cells](image)

Figure 26: Representation of the period distribution over the fraction of oscillating cells.

### 3.3.2.3 Western blot experiments on MCF10A confirmed changes in p53 dynamics and showed that IKK2i (Calbiochem IV) is a strong inhibitor

Western blot was performed in untreated and IKK2i treated MCF10A cells prior 10 Gy irradiation. The time course from time point 0h to time point 10h, for either untreated or treated cells, was blotted together with time points 0h, 1h, 3h, 6h and 9h of the other condition.

By comparing p53 dynamics of the time course under both conditions, I observed that in treated cells the second p53 pulse started at time point 8h against time point 6h in untreated cells; moreover the width of the second pulse in treated cells was more defined and limited to a precise time frame (8h to 10h) if compared to the second pulse of untreated cells (Figure 27 upper and lower membrane). These results are consistent with the microscopy data showing that treatment with IKK2 inhibition seems to make the p53 induction after irradiation more regular by increasing the percentage of oscillating cells.

By comparing p53 levels of the two conditions blotted in the same membrane, no difference in the amplitude of the first p53 (time point 3h) was observed confirming the data analysis of the microscopy data where no differences in amplitude were observed either.
Figure 27: Western blot showing p53 level for MCF10A cells treated with either DMSO or 15 µM IKK2i 1h prior 10 Gy irradiation. Cells were harvested every hour for 10 hours after irradiation.

### 3.3.3 Comparisons of different IKK2 inhibitors show that the p53 changes in dynamics are not side effects of the inhibitor

The changes in p53 dynamics observed in cells treated with the IKK2 inhibitor IV from Calbiochem prior to irradiation could be due to unspecific effects of the inhibitor. For this reason p53 dynamics of A549 cells treated with three additional IKK2 inhibitors (Calbiochem sc-514, PS1145 and IMD-Sigma-0354) prior irradiation were compared. The features of p53 dynamics that were changed in A549 cells when treated with IKK2i CB IV (period, timing, p53 slope increases and width of the first pulse) were analyzed.

In Figure 28 the median trajectories of p53 induction for each condition are plotted. Different IKK2 inhibitors showed similar effects on p53 dynamics, specifically a shift to longer periods (Figure 28 B, C, D). A slower accumulation rate of p53 for the first pulse was also detectable in the median p53 trajectories of cells treated with IKK2i sc-514 CB (Figure 28, B) and IMD Sigma-0345 (Figure 28, D).
Figure 28: Median of p53 induction and interquartile range of all data are shown for each condition. Comparison between untreated A549 cells after 10 Gy irradiation and cells treated with IKK2i CB IV, A) Comparison between untreated cells and cells treated with IKK2i sc-524 CB, B) Comparison between untreated cells and cells treated with IKK2i PS1145, C) Comparison between untreated cells and cells treated with IKK2i IMD Sigma-0354 D).

From the quantification of the previously listed key features (Figure 29), I observed that cells treated with any IKK2 inhibitors showed a shift towards longer period and longer width of the first pulse. The slope of increase of the first pulse appeared to be slower except for cells treated with IKK2i PS1145. Also the slope of increase for the second pulse appeared to be slower except in cells treated with IKK2i PS1145 and IKK2i sc-514 CB.
Figure 29: Box plot representation of the analysis of the p53 trajectory features for irradiated A549 cells untreated and treated with 4 different IKK2is. Ns (non significant) * p ≤ 0.05 ** p < 0.01 *** p < 0.001, Wilcoxon rank-sum test comparing treated population cells to the untreated cells (light blue), null hypothesis the two population are the same.

Taken together these data show that the changes observed in p53 dynamics in A549 cells are specific for inhibiting IKK, and not caused by off-target effects. Although in cells treated with
IKK2i CB IV the changes appeared to be slightly more pronounced than in cells treated with the other IKK2 inhibitor molecules, the trend was the same.

3.3.4 Inhibition of TAK1 greatly affects p53 dynamics in single cells

TAK1 is necessary to activate IKK2 via phosphorylation upon DNA damage. In order to study the role of TAK1 in modulating the dynamics of p53 activation after irradiation, A549 cells were treated either with IKK2i CB IV or with a TAK1 inhibitor ([5Z]-7-Oxozeaneol) prior irradiation and imaged for 24h. The p53 median trajectory in TAK1i treated cells prior irradiation showed a big p53 first pulse and high variability (wide interquartile range), pointing out that TAK1 inhibition may be a stronger perturbation than IKK2 inhibition for the p53 pathway (Figure 30).

![Figure 30: Median of p53 induction and interquartile range of all data is shown for each condition. Comparison between untreated A549 cells after 10 Gy irradiation and cells treated with IKK2i CB IV, A) Comparison between untreated cells and cells treated with TAK1i, B).](image)

Treatments with either IKK2 inhibitor or TAK1 inhibitor showed a shift towards longer period, longer width and timing of the first pulse. The slope of increase in cells treated with TAK1i was either not changed (first pulse), or increased (second pulse), (Figure 31).

Cells treated with TAK1i showed a different p53 median trajectory, while the features seemed to follow the trend observed for IKK2i treated cells despite being more pronounced.
Figure 31: Box plot representation of the analysis of the p53 trajectory features for irradiated A549 cells untreated and treated with IKK2i CB IV and TAK1i. Ns (non significant) * p ≤ 0.05 ** p < 0.01 *** p < 0.001, Wilcoxon rank-sum test comparing treated population cells to the untreated cells (light blue), null hypothesis the two population are the same.

Analyzing the single cell example trajectories revealed that treatment with TAK1 inhibitor affected mainly the first p53 pulse that was more pronounced while the following pulses showed the same characteristic as the one observed in untreated cells (Figure 32). These observations were confirmed by the feature analysis, where the amplitude of the first pulse is doubled in cells treated with the TAK1i while the amplitude and the width of the second pulse resembled the measurements of the other conditions (Figure 33).
A549 cells treated with IKK2i CB IV prior 10 Gy IR

Figure 32: P53 trajectory phenotypes in A549 cells treated with 15μM IKK2i CB IV 1h prior 10 Gy irradiated (upper panel), and treated with 3 μM TAKi 1h prior 10 Gy irradiation (lower panel).

A549 cells treated with TAK1i prior 10 Gy IR

Figure 33: Box plot representation of the analysis of the p53 trajectory features for irradiated A549 cells untreated and treated with IKK2i CB IV and TAK1i.

49
Comparing the timing between pulses (Figure 34) revealed that TAKi treated cells did not show an increase in the period (timing between peaks). By focusing for example on the timing between the second and the third pulse, no differences in time were observed in TAKi treated cells when compared to untreated cells. Therefore, the increase in period observed over the overall TAK1i treated population was due mainly to a shift to later timing of the first pulse (Figure 31).

Inhibiting TAK1 affects greatly p53 dynamics by increasing the amplitude and width of the first pulse. TAK1 inhibition may perturb p53 system greatly as this kinase is involved in the activation of several other important pathways such as p38/JNK and TGFβ.

3.3.5 P65’s transcriptional activity is necessary to modulate p53 dynamics after γ-irradiation

In order to understand whether p53 dynamics were regulated via p65 transcriptional activity or by the activity of IKK2, I transfected A549 cells via lentiviral infection with a construct expressing IκBα super-repressor (IκBα-SR) (Figure 35).
Figure 35: Schematic representation of the IκBα-SR lentiviral construct used to infect A549 cells. IκBα-SR is fused to RFP to select transfected cells and expressed by a strong constitutive promoter EF1α.

IκBα-SR is made by either substitution of the two N-term serines or deletion of the N-terminal part. This truncated form of IκBα can no longer be phosphorylated, ubiquitinated or degraded, thereby preventing NFκB nuclear translocation and NFκB-dependent transcription (Brown et al., 1995; Traenckner et al., 1995). For this study I used IκBα-SR that lacked the N-terminal (generous gift from Dr. C. Scheidereit, Max Delbrück Center, Berlin).

