Single molecule tracking of transcription factors in live cell nuclei

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Abstract

The regulation of gene expression depends on many cellular processes. An important factor in eukaryotic cells is the initiation of transcription, which is regulated by chromatin structure within promoter and enhancer regions, and also by large factors that interact with these regions. These interaction factors are transcription factors (TF), which are thought to be the first to bind to specific regulatory sites of genes and help to recruit other regulatory factors for the gene. Chromatin binding in living cells, can be dispositived by detecting the retardation of a fluorescently tagged protein, as it interacts with the comparatively immobile scaffold. In single-molecule tracking (SMT), the retardation is determined by tracking particular molecules and directly identifying bound molecules that stop moving.

It is not known what domains in the transcription factor regulate its residence time on chromatin so one of the goals in my master thesis was to start investigating this question, in particular to determine whether the residence time is determined solely by the DNA binding domain or whether other parts of the transcription factor protein are also involved.

To generate mutations of the transcription factor cloning was performed. For this purpose I mutated different domains of p53, and its core DNA binding domain which is supposed to be involved in specific-site binding of p53 to DNA targets. And a separate mutant in the tail domain delta30 was generated, which has been hypothesized to mediate non-specific DNA binding. For single molecule tracking in live cell, the constructs were labeled with the Halo tag marker. Halo tag is a ~30kD protein that was linked onto the end of the p53 protein and covalently binds the small fluorescent ligand TMR that is bright and does not photobleach too fast. This fluorophore is membrane permeable and is added for Halo tag labeling prior to molecule microscope. Movies of single molecules in the nucleus were made and then used for tracking the motion of individual molecules. Molecules making large jumps there supposed to be mobile, whereas molecules make tiny jumps or no jump at all were supposed to bind to a DNA site.

This study aimed to understanding whether the proteins just moved by simple diffusion or whether they run into trapes which restrict their motion. For bound molecules, we measured the residence time and their correlation to amount of transcript that was produced from a gene.

Zusammenfassung

Die Regulation der Genexpression ist von vielen zellulaeren Prozessen abhaenig. Ein wichtiger Fakor in eukaryotischen Zellen ist die Initiation der Transkription. Diese wird durch die Chromatinstruktur innerhalb der Promotoren und Enhancer Region reguliert, sowie durch zahlreiche Faktoren die mit diesen Regionen interagieren. Diese Faktoren bezeichnet man als Transkriptionsfaktoren. Sie binden zuerst an der genspezifischen regulatorischen Stelle und veranlassen andere regulatorische Faktoren für dieses Gen zu rekrutieren.
Die Bindung von Chromatin in lebenden Zellen kann duch die Erfassung der Retardation eines Fluoreszenz markierten Proteins durch Bindung an ein „immobile scaffold“ bestimmt werden.Diese Retardation kann durch Single molecule tracking (SMT) fluoreszenz markierter Moleküle bestimmt werden.
Ziel dieser Arbeit ist es, zu verstehen wie sich die Transkriptionsfaktoren (p53 Mutanten) im Zellkern bewegen und wie diese Bindung die DNA Transkription beeinflussen kann.
Während meiner Master Arbeit habe ich unterschiedlich Transkriptionsfaktoren von p53 Mutanten kloniert, die es erlaubten Single molecule tracking von geeigneten modifizierten Transkriptionsfaktoren in lebenende Zellkern durchzuführen. In diesem Zweck wurden Konstrukte von p53 mit einem Halo tag exprimiert. Letzteres ist ein ~30kD grosses Protein, das kovalent mit einem relativen membrandurchlässigen Fluorophor (Tetramethylrhodamin) in der Zelleeingebracht werden kann. Mit einem Single Molecule Mikroskop wurden Filme von Einzelmolekülen im Zellkern angefertigt, um die Bewegung der individuellen Moleküle zeitabhängig zu verfolgen. Moleküle, die grosse Sprünge von einem Zeitpunkt zum naechsten vollziehen werden als bewegliche Moleküle angesehen, wogegen Moleküle die sehr kleine Sprünge oder keine Sprünge vollziehen, DNA gebunden sind. Für die gebunden Moleküle wird die Analyse dieser besimmt und diese mit der Menge an Transkriptions Produkt bestimmter Gene korreliert.