IκBα-SR was fused with RFP in order to be able to detect infected cells during automated analysis. The resulting cell lines were first used to validate the inhibitory effect of the super-repressor via immunofluorescence (Figure 36). The quantification analysis of the immunofluorescence shows that IκBα-SR successfully inhibits p65 nuclear translocation 30 minutes post stimulation with TNFα (Figure 37).
Figure 36: Immunofluorescence performed on A549 control cells and IkBα-SR transfected cells untreated (upper panel) and treated with 10ng/ml TNFα (lower panel). Cells were harvested 30 minutes after stimulation.

Figure 37: Quantification of the immunofluorescence for A549 and A549 IkBα-SR cells treated and untreated with 10ng/ml TNFα. In the left panel the distribution of the nuclear-cytosolic intensity ratio for p65 is shown for time point 0 and 30min. In the right panel the mean nuclear-cytosolic intensity ratio for p65 is plotted over 0, 30, 60 and 90 min.

Infected cells with the IkBα-SR together with control treated and untreated cells were irradiated with 10 Gy and imaged for 24h. P53 dynamics were greatly affected by inhibition of p65 nuclear translocation; by comparing the median p53 trajectory for transgenic cells and IKK2i treated cells we see that although the first p53 pulse showed similarities between the two conditions, the presence of IkBα-SR induced changes that were not seen in IKK2i treated cells (Figure 38).
Figure 38: Median of p53 induction and interquartile range of all data are shown for each condition. Comparison of untreated A549 cells (in violet), transfected cells with the IκBα-SR (in green) and treated cell with IKK2i CB IV (in orange) after 10 Gy irradiation.

By analyzing single cell trajectories for p53 (Figure 39) in transgenic cells, we can observe: cells that oscillate, cells that show only a pulse and cells that do not respond to DNA damage; these trajectory phenotypes were also observed in IKK2i treated cells (Figure 18). I quantified the changes in p53 dynamics by analyzing the trajectory features in IκBα-SR cells and I compared with control untreated cells (Figure 40). The presence of the IκBα-SR had a strong effect on the shape of p53 pulses: the peak amplitudes were decreased as well as the rate of p53 accumulation and degradation (Figure 40A, B and C); the width of the pulses was increased as well as the timing of the first pulse (Figure 40D and E). The percentage of not reacting cells (not pulsing or showing only one pulse) was increased 4-fold, suggesting that inhibition of p65 nuclear translocation can not only modulate but also inhibit the p53 response upon irradiation (Figure 40G). The period calculated over the whole pool of tracked cells for both conditions was also increased in IκBα-SR expressing cells (Figure 40F).
Figure 39: Examples of p53 trajectories in single cells for A549 cells transfected with IκBα-SR.

Figure 40: Box plot representation of the trajectories properties for untreated cells in light blue and IKK2i treated cells in orange. * p ≤ 0.05 ** p < 0.01 *** p < 0.001, Wilcoxon rank-sum test comparing IKK2i treated population cells to the control cells, null hypothesis the two population are the same.
In order to determine if the difference in period was due to a real shift in timing or to the fact that IkBα-SR expressing cells showed high p53 dynamic variability, we selected pulsing cells and plotted the distribution of their periods (Figure 41). The period in IkBα-SR expressing cells was only slightly shifted towards longer timing.

**Figure 41:** Representation of the period distribution over the fraction of oscillating cells for untreated cells and IKK2i treated cells.

Taken together these data showed that loss of p65 transcriptional activity greatly affected p53 activation, as 40% of cells did not react to irradiation in cells expressing IkBα-SR. Moreover lack of p65 activity altered p53 dynamics by lowering the amplitude and the accumulation / degradation rate of p53, leading to an increased peak width. Period and timing of the peaks were also slightly affected by the loss of transcriptional activity of p65.

If we compare these results with the one obtained by treating the cells with the IKK2i we can observe similarities: slope increase of p53 was decreased and the width was increased (Figure 19, Figure 23), percentage of non-reacting cells was increased. Although there were similarities the two conditions did not phenocopy one another. The differences between cells treated with IKK2i and cells expressing IkBα-SR may be due to differences in the type of perturbation and strength of perturbation. For cells treated with the IKK2i, the perturbation is fast (ca 1h) and not permanent, while cells expressing IkBα-SR have a stronger perturbation that is permanent and may lead to cellular adaptation.

### 3.3.6 Wip1 may contribute to linking the p53 and NFκB pathways

In order to understand how the NFκB pathway could regulate p53 dynamics, I made an educated guess that Wip1 could be a good candidate. Wip1 is a negative feedback loop regulator of p53 and its levels influence p53 dynamics (Batchelor et al., 2008). Moreover, it has been reported to regulate p65 activity by dephosphorylation, preventing p65 binding to target genes promoters (Chew et al., 2009). In addition, p65 may also play a role in regulating
the transcription of Wip1 (Löwe et al., 2009). For this reason I tested if the level of Wip1 may change upon activation or inhibition of p65 in undamaged cells. Stimulating MCF10A cells with 10ng/ml TNFα did not affect the expression level of Wip1, while inhibiting p65 activation by treating the cells with IKK2i led to an increase of Wip1 expression in MCF10A under normal conditions (Figure 43). These results were consistent with the previous one showing that stimulating cells prior or post irradiation with TNFα did not affect p53 dynamics while treating cells with the IKK2i greatly affected p53 dynamics.

In order to understand if Wip1 could be the cross talk player between the two pathways I transfected A549 cells via lentiviral infection with a construct bearing Wip1 fused to RFP expressed by a constitutive promoter (EF1α) (Figure 42).

Figure 42: Schematic representation of the Wip1 lentiviral construct used to infect A549 cells. Wip1 is fused to RFP to select transfected cells and expressed by the strong constitutive promoter EF1α.

Figure 43: QPCR results on MCF10A treated with 10ng/ml TNFα and harvested after 4h or treated with IKK2i and harvested after 2h, 4h, 7h and 17h. Error bars represent the standard deviation.

Transfected cells together with control cells and IkBα-SR expressing cells were irradiated with 10 Gy and imaged for 24h every 15 minutes. In Figure 44 are depicted the median p53 trajectories for each condition.

P53 dynamics were greatly affected by higher levels of Wip1 as expected from its role as a negative feedback regulator. However, comparing p53 dynamics in cells over-expressing Wip1 and IkBα-SR revealed that the median p53 behavior was not the same. The first p53 pulse was not affected by the presence of high levels of Wip1 and the majorities of the cells...
tracked showed either no peak or only one peak that was not modulated by the presence of Wip1 (Figure 45).

Figure 44: Median of p53 induction and interquartile range of all data are shown for each condition. Comparison of untreated A549 cells (in violet) with A549 cells overexpressing Wip1 (in red) and overexpressing IκBα-SR (in green) after 10 Gy irradiation.

Figure 45: Examples of p53 trajectories in single cells for A549 cells over expressing Wip1.

In order to quantify the changes of p53 dynamics provoked by high level of Wip1 I analyzed the features of all trajectories (Figure 46). As already observed in the median trajectory, the first pulse was not altered (Figure 46A, B, D), only the width is slightly increased (Figure 46D). What was indeed strongly changed was the overall p53 induction: the percentage of oscillating cells was decreased 3-fold (Figure 46F), the average number of peaks per trajectory 2-fold (Figure 46G) and the percentage of non-reacting cells or those showing only
one pulse was increased 5-fold (Figure 46H). Also the period calculated over the overall pool of cells seemed increased if compared to the control cells.

As previously done, I proceeded to calculate the distribution of the period only for cells that showed pulses (Figure 47). The period distribution was still shifted to longer values as a result of the high variability of the timing of the second peak and not to the fact that the pulsatile cells are really showing a regular longer period (Figure 48).
Figure 47: Representation of the period distribution over the fraction of oscillating cells for untreated cells and IKK2i treated cells.

Figure 48: Distribution of the timing of the peaks for A549 control cells and A549 Wip1 over-expressing cells irradiated with 10 Gy.

Taken together these data show that high levels of Wip1 indeed affect p53 dynamics. Wip1 acts as a negative feedback regulator in the DNA damage response and dephosphorylates not only p53 but also ATM, thereby turning off the whole response. This effect can be seen in the high level of non-reacting cells upon irradiation. Although many cells expressing IkBα-SR did not show p53 activation as well, the resulting p53 behavior did not phenocopy the one observed in Wip over-expressing cells, pointing out that Wip1 may only partially contribute to the crosstalk between the p53 and NFκB pathway and that other molecular mechanisms may play a role in linking the two pathways.
3.3.7 Transcriptional activity of p53 is changed by inhibiting IKK2 prior γ-irradiation

Previous studies have shown that changes in p53 dynamics affect also p53 target genes transcription (Purvis et al., 2012, Chen et al., 2013). In this work, it was shown that differential p53 dynamics at different temperatures affect target genes expression levels as well.

MCF10A cells treated with IKK2i showed a more regular pulsatile median p53 trajectory after irradiation compared to untreated cells (Figure 24). Moreover, I calculated that a higher percentage of cells showed regular pulses when perturbed with the inhibitor and that the overall population of perturbed cells has a more regular behavior. I wanted to test if the differences in p53 dynamics induced by treatment with IKK2i were enough to trigger different p53 transcriptional activity. For this reason I performed qPCR in MCF10A cells treated and untreated with IKK2i 1h prior 10 Gy irradiation and harvested after 7h, 17h and 24h. Among the apoptotic genes, NOXA was up-regulated 3-fold by the treatment with IKK2i, while BAX and PUMA showed only differences at 17h post damage. Genes regulating cell cycle arrest like p21 and GADD45a were slightly up regulated in cells treated with the IKK2i. PML, a gene involved in inducing senescence, was not changed by IKK2i treatment, while MDM2 gene (p53 negative feedback regulator) was up-regulated.
Figure 49: QPCR results in MCF10A cells untreated and treated with IKK2i 1h prior 10 Gy irradiation. Cells were harvested 7h, 17h and 24h after irradiation. Error bars represent the standard deviation.

### 3.4 Cellular state affects p53 dynamic after γ-irradiation

In this study we have explored how p53 dynamics after irradiation can be affected by a variety of factors such as temperature or the state of other pathways involved in the DNA
damage response such as NFκB. In this chapter we studied how cellular proliferation state can affect p53 dynamics after irradiation. MCF10A cells are a good model to study the interdependency between p53 induction and cellular proliferation state as this cell line is dependent on a defined medium containing EGF, insulin and hydrocortisone together with 5% horse serum (Debnath, J et al., 2003). We cultivated cells under different media conditions for 12h before 10 Gy irradiation, in order to study how much the dynamics of p53 are influenced by the presence or absence of a specific component. We compared p53 induction in cells cultivated in 6 different media combinations: normal media, media where horse serum has been exchanged for 0.5% BSA (Bovine Serum Albumin), minimal media containing BSA and EGF, minimal media containing BSA and insulin, minimal media containing BSA and hydrocortisone and minimal media containing only BSA (Figure 50). From the median trajectories we can see that presence or absence of horse serum does not affect the p53 response (Figure 50A, B). A minimal media with BSA and EGF proved to be sufficient to reproduce p53 induction (Figure 50C). P53 median dynamics in cells cultured in only BSA or BSA and insulin show lower level of p53 but overall conservation of the dynamic pattern (Figure 50D, F). When cells are cultured with BSA and hydrocortisone they show a different p53 response and overall lower level of p53 accumulation (Figure 50E).
Figure 50: MCF10A cells irradiated with 10 Gy under 6 different media conditions. For each condition p53 median trajectory, depicted in blue (solid line), is plotted together with 150 single cells trajectories (light blue).

Cells cultured in normal media, normal media without horse serum and minimal media containing BSA with EGF showed the same characteristics.

Cells cultured with BSA only have lower amplitude and slower accumulation and degradation of p53 (Figure 51B, D, E). Cells cultured with insulin and BSA show only slightly lower amplitude and slower p53 accumulation rate.
Cells cultured only in BSA with hydrocortisone showed lower p53 amplitude of the first peak, slower p53 accumulation and degradation (Figure 51B, D, E), lower percentage of pulsatile cells and higher percentage of not reacting cells (Figure 51G, H). The period was increased (Figure 51C) as a result of high cellular variability and low number of pulsatile cells.

The percentage of cells dividing was higher if EGF was present in the media and slightly increased by horse serum (Figure 51I). However, the overall percentage of dividing cells was low because cells after irradiation go into cell cycle arrest.
Figure 51: Box plot representation of the trajectory properties for MCF10A cells cultured for 12h prior irradiation in 6 different media conditions. Error bars represent standard deviation.

Taken together these results show that the presence of EGF affects p53 induction level (amplitude) but not the rate of p53 accumulation and degradation. The presence of hydrocortisone in the media suppresses p53 activation and changes the dynamics of p53 induction, only if EGF and insulin are not present. EGF presence may trigger activation of p53 via PI3K/AKT (Wierød et al., 2008; Leena Latonen et al., 2003) and counteract the effect of hydrocortisone that inhibits p65 activation (Aljada et al., 1996) and therefore also inhibits p53 induction (this study).
4 Discussion

4.1 P53 dynamics in single cells are modulated by a variety of perturbations

P53 is a key transcription factor that is involved in the response to a great variety of stresses and its function is required for the maintenance of cellular homeostasis. Considering the important role of p53 in the cell, we wanted to assess how and to which degree, p53 activation was affected after DSBs by different perturbation types. Measurement of p53 activation was performed in single cells (A549 cells and MCF10A cells) by time-lapse live-cell fluorescence microscopy. Physical perturbations were obtained by varying the temperature, molecular network perturbation by inhibiting the NFκB pathway and cellular state perturbation by changing growth factors composition in the culturing medium.

4.2 Temperature changes and p53 dynamics

Temperature usually has a major effect on chemical reactions. At higher temperature the molecules have more thermal energy and the probability of collision is higher. Following Arrhenius equation increase of the temperature of 10°C doubles the reaction rate. Like most chemical reactions, also the rate of an enzyme-catalyzed reaction increases as the temperature is raised. The reaction rate increases with temperature to a maximum level then abruptly declines with further increase of temperature as the enzymes denaturation process starts. In humans the body temperature is usually kept constant at 37°C except in the case of fever where temperature may raise up to 42°C and in case of bodies extremities that may have a temperature lower than 37°C depending also on external climatic conditions.

Fundamental biological processes like circadian rhythm are temperature compensated so that the night and day cycle is determined only by the light cycle and not from the weather conditions (Barrett at al., 1995, Sorek and Levy, 2012). In this study I observed that p53 response to DNA damage is not temperature compensated although p53 is a fundamental component for maintaining cellular homeostasis in mammalian cells.