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1 Introduction

The accurate regulation of gene expression depends on a lot of cellular processes. An important factor in eukaryotic cells is the initiation of transcription. This is regulated by the chromatin structure within promoter and enhancer regions, and also by large factors that interact with these regions. This interaction factors are the transcription factors (TF), which are thought to be the first to bind to the gene specific regulatory sites and help to hire other regulatory factors for the gene.

Binding to chromatin can be dispositived in living cells by detecting the retardation of a fluorescently tagged protein as it interacts with a comparatively immobile scaffold. In single-molecule tracking (SMT), the retardation is determined by tracking particular molecules and directly identifying bound molecules as those that stop moving.

There are several live cell binding studies using SMT that produce widely aberrant estimates with no consensus. These studies show results of chromatin-bound fractions of transcription factors that range from 20 – 99%, leading to diverse predictions about regulatory-site occupancy. This is not limited to bound fractions, as estimates for transcription factors residence times on chromatin have reached from 0.005 sec to 4.5 sec. Many of these discrepancies are results of technical errors, because the measuring of live cell binding has limitations. It is important to resolve this discrepancies due to in vivo measurements are crucial for determining how cellular reactions flow in the complex milieu of the live cell.

The binding ratio for SMT is more important since bound molecules can be visualized, although the measurement is quite complex because a stationary molecule will appear to move based on the precision limit of localization and a freely diffusing molecule will bound transiently if it undergoes some small displacements. Eventually, identifying bound molecules remains a key question in single molecule tracking.

1.1 Transcription

Transcription is the key factor in the regulation of gene expression. The transcription process contains several different steps and starts when the RNA polymerase (RNA plo) binds the template DNA strand and starts to catalyze production of complementary RNA(Hager et al. 2009). There are three different types of RNA polymerases in eukaryotic cells. RNA pol I in eukaryotic cells transcribes genes that encode most of the ribosomal RNAs (rRNA). RNA pol III encodes small rRNA and transfer RNAs, as well as other small regulatory RNA molecules. RNA pol II transcribes messenger RNAs, which conduce as the templates for production of protein molecules. The first step of transcription is initiation, which is the binding of RNA pol to the DNA upstream of the promoter.

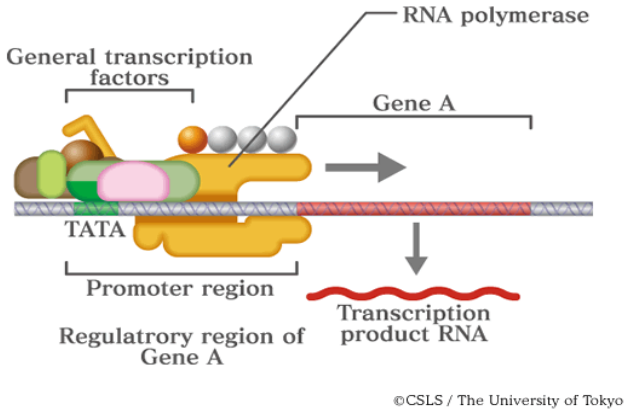


Figure 1: Initiation of transcription from a eukaryotic promoter (http://csls-text.c.u-tokyo.ac.jp/active/04_03.html)

In eukaryotic cells the core promotor is often found shortly upstream of the start site of the gene, which is transcribed by pol II. The transcription rate and localization of the start site can be affected by pol II genes which have a TATA box 25 to 35 bases upstream of the initiation site. RNA polymerases in eukaryotic cells benefit transcription factors (TF). One of these factors is TFIID, which recognizes the TATA box and assures that the correct start site is being used. The enhancer sequences control the level of gene transcribing. Enhancers can be thousands of nucleotides distant from the promoter with which they interact, but they are brought into proximity by the looping of DNA. The result of this looping is the interaction between the proteins bound to the enhancer and those bound to the promoter. Proteins that inhibit looping are

called repressors and those that boost are called activators. Once the transcription is initiated, the RNA polymerase reads the single strand and adds nucleotides on the 3' end of the growing chain (Izban and Luse, 1992 and Clancy, 2008).