Contrarily to the expectations that at lower temperature the overall p53 activation process might be slower, by decreasing the temperature down to 30°C we observed increased p53 intensity, pulse width and longer timing between pulses, while the rate of p53 accumulation and degradation stays the same. At 40°C a decrease in the period is observed together with increase in the rate of p53 accumulation and degradation, indicating that an overall p53 activation pathway and DNA repair may be accelerated.
It is hard to predict how the biochemical process are affected by temperature, for this reason a mathematical model would be of great help to identify which reactions within the pathway may affect p53 dynamics in the same manner observed at different temperatures. A hypothesis could be that the destabilization of Mdm2 is delayed at lower temperature than the physiological one; this would explain why at 30 degrees p53 activation starts at later points while the rate of p53 accumulation and degradation stays the same. This can be tested in future experiment by checking the degradation and production rate of Mdm2 after IR at different temperatures. Another hypothesis would be that the Mre11-Rad50-Nbs1 (MRN) complex that acts as a DSB sensor is slowed down; this hypothesis may be tested by checking the number of DNA damage foci over time after IR at different temperatures. DNA repair after 10 Gy irradiation dose is active at least for 24h due to the large amounts of DSBs created. For this reason I discard the hypothesis that the rate of DNA repair might be involved in shaping p53 dynamic at different temperatures.

Together with changes in p53 dynamics, also the expression of p53 target genes was affected. Expression of p53 target genes was affected differently to what was expected from previous publications where high level of p53 (reached by using Nutlin or high doses of Etoposide) were associated with higher level of target genes transcription (Chen et al., 2013; Purvis et al., 2012). In our case higher level of p53 (30°C condition) did not always associated with higher level of p53 transcription. There are several reasons why this can be: first of all although the level of p53 are higher, p53 is still oscillating contrarily to the previous studies where p53 maintained a sustained high level over time. Second, p53 dynamics were modified by varying the temperature and not by altering the damaging stimulus or directly interfering with p53 dynamic. In the study from Purvis et al. Nutlin was used to maintain sustained high p53 level over time, while in the study by Chen et al. increasing amount of DSBs, obtained by treating the cells with higher doses of Etoposide, were said to keep the p53 level sustained. Increasing or changing the damaging dosage may affect the overall DNA damage response in a way that could affect p53 target gene expression. By keeping the damaging stimulus constant and only varying the temperature we uncoupled the DNA damage response activation status and the p53 dynamics. We could see that indeed p53 dynamics affect gene transcription but very heterogeneously and in a gene specific way. Previous studies have shown that hyperthermia together with anti-cancer treatment enhances the effect of such treatment (Wiedemann G.J., 1993; Zhu W.G., 1995; Murata 2001; Ahmed et al., 2013). Coupling hyperthermia with cancer treatment resulted in increased apoptosis and decreased cell proliferation and metabolism (Li et al., 2003). Other studies on neutrophils and CHO cells have shown that
hypothermia has the opposite effect, it delays apoptosis together with making cells accumulate in G1 arrest (Moore A. et al., 1997; Pryde et al., 2000). There are no evidences that temperature-induced apoptosis is p53 dependent (Yonezawa et al., 2002; Jara et al., 2002; Gu et al., 2014); nevertheless there are evidence that hyperthermia could be an effective radiosensitizer as it can induce several steps associated with IR signaling such as γ-H2AX foci formation, ATM phosphorylation but not 53BP1 and SMC1 foci formation (Guan et al., 2002; Hunt et al., 2007). Apoptosis due to hyperthermia seems to be triggered via CASP3 and ER stress pathway (Shellman et al., 2008; XU et al., 2008). Having a better understanding of the interplay between hyperthermia with p53 activity at the single cell level may help in cancer therapy. Studies at the single cell level could help shed some light on how dynamics of p53 could play a role in the cellular response under different temperatures. As past studies never considered dynamics of p53 but only protein levels at defined time points, their approach could have been insufficient to determine p53 activation and role for cellular outcome.

Studying p53 dynamics under various temperatures may be also an interesting tool to understanding the correlation between dynamics and gene transcription, for example how p53 pulse frequency relates to gene transcription. Coupling single cell microscopy experiment with RNA seq could be useful to investigate this correlation. With this approach we could also compare gene transcription profiles of cells where p53 dynamic has been altered by temperature to cells where p53 dynamic has been altered by other perturbations, for example IKK2 inhibition. This comparison would permit to assign which pool of genes is affected by the altered p53 pulse frequency, which is affected by the inhibition if IKK2 and which from the temperature shift.

**4.3 NFκB affects the dynamics of p53 in irradiated cells**

In this study I investigate the role of NFκB in shaping the dynamics of p53 in irradiated cells. Stimulation with TNFα in both A549 and MCF10A cells did not affect p53 activation. On the other hand inhibition of IKK2i as well as direct inhibition of p65 nuclear translocation led to changes in p53 dynamics in both cell lines after irradiation.

**4.3.1 TNFα stimulation does not interfere with p53 activation upon irradiation in A549 and MCF10A cells.**

Inflammation is tightly related to cancer (Hanahan D., et al., 2011, Demaria S., et al. 2010). In the tumor microenvironment cytokines like TNFα regulate not only immune cells response but also cancer development (Grivennikov S.I., et al., 2010). Previous studies have also
reported that cancer cell lines sensitive to TNFα (like MCF7, LNCaP and ME180) show p53 activation after stimulation with TNFα that leads to p21 accumulation and apoptosis (Lewandowski et al., 2005; Akca H. et al., 2002; Rokhlin et al., 2000; Donato and Perez 1998). In the case of this study, stimulation with TNFα at various time points, before and after irradiation, did not change p53 dynamics in γ-irradiated A549 and MCF10A cells. These results point in the directions that in both cell lines TNFα stimulation and activation of the canonical NFκB pathway do not initiate a p53 response, despite activating NFκB nuclear translocation.

Studying p53 dynamics in single cell level may be an important tool to unveil interaction between inflammation and p53 activations in cell lines where this relation has been observed, in order to create drugs that could uncouple the positive influence of p53 on activation of inflammatory genes.

4.3.2 IKK2 inhibition changes p53 dynamics in A549 and MCF10A cell lines and also affects p53 target genes expression

A549 cells after γ-irradiation show a very regular pulsatile p53 behavior in single cells. This characteristic makes them very suitable to analyze p53 dynamic features of single cells trajectories such as: period, width of the pulses, amplitude, rate of accumulation and degradation. This regular p53 behavior after γ-irradiation facilitates the analysis of the NFκB-mediated perturbation of p53 dynamics. Treating A549 cells with IKK2 inhibitor at different time points, prior or after 10 Gy γ-irradiation, changes p53 dynamical characteristics. The timing of p53 pulses is delayed and the rate of p53 nuclear accumulation is slowed down while the degradation rate is maintained. The width of the pulses is increased while no difference in the amplitude was observed. The same effects on p53 changes in dynamics were observed with 4 different IKK2 inhibitors confirming that these changes are due to IKK2 inhibition and not to some unspecific cross-reaction of the inhibitor.

To validate the observed modulation of p53 dynamics in another cell line I used MCF10A cells. Contrarily to A549 cells, p53 response in MCF10A cells after γ-irradiation is very heterogeneous and it can range from a pulsatile p53 behavior, to p53 accumulation, to no response (D. Friedrich, 2014 Master thesis). Such diverse p53 activation patterns can make the effect of NFκB perturbation more challenging to be studied. As result of IKK2i treatment prior 10 Gy irradiation, twice the number of cells showed pulsatile behavior. The overall p53 response appeared to be less variable and consistent with what we observed in A549 cells, the
timing of the pulses was delayed in the fraction of oscillating cells and the accumulation rate for the first p53 pulse is slowed down.

Inhibition of IKK2i in both cell lines affects p53 dynamics in a similar way. The reduced p53 accumulation rate, observed in cells treated with IKK2i, may be the cause of bigger pulse width and also longer timing between peaks. Previous studies regarding the interplay between p53 and IKK2 have reported that IKK2 upon DNA damage can phosphorylate p53 on Ser 362 and 366 promoting p53 degradation via SCF complex and independently from Mdm2 (Xia et al., 2009). Moreover other studies have shown that IKK2 inhibition can stabilize p53 accumulation and acetylation with subsequent activation of p21 and apoptosis (Yang et al., 2010). However, in this study inhibition of IKK2 did not lead to higher p53 pulse amplitude or to faster p53 accumulation or to slower p53 degradation, all changes that were expected in case of strong p53 stabilization due to IKK2 inhibition.

4.3.3 Nuclear translocation of p65 is necessary to shape p53 activation after irradiation

A549 cells were transfected with IκBα-SR in order to study the role of p65 nuclear translocation and activity in modulating the p53 response to DNA damage and compare it with the effect of IKK2 inhibition. Surprisingly inhibiting p65 translocation also decreases p53 activation; the data showed 40% of cells do not react to irradiation in cells expressing IκBα-SR. Lack of p65 activity alters also p53 dynamics by lowering the amplitude and both accumulation and degradation rate of p53. Width of the peaks is increased and also the period and timing of the peaks are also slightly shifted towards later time points. Two previous studies have already reported that NFκB inhibition obtained by stable expression of IκBα-SR, inhibits p53 activation and function after treatment with Doxocyclin. Decrease of p53 protein level and absence of G1 cell-cycle arrest and induction of p53 target genes have in fact been reported by both studies (Fujioka et al., 2004; Zhou et al., 2003). In both works p53 level was measured with Western blot after 12h or 24h from Doxocyclin stimulation.

In this study I confirmed that in A549 cells inhibition of NFκB activity leads to p53 dampening after DNA damage. This approach focused on studying perturbation of p53 pathways by measuring dynamic changes in p53 with high time resolution in single cell. This new approach may help to shed light on the possible mechanism that can be involved in this process by measuring the dynamic features of p53 activation in cells being perturbed under various stress strengths. P53 dynamics of cells treated with IKK2i and cells expressing IκBα-SR show some similarities like: the accumulation of p53 is decreased and the pulse width is
increased, percentage of non-reacting cells is increased. Although there are similarities the two conditions do not phenocopy one another; most importantly, in cells expressing IκBα-SR p53 activation is strongly damped as discussed above. The differences between cells treated with IKK2i and cells expressing IκBα-SR may be due to differences in the type and strength of the perturbation. For cells treated with IKK2i, the perturbation is fast (1h) and not permanent, while cells expressing IκBα-SR have a stronger perturbation that is permanent and may lead to cellular adaptation. Another possible explanation for differences between the two perturbations types might be that in cells treated with IKK2i the overall p53 response may be shaped by the balance between p65 inhibition leading to dampened p53 activation and IKK2 inhibition leading to p53 stabilization. Contrarily in cells expressing IκBα-SR, the overall p53 response is shaped by inhibition of p65 transcriptional activity. Further experiments need to be performed to understand the relationship between p65 and p53 activation.

In many cancers NFκB is up regulated and leads to inflammation and survival gene up-regulation (Hanahan D. et al., 2011). Many treatments involving inhibiting NFκB together with cancer therapies lead to tumor cell death or growth inhibition. Inhibition of NFκB can occur by targeting different molecules: by IKK2 inhibition, by the use of glucocorticoids (promoting IκBα production) or by the use of proteasome inhibitors (preventing IκBα degradation). (Yamamoto and Gaynor, 2001). As suggested from this study, targeting IKK2 instead of direct p65 translocation and activity may bring advantages, as the function of p53 is not abrogated.

4.3.4 Wip1 may play a partial role in the p53 and NFκB crosstalk after irradiation

Form this study emerged that inhibition of p65 nuclear translocation in A549 cells expressing IκBα-SR leads to dampening of p53 activation. Transcriptional p65 activity seems to be involved in shaping p53 dynamics after DNA damage. An educated guess was to test if Wip1 might be involved as crosstalk player between p65 transcriptional activity and p53 activation. Wip1 is involved directly in shaping p53 dynamic as a negative feedback regulator. Moreover, one previous study has observed a relationship between Wip1 and p65 activity. More in detail Wip1 may regulate the phosphorylation status on Ser 536 of p65 that is needed for the recruitment of the transcriptional co-activator p300 (Chew et al., 2009).

In this study I hypothesized that lack of NFκB activity may correlate with higher Wip1 level that can in turn dampen p53 activation after DNA damage. I tested this hypothesis by measuring the p53 response after γ-irradiation in A549 cells over expressing Wip1. The
majorities of the cells do not show p53 activation, and cells that show p53 activation bear only the first p53 pulse which shape is not changed from the control cells (irradiated A549 wt cells). Taken together these data show that p53 dynamics in Wip1 over expressing cells do not phenocopy exactly the p53 dynamics observed in IκBα-SR expressing cells. For this reason we may think that Wip1 play a role in dampening p53 activation but that at the same time it’s not the only player involved in the process.

4.3.5 TAK1 inhibition changes the characteristic of the first p53 pulse

TAK1 is involved in activating the canonical NFκB pathway as well as NFκB DNA damage response by direct phosphorylation and activation of IKK2 (Hinz et al., 2010). By inhibiting TAK1 therefore we also inhibit IKK2. In this study I tested the effect of TAK1 inhibition on p53 dynamics after irradiation in A549 cells and compared it to the perturbation obtained by IKK2i treatment. Inhibition of TAK1 in A549 cells provokes a strong p53 response with the first p53 pulse showing bigger amplitude and broader width. The dynamics features of the following pulses are not changed by the effect of TAK1 inhibitor. This may be due to the fact that the inhibitor activity is compromised after a while at 37°C.

The effect on p53 dynamics is stronger than the one obtained with IKK2i treatment and consistent with previous studies that indicate that inhibition of TAK1 leads to an increase of p53 mediated senescence and sensitization of cancer cells to chemotherapeutic drugs (Dvashi et al., 2014; Fan et al., 2013). TAK1 inhibition may perturb p53 system more strongly than IKK2 inhibition as the former kinase is involved in the activation of several other important pathways such as p38/JNK and TGFβ. Overall we can say that inhibiting TAK1 prior DNA damage trigger a stronger p53 response and it may be useful in cancer treatment.

4.3.6 P53 target gene transcription level are affected by IKK2 inhibition

Inhibition of IKK2 in MCF10A cells resulted in a cell population switch towards p53 pulsatile behavior. Previous studies have shown that changes in p53 dynamics affect also p53 target genes transcription (Purvis et al., 2012, Chen et al., 2013). In this study, inhibition of IKK2 in MCF10A cells leads to different p53 target genes transcription, more precisely treatment with IKK2i leads to an increase in some p53 target genes expression like: NOXA, P21, GADD45a and MDM2. Although a shift in p53 target gene transcription is observed, it is not known if it is determined solely by p53 dynamics or by IKK2 inhibition or by a joint contribution of both factors. There are studies suggesting that lack of IKK2 in cells leads to acetylation, stabilization and accumulation of p53 promoting p21 expression, cell cycle arrest, apoptosis
and decreased cell viability (Yang et al., 2010) and this may explain the increase observed in gene expression.

In order to uncouple the contribution from the dynamic (bigger timing between pulses and slower p3 accumulation) and the direct effect of IKK2 inhibition we could use the knowledge obtained from this work about the effects of temperature on p53 dynamics and perform transcriptome analysis in cells being irradiated and treated with IKK2i and cells irradiated under slightly lower temperature that has been shown to increase the timing between pulses.