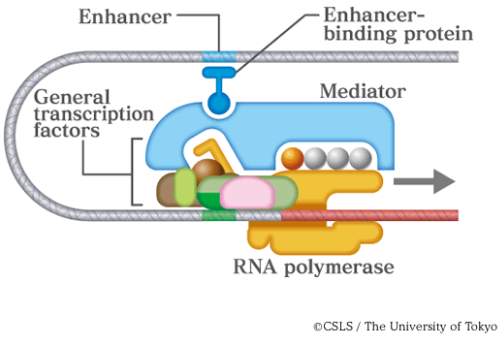


Figure 2: Transcriptional activation by an enhancer (http://csls-text.c.u-tokyo.ac.jp/active/04_03.html)

The termination of transcription in eukaryotes is initiated by different processes, depending on the utilization of polymerase. With in genes transcribed by polymerase I, the transcription stops using a termination factor. The transcription stops after transcribing a terminator sequence, which contains a polyuracil stretch, by a mechanism resembling rho-independent prokaryotic termination. The transcription of pol II genes can last for hundreds or thousands of nucleotides exceeding at the end of a coding sequence by RNA polymerase cleavage. Cleavage associates to be linked with termination of transcription and occurs at a consensus sequence. The 3'-end of the mature pol II mRNAs is polyadenylated, which is resulting in a poly (A) tail. This event is followed by cleavage and is also coordinated with termination (Kritikou, 2005 and Clancy, 2008).

The process of transcription is actually dynamic but that is known about the transcription machinery is pre-built, from static biochemical analysis (Hager et al. 2009). These methods have been beyond price in specifying the key factors involved in the transcription process and also their interactions but they are not perfectly adequate to gain insight into the real-time kinetics of transcription. The use of newly developed cell biological methods, usually based on *in vivo* imaging, to the study of transcription in its natural correlation and in real-time has conquer some of these limitations (Misteli, 2001 and Darzacq et al., 2009). These

new technologies are now supplying first insights about how transcription is arising in a live cell nucleus (Hager et al. 2009).

1.2 Transcription Factors

1.2.1 Overview

The process of transcription comprises multiple distinct steps. The core promoter structure bears sequences that attend to keep a set of protein complexes fundamentally referred to as the common transcription factors (Kornberg, 2005).

The regulation of transcription in eukaryotes is the result of shared effects of structural behavior of the DNA and the interaction of proteins, so called transcription factors. There is one important structural difference between eukaryotic and prokaryotic DNA, which is the development of chromatin in eukaryotes. The general function of these regulatory proteins is to activate or inhibit transcription of DNA by binding to specific DNA sequences. Transcription factors have specific DNA-binding domains with an up to 10^6-fold higher affinity of their target sequence than of the DNA-strand. On the basis of these highly conserved sequences the transcription factors can be classified into several families, such as SOX proteins, MADS box-containing proteins, and POU factors (Remenyi et al., 2004). They can also be classified by their three-dimensional protein structure, including helix-turn-helix, helix-loop-helix, and zinc finger proteins. These different kinds of motifs result in transcription factor accuracy for the consensus sequences to which they bind (Philips, 2008). Sequence-specific transcription factors are the major and most different mechanisms of gene regulation in prokaryotic and eukaryotic cells (Pulverer, 2005). Many activating transcription factors are bound to DNA until removed by a signal molecule, whereas others might only bind to DNA, once influenced by a signal molecule. Also the binding of one transcription factor type can affect the binding of others. The gene expression is highly variable addicted to the type of activators participating and to signals that are currently control bindings (Philips, 2008).

1.2.2 3D genome scanning

The recruitment of transcription complexes to target genes constitutes the basis of all transcriptional activity and regulation. This machinery relates to well-defined binding sites in promoter regions, as regulatory factors bind to specific sites in control elements in the proximity and, at times, at long excision away, from target. For gene-specific regulators and for the basal machinery, the specific binding sites are acutely rare in the genome in comparison to the number of nonspecific binding sites which may have an effect on defined transcription factors. The average size of a mammalian core promoter is 150 nt and the promoter area itself is smaller than 0.1 % of the human genome, which means there are only a couple of specific binding sites in the genome. To find the rare specific binding sites in the genome, transcription factors have two essential dynamic properties. The first is capability to quick diffusion through the nucleus and second their addiction to high transiently bind to chromatin (Gorski et al., 2006, Hager et al. 2009 and Misteli, 2001). FRAP (Fluorescence recovery after photobleaching) experiments of transcription factors have detected that most of the TF move fast within the nucleus (Sprague et al., 2004). The diffusion coefficient of a transcription factor ranges from 0.5 to 5 $\mu m^2 s^{-1}$ which depends on the size, shape, and its interaction with chromatin. This diffusion behavior allows a molecule to transit the entire length of a mammalian nucleus in a few seconds and makes it also possible for a single molecule of a transcription factor to visit the area of the nucleus in a matter of minutes. This high mobility is the basic for their efficiency to find their few specific binding sites in the genome. The second attribute that contributes to a capable transcription factor targeting is the highly dynamic and transient nature of their binding to chromatin in vivo (Hager et al. 2009). The quick movements through the nuclear space and the much transient interactions of transcription factors with chromatin along the way point towards a 3D scanning model to find their specific target-binding sites in the genome (Misteli, 2001). When transcription factors diffuse with in the nucleus, they often interact physically and build conjunction with chromatin fibers along the way (see figure 3). This conjunction often happens with off-target sites and does not result in any functional interaction, due to the scarcity of specific binding sites. After a brief immobilization, the transcription factors separates and sustain its random walk through the nucleus until it meets another chromatin fiber, where it experience another interaction. The 3D

hopping will sustain till the molecule finds a specific target site where it binds and activates a functional response (see figure 3).

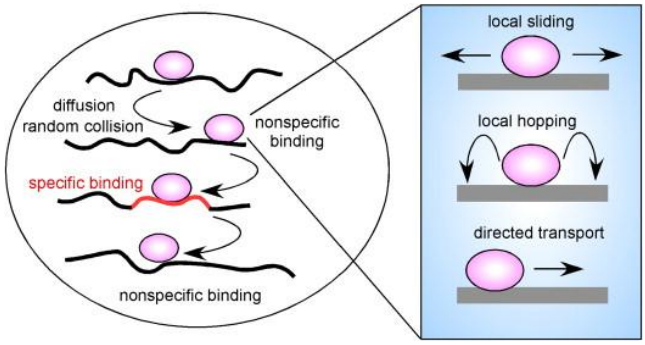


Figure 3: Transcription factors find their specific binding sites by random scanning of the genome in 3DA. The TF (purple) diffuses through the nuclear space and by random collision associates with chromatin. Most encounters are at nontarget sites resulting in highly transient interactions. Occasionally, a specific binding site (orange) is encountered, and prolonged binding occurs. At each encounter a TF might undergo local motion on the chromatin fiber by either sliding along the DNA, hopping locally, or by directed, motor driven motion.

An essential implication of the 3D model is that the huge majority of molecules of a transcription factor are bound to chromatin at any time, although at nontarget sites (Hager et al. 2009). Single-molecule monitoring on the lac-repressor in living bacteria shows that a single lac repressor molecule spends most of its time bound to chromatin even though it's not linked with specific target site (Elf et al., 2007). The interaction of transcription factors with nonspecific sites will not generate any functional result since other factors necessary to generate a transcriptional response are missing. It is fewer clear if nonspecific interactions of others, for instance histone-modifing enzymes or chromatin modelers, do bring to a functional response. It seems that the 3D hopping is complemented by local scanning of the DNA (Gorman and Greene, 2008). It's important to now that once a transcription factor associates with the chromatin fiber, it can also go into a local search type, and interact with chromatin fiber in the neighborhood. Using one-dimensional diffusion coefficients from in vitro studies and rating of well times on the consequence of few seconds deduced from photobleaching experiments, it is theoretically possible that a transcription factor searches multiple hundred base pairs before separation again. The local search of the

transcription factor could be developed via one-dimensional sliding along the chromatin fiber. This can be complicated at the complex higher-order folding of the chromatin fiber and the areal barrier generated by the attendance of a large number of architectural chromatin proteins that blanket the chromatin fiber. To analyze its direct environments, it is also possible that a transcription factor might undergo local motion through local hopping, directed, motor driven motion or handover between chromatin segments (Gorman and Greene, 2008).