4.3.7 How can we understand the p53 and p65 interaction?

In this study I demonstrated that a strong cross talk between p53 and p65 exists after DNA damage. It emerged that both IKK2 and p65 play distinct roles in shaping p53 dynamic: p65 inhibition leads to a strong dampening of the p53 response consistent with previous studies (Fujioka et al., 2004; Zhou et al., 2003), while IKK2 inhibition leads to a shift towards longer period between pulses and slower p53 accumulation. The latter observed phenotype may be resulting from the combination of IKK2 inhibition contribution together with p65 repression contribution. IKK2 inhibition contribution should lead to increase in p53 stability (Xia et al., 2009) that would be balanced by the dampening effect of P65 repression. Nevertheless P65 activation in the presence of IKK2i might not be totally abrogated, and this would explain why cells treated with IKK2i show still p53 activation. A possible reason for incomplete p65 repression could be that IκBα phosphorylation may be compensated by IKK1. This hypothesis can be also applied for interpreting the results obtained by treating the cells with TAK1 inhibitor prior irradiation. TAK1 inhibition leads to inhibition of IKK2 and possible attenuation of the p65 response. This would explain the higher p53 activation at least for the first hours after IR. For this matter, treating cells with TAK1 inhibitor together with IKK1 inhibitor or with IKK2 together with IKK1 inhibitors prior γ-irradiation may be interesting to determine the contribution of IKK1 on p65 activation and how p65 activation influence p53 dynamics. Another possible experiment would be to compare the effect, in irradiate cells, of IKK2 siRNA with IKK2i and TAK1 siRNA with TAKi. These experiments might reveal any differences between the two perturbations types at the level of p53 dynamics and at the level of p65 activation after DNA damage.

In this study I also tested the hypothesis that p65 inhibition may lead to increases of Wip1 activation and consequently p53 dampening. We observed that Wip1 may participate in dampening p53 activation but it is not the only player. A transcriptome analysis of cells expressing IκBα-SR (or treated with p65 siRNA) could help to understand which are the
genes regulated by p65 activity that have the capacity to change also p53 dynamics. Moreover comparing cells treated with IKK2 inhibitor (or siRNA for IKK2) with cells expressing IκBα-SR (or treated with p65 siRNA) could help to understand which genes are regulated solely by p65 activity (among which some may affect p53 dynamics) and which genes may be indirectly affected by IKK2 activity. A cell line expressing both p53 and p65 fluorescent reporters could be established to study how p53 and p65 are dynamically interconnected. By perturbing this system with small molecules like nutlin or inhibitors for upstream p53 kinases like ATM, Chk2, ATR or inhibitors of p65 activation kinases like IKK1, IKK2 TAK1, RIP1, we could alter selectively the p53 and p65 pathways and observe how these changes would affect also the dynamics of the other pathway.

4.4 Cellular state affects p53 dynamics

MCF10A cell line is a suitable model to study the relationship between p53 induction after DNA damage and cellular state. MCF10A cells are in fact dependent on specific growth factors in order to proliferate. This study showed that the presence of EGF affect p53 induction level (amplitude) but not the rate of p53 accumulation and degradation. Surprisingly the presence of hydrocortisone in the media suppresses p53 activation, but only if EGF and insulin are not present. MCF10A cells cultured in only BSA before irradiation, showed lower p53 amplitude, slower accumulation and degradation rate. Taken together these results show that p53 dynamic in irradiated MCF10A cells are shaped by the interplay of EGF/insulin that can trigger activation of p53 via PI3K/AKT (Wierød et al., 2008; Latonen et al., 2003) and the presence of hydrocortisone that can inhibit p65 activation (Aljada et al., 1996) and consequently also inhibit p53 induction (this study). Glucocorticoids such as hydrocortisone promote IκBα expression that sequestrates p65 into the cytosol (Auphan N. et al., 1995). Further tests should be performed to confirm the hypothesis that hydrocortisone inhibits p65 nuclear translocation. Moreover in this work cells were starved for 12h; further studies may be done in performing the same experiments at increasing starvation times to see if the results may change due to the fact that more time is needed to completely reset the signaling pathways. Another interesting experiment could be to study at the single cell level the effect of treating serum starved cells with EGF after being irradiated. This experiment would allow observing how fast is the interaction between EGF activation and p53 activation.

This work also pointed out that treating patients with hydrocortisone in order to alleviate cancer symptoms such as inflammations, swelling and low appetite might be contra
productive if provided together with chemotherapy and radiotherapy treatments. Hydrocortisone in fact dampens p53 activation and therefore may interfere with cancer treatments in tumor where p53 is wt and also can dampen p53 response in normal cells after radiation or chemotherapy that can lead to the developing of secondary tumors due to the treatments. Another point of reflection brought out by this study is that depending on the cell line and the type of research that is intended to pursuit it is very important to understand if the culturing conditions used are adequate in order to obtain results that may mirror the in-vivo reality. The usual culturing media conditions that implicate 10% of serum are certainly very artificial. In-vivo tissue growth is very slow; moreover in tumor tissue the access of nutrients, growth factors and also oxygen may be limited. Using the canonical culturing media condition as a general model can in fact be misleading as it pushes the cell towards high proliferation rates and boosts the activation of signaling pathways like MAPK, AKT/PI3K.

5 Conclusions

In this study I focused on the effects of perturbations of the p53 pathway activated after γ-irradiation. I determined that p53 dynamics and consequently p53 target gene transcription could be extremely affected by physical perturbation like temperature changes or by varying culturing conditions. Interestingly I observed also that the state of other pathways like NFκB and EGF could have huge effects on the activation signature of p53. From this study emerged that there may not even be a standard p53 activation dynamic pattern; depending on the cell line, the activation state of the respective signaling pathways could vary greatly and this may eventually lead to different p53 activation signature (as observed in MCF10A and A549 cells). Moreover different culturing conditions can further affect p53 activation.

In a great variety of cancer types, activation of p53 by means such as radiotherapy and chemotherapy is used as a possible cure. Since many factors ranging from oncogene activation, in vivo condition like accessibility to nutrients or growth factors, or even pharmacological treatments can potentially affect p53 activation we need to study more systematically the relationship between perturbations and p53 response in order to make cancer therapy more useful and effective.
6 Materials and methods

6.1 Materials

If not listed below, chemicals and materials were obtained from Carl Roth GmbH & Co. KG.

6.1.1 Antibodies, chemicals and kits

Puromycin (10mg/mL); Carl Roth GmbH
Geniticindisulfate (G418); Carl Roth GmbH
Hygromycin (HY061-221); Invitrogen, Life Technologies
Human EGF (AF-100-15); Peprotech
Hydrocortisone H-0888; Sigma Aldrich
Cholera Toxin (C805.2-1MG); Sigma Aldrich
10 µg/ml Insulin (19278); Sigma Aldrich
Pen/Strep (15140-122); Gibco, Life Technologies
GlutaMAX (35050038); Gibco, Life Technologies
Hepes (15630056); Gibco, Life Technologies
DMEM/F-12 Medium; Gibco, Life Technologies
McCoy’s 5A medium; PAA
Horse Serum (P30-0702); PAN Biotech
Fetal Calf Serum; Life technologies
Goat serum; PAN
Bovine Serum Albumin; Sigma
Penicillin/Streptomycin (15140-122); Gibco, Life Technologies
p53 DOI mouse monoclonal IgG; Santa Cruz Biotechnology, Inc.
GAPDH rabbit monoclonal IgG (G9545); Sigma Aldrich Co.
NFκB p65 (C-20) rabbit polyclonal IgG (sc-372); Santa Cruz Biotechnology, Inc.
IkBα (C-21) rabbit polyclonal IgG (sc-372); Santa Cruz Biotechnology, Inc.

Peroxidase conj. goat anti rabbit IgG (31460); Thermo Scientific
Peroxidase conj. goat anti mouse IgG (31430); Thermo Scientific
Alexa Fluor goat 488 anti-rabbit IgG (A-11034); Life technologies
Prolong Antifade; Life Technologies
Hoechst 33342, Trihydrochloride; Life technologies
Trypsin; Life technologies
M-MuLV reverse transcriptase and Oligo-dt Primers; NEB
High Pure RNA Isolation kits; Roche
SYBR Green reagent; Roche
Gateway BP Clonase II; Invitrogen
Gateway LR Clonase II plus enzyme; Life Technologies
Gibson Assembly Master Mix; NEB
QIAprep Spin miniprep kit; QIAGEN
QIAquick PCR Purific. Kit; QIAGEN
QIAquick Gel Extraction Kit; QIAGEN
401481 IKK-2 Inhibitor IV - Calbiochem; Merk-Millipore
401479 IKK-2 Inhibitor VI, sc-514 Calbiochem; Merk-Millipore
PS-1145 IKK-2 Inhibitor; Cayman Chemical
IMD-Sigma-0354 IKK-2 inhibitor; Sigma
TAK inhibitor (5Z-7 Oxozaenol); Sigma
TNFα; Enzo Life Science

6.1.2 Devices
Cesium-137 γ-ray source irradiator
CO2 Incubator Binder; GmbH (Tuttlingen, Germany)
Vertical gel running systems: Novex Mini-Cell; Life Technologies (Carlsbad, USA)
Fluorescence microscope Eclipse Ti; Nikon (Tokio, Japan)
Lumen200 Fluorescent Lamp; Prior Scientific (Rockland, USA)
Shutter Lambda SC; Sutter Instruments (Novato, USA)
Automated Stage ProScan3 H117; Prior Scientific (Rockland, USA)
Orca R2 CCD Camera; Hamamatsu Photonics (Hamamatsu, Japan)
Pecon NL2000 Incubator; Pecon (Erbach, Germany)
NIS-Elements AR imaging Software; Nikon (Tokio, Japan)
Matek glass bottom dishes (Matek Corp., Ashland, USA)
NuPAGE SDS-PAGE system; Life Technologies (Carlsbad, USA)
Molecular Imager Chemi Doc™ XRS+; Biorad Laboratories GmbH (Munich, Germany)
Matlab 2012b Mathworks (Ismaning, Germany)
Image Lab Software; Biorad Laboratories GmbH (Munich, Germany)
EL 800 Universal Microplate Reader; Bio-Tek Instruments (Friedrichshall, Germany)
Trans-Blot Turbo Transfer System; Biorad Laboratories GmbH (Munich, Germany)
TC10 Automated Cell Counter; Biorad Laboratories GmbH (Munich, Germany)
StepOnePlus PCR machine and Software; Applied Biosystems

6.1.3 Solutions, buffers and cell culture media

SDS Page Running Buffer
1x NuPAGE MES SDS Running Buffer (Life Technologies)
RIPA lysis buffer
50 mM Tris pH 7.4, 100 mM NaCl, 1% Triton X-100, 0.5%Na-Deoxycholate, 0.1% SDS, 1:100 Protease inhibitor cocktail Plus (#3751.1, Roth), 1:100 Phosphatase inhibitor cocktail (P0044, Sigma)
TBS-T
50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween-20
TBS
50 mM Tris pH 7.4, 150 mM NaCl
PBS
10 mM Na2HPO4, 2 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4
Transfer Buffer
25 mM Tris, 192 mM Glycine, 20% Methanol
Blocking solution
5% Skim Milk Powder (Carl Roth GmbH & Co. KG) in TBS-T

Mild stripping Buffer

25 mM Glycine pH 2.0, 2% SDS

Growth Medium and Assay Medium MCF10A

5% Horse serum, 20 ng/mL EGF, 0.5 µg/mL Hydrocortisone, 100 ng/mL Cholera toxin, 10 µg/mL Insulin, 1:100 Pen/Strep in DMEM/F12 medium

Resuspension Medium MCF10A

20% Horse serum, 1:100 Pen/Strep in DMEM/F12 medium

Microscopy Medium MCF10A

5% Horse serum; 20 ng/mL EGF, 0.5 µg/mL, Hydrocortisone, 100 ng/mL Cholera toxin, 10 µg/mL Insulin, 1:100 Pen/Strep, 1% Glutamax, 1 % HEPES in RPMI 1640 medium (without phenol red)

Growth Medium and Assay Medium A549

10% Fetal Calf Serum and 1:100 Pen/Strep in McCoy’s 5A Medium

Microscopy Medium A549

10% Fetal Calf Serum, 1% Glutamax, 1% Heps and 1:100 Pen/Strep in RPMI 1640 medium (without phenol red)

6.1.4 qPCR Primers

Bax_up: CTGACGGCAACTTCAACTGG

Bax_down: GATCAGTTCGGGCACCTTG

GADD45a_up: GCA ATA TGA CTT TGG AGG AAT TCT C

GADD45a_down: TGA CTC AGG GCT TTG CTG

Mdm2_up: GATGAAAGCCTGGCTCTGTGTGT

Mdm2_down: TTCGATGGCGTCCCTGTAGATTCA

NOXA_up: GGA GAT GCC GCC TGG GAA GAA G

NOXA_down: TGC CGG AAG TTC AGT TTG TC

p21_up: TGGACCTGTCACTGTCTTGT

p21_down: TCCTGTGGGGCGGATTAG
6.2 Methods

Cell culture

A549 cell line (human non-small cell lung cancer) was cultured in McCoy’s 5A medium supplemented with 10% fetal calf serum; MCF10A (non-transformed human breast epithelial) cell line was maintained in DMEM/F-12 medium supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin and 10 µg/ml insulin (Debnath et al., 2003). All media contained penicillin and streptomycin, and all cells were grown at 37 °C and 5% CO₂. When appropriate, selective antibiotics (400 µg/ml G418, 50µg/ml hygromycin or 0.5µg/ml puromycin) were added to maintain transgene expression.

The A549 p53-Venus reporter cell line have been already described (Chen at al. 2013) and they were a generous gift from Dr. Jue Shi, Center for Quantitative Systems Biology and Department of Physics, Hong Kong Baptist University. A nuclear marker was added in our lab to allow automated tracking by infecting with lentiviruses expressing histone 2B fused (H2B) to cyan fluorescent protein (CFP) under the control of the human Ubiquitin C promoter (UbCp).

MCF10A p53-Venus/H2B-CFP reporter cell lines were created by infecting cells with lentiviruses encoding a construct where p53 was fused to Venus fluorescent protein under the control of UbCp and with a construct encoding H2B fused to CFP behind the human elongation factor 1α (EF1α) promoter. Stable MCF10A reporter cell line was established by antibiotic selection of stable integrations through G418 (genticin disulfate) and hygromycin.
resistance by PhD Henriette Strasen. Validation of MCF10A reporter cell line was performed by Dhana Friedrich (D. Friedrich, 2014 Master thesis).

The A549 p53-Venus/H2B-CFP/IκBα-SR-RFP and A549 p53-Venus/H2B-CFP/Wip1-RFP cell lines were created by infecting the A549 p53-Venus/H2B-CFP reporter cell line with lentiviruses encoding either a construct where IκBα-SR was fused to red fluorescent protein (RFP) under the control of EF1α promoter or a construct where Wip1 was fused to red fluorescent protein (RFP) under the control of EF1α promoter.

**Treatment with radiation**

γ-irradiation was applied using a Cs-137 source with 10 Gy corresponding to 86.7 sec irradiation calculated from the actual dose rate. Controls were treated equivalent except of irradiation procedure.

**Inhibitor and TNFα treatments**

Media was replaced 1h before γ-irradiation (if not specified otherwise by the experiment conditions) with fresh one containing DMSO (control) or containing 15 μM of Calbiochem IV IKK2 inhibitor (IKK2i); 2 μM PS-1145 IKK2i; 2 μM IMD-0354 Sigma IKK2i; 48 μM Calbiochem VI, sc-514 IKK2i; 3 μM TAKi (5Z-7 Oxozaenol) or 10ng/ml TNFα. After γ-irradiation cells were harvested (for western blot, RT-qPCR or immunofluorescence) at the indicated time points or imaged by fluorescence microscopy.

**Different temperature treatments**

After γ-irradiation A549 cells were promptly incubated at the desired temperature and imaged by fluorescence microscopy or harvested for RT-qPCR at the indicated time points.

**Starvation treatments**

Normal growth medium was replaced 12h before γ-irradiation by five different serum-starvation media and by fresh normal growth medium (control). After γ-irradiation MCF10A cells were imaged by fluorescence microscopy. In the five different starvation media horse serum was replace with 0.5% BSA (Bovine Serum Albumin) and all contained 100 ng/ml cholera toxin and penicillin/streptomycin. The different media contained: 0.5% BSA and 20ng/ml EGF; 0.5% BSA and 10 μg/ml insulin; 0.5% BSA and 0.5 μg/ml hydrocortisone; BSA only

**Culturing of cells for microscopy and coating of microscopy plates**
A549 and MCF10A cells were seeded on poly-lysine covered Matek glass bottom dishes two days prior microscopy imaging. The day of the experiments, medium was changed to the respective microscopy medium that contained no phenol red.

**Live-cell time-lapse microscopy**

For live-cell time lapse microscopy cells were imaged on a Nikon Ti inverted microscope with perfect focus system. Imaging was performed with a 20x plan apo objective (NA 0.75) and Hamamatsu Orca R2 camera. Fluorescent images were detected with the following filter sets: Venus - 500/20 nm excitation, 515 nm dichroic beam splitter and 535/30 nm emission; CFP - 436/20 nm excitation, 455 nm dichroic beam splitter and 480/40 nm emission (Chroma). Images were acquired every 15 min for 24h. Image acquisition was controlled by Nikon NIS Elements AR software. The microscope was enclosed with an incubator chamber to maintain 37°C, 5% CO2 and humidity.

**Single cell analysis and image analysis**

A custom written Matlab based cell tracking software was used for segmentation of each nucleus according to H2B-CFP fluorescence. Flat field images were acquired at increasing exposure time in order to create a calibration mask. This mask was applied to raw images to normalize differences of fluorescence illumination and eliminate background noise from the camera. Nuclei were segmented using an adaptive or global threshold determined by Otsu’s method and touching nuclei were separated by a watershed algorithm. Based on nuclear segmentation, the coordinates of the center of mass of each nucleus were calculated and the cells were tracked from one time point to the next using a custom algorithm. This algorithm, based on “greedy matches”, connects cells based on distance, area and fluorescence intensity of the nuclear marker. For each nucleus that was identified during segmentation, a doughnut shape like area of a given width was placed around it. This area was used to estimate fluorescence in the cytoplasm. The algorithm ensured that the estimated cytoplasmic area did not overlap with cytoplasm or nuclei from neighboring cells. In this manner we were able to measure the absolute fluorescence intensity of proteins fused with fluorophores localized both in the nucleus and in the cytoplasm at each time point.

P53 mean trajectories extracted from each tracked cell are synchronized *in-silico* to cell division. Cell division is detected in the nuclear marker channel as the fluorescent intensity of the nuclear marker double during the S phase of mitosis and drop back to initial level after cellular division has taken place. Single cell analysis was done by a MATLAB based custom written image analysis software. For single cell analysis, tracking data was taken as absolute
fluorescence intensity normalized to the area. Cells leaving the field of view were excluded to ensure that the data is not biased by traces of cells lost early in the experiment. Finally the resulting single-cell trajectories were computationally analyzed to extract features of p53 dynamics, e.g. amplitude or duration of protein accumulation pulses.

**Protein purification and Western blotting**

For immunoblotting $6 \times 10^5$ cells of MCF10A and A549 were seeded 2 days prior to the experiment in growth medium and harvested at the opportune time points after 10 Gy of $\gamma$-irradiation. Cells were subsequently lysed in the presence of protease and phosphatase inhibitors and proteins were extracted and quantified using the BCA assay. Equal protein amounts were separated by electrophoreses on 4–12% Bis-Tris gradient gels and transferred to nitrocellulose membrane by electroblotting. We blocked membranes with 5% non-fat dried milk according to manufacturer's recommendations and incubated them overnight with primary antibody. The next day the membranes were washed in TBS-T and incubated with the secondary antibody coupled to peroxidase, and washed again. The protein levels were detected using chemoluminiscence (ECL Prime, Amersham).

**RT-qPCR**

For RT-qPCR $6 \times 10^5$ cells of MCF10A and A549 cells were seeded 2 days prior to the experiment in growth medium and harvested at the opportune time points after 10 Gy of $\gamma$-irradiation. Cells were harvested and mRNA extracted using High Pure RNA Isolation kits. Complementary DNA was generated using M-MuLV reverse transcriptase and oligo-dT primers. Quantitative PCR was performed in triplicates using SYBR Green reagent on a StepOnePlus PCR machine. Sequences of primers used are listed in the chapter above 6.1.4.

**Immunofluorescence**

Cells were grown on coverslips coated with poly-L-lysine; cells were treated as appropriate (TNFα, IR etc) and fixed at the right timing with 2% paraformaldehyde, and permealized with 0.1% Triton X-100 in PBS. Cells were then blocked with 10% goat serum and incubated with p65 antibody in 1% BSA for 1h at room temperature. Cells were washed in PBS, incubated with secondary antibody coupled to Alexa Fluor 488 in 1% BSA, and washed again. Finally, they were stained with Hoechst, and embedded in Prolong Antifade. Images were acquired with a 20x plan apo objective (NA 0.75) using appropriate filter sets. Automated segmentation was performed in MATLAB (MathWorks) using a costume algorithm.
7 References


Borchers et al. as well, as show their that genetic Disorder and residual helicity alter p53-Mdm2 binding affinity and signaling in cells. (2014) Nature Chemical Biology 10, 1000–1002.


Leontieva O.V. and Blagosklonny M.V. DNA damaging agents and p53 do not cause senescence in quiescent cells, while consecutive re-activation of mTOR is associated with conversion to senescence. (2010) Aging 2(12), 924-935.


Wu Z.H., Shi Y., Tibbetts R.S., Miyamoto S. Molecular linkage between the kinase ATM and NF-κB signaling in response to genotoxic stimuli. (2006) Science, 311, 1141–1146


Xu et al. Hyperthermia Induces the ER Stress Pathway. (2011) Plos One, 6 (8).


89
8 Acknowledgment

I would like to express my thanks to Prof. Alexander Löwer for giving me the opportunity to do my PhD in his research group and his willingness to conduct the examination of this thesis. I thank the group of Prof. Claus Scheidereit especially Dr. Michael Hinz for the help with materials and advices.

I thank all my colleagues Ana, Andrea, Caibin, Gitta, Ilias, Ines, Jette, Marcel, Manuela and Silke for the lively atmosphere in the lab and the support.

I specifically thank Ana and Jette with whom I started the PhD and lived many adventures, laughed a lot and learned a lot. They also helped me to remain sane during the all four years.

A special thank goes to my family and friends especially Vanessa and Giorgia that have always supported me all the way and helped me to finish the work. I especially thank Tiago for the care and the patience in supporting me through the writing.

“To live is the rarest thing in the world. Most people exist, that is all.”

O.W.
Hiermit bestätige ich, dass die vorliegende Arbeit von mir selbständig verfasst wurde und ich keine anderen als die angegebenen Hilfsmittel, insbesondere keine im Quellenverzeichnis nicht benannten Internet-Quellen, benutzt habe und die Arbeit von mir vorher nicht zu einem anderen Prüfungsverfahren eingereicht wurde.

Berlin, den 28.9.2015__________________________

Elena Cristiano