1.2.3 Binding of transcriptions factors in the genome

All DNA in eukaryotic cells is organized in nucleosomal arrays. This iterant chromatin fiber is organized in sequence into congeries higher-order structures that conduce to compact the large amount of DNA. If transcription factors transit the nuclear space, they collaborate repeatedly with DNA, especially with chromatin. Because nucleosome octamers sequester plenty of the DNA from direct contact, the reorganization of these structures must connect efficient interactions with regulatory elements (Hager et al. 2009). Some studies showed that a lot of the specific sites, where transcription factors bind, have regional chromatin structures that are subset sensitive to nucleolytic representatives, especially DNase I and microcrococcal nuclease (Wu et al., 1979). These sites, often referred to as DNase I hypersensitive sites (DHS), incorporate areas with tattered nucleosome structures. Lately studies showed that a high number of DHS sites are present in eukaryotic genomes with are approximately 2 % (Boyle et al., 2008 and Hesselberth et al., 2009).

Other studies also show that a large fraction of transcription regulatory elements is linked with these DHS sites (Hager et al. 2009). The interaction of site-specific DNA binding proteins with chromatin is nearly universally linked to chromatin remodeling. Transition between two comparative stable sites occurs due to sliding of nucleosoms of exchange position, cleavage, or distance of core structures (Mellor, 2005 and Hager et al., 2009). In comparison to this, divers studies have implied that the remodeling of nucleosome structures at DHS sites is a running and dynamic process. Beside these, other characteristics of regulatory transcription factor binding sites affect their general dispersal throughout the genome. A large majority of response elements are located at appreciable distances from target promoters. New studies indicate that interacting

elements of transcription factors can be monitored at distances of 200 kb or greater (Hakim et al., 2009; Gondor et al., 2008 and Simonis et al., 2006). These long-range interactions between regulatory sites and promoters seem to be a widespread mechanism in eukaryotes. The fast interaction of factors with chromatin in living cells is left with a conundrum. There are several mechanisms that can be proposed. For example CTCF, anchoring proteins, which is often found at domain boarders or cohesions, may offer stability for long-range interactions. There are also other architectural elements that assist with interacting elements in local proximity. The idea of a transcription factor asserts a local domain with elementary components of the transcription apparatus tied at subnuclear sites. Those structures could form accumulation sites for elevated concentrations of transcription factors and factor template interactions in these areas could be highly transient, as obtained in FRAP experiments. In fact, the work of Fraser and colleagues showed the dynamic nature of these structures (Hager et al. 2009 and Osborne et al., 2004).

1.2.4 The role of chromatin

The structure of chromatin collaborates with the different levels of complexity in gene regulation. It allows synchronous regulation of functionally or structurally similar genes that tend to be present in largely spaced clusters or domains on eukaryotic DNA (Sproul et al., 2005). The chromatin domains have plenty of sizes and several dimensions of stability. These variations are solely found in eukaryotes, such as epigenetic memory, throughout cell division cycles and transcription at different stages of development. They are also enabling the perpetuation of differentiated cellular levels, which is essential to the survival of multicellular organisms (Philips, 2008 and Struhl, 1999).

Even if transcription factors are present in a cell, transcription does not always happen, because often the transcription factors cannot catch their target sequences. The combination of proteins and the DNA molecule is the first step in its silencing. These adjunctive DNA and histone proteins are collectively called chromatin. This complex is strong bounded by adduction of the negatively charged DNA to the positively charged histones (Philips, 2008).The nucleosome is the structural unit of chromatin; it consists of a central protein complex, the histone octamer, and two turns of DNA, of about 146 base pairs (bp), which are wrapped around the histone octamer

complex. The octamer has four different types of core histones which carry two copies each of H2A, H2B, H3 and H4 (www.nobelprize.org).

The contact of transcription factors and RNA polymerase to DNA promoters can restrict the state of chromatin, contributing to the constrictive ground state of gene expression. In order to gene transcription to happen, the structure of chromatin requisite is unwound (Philips, 2008). The chromatin interaction with activators and repressors can occur in domains of chromatin that are open, closed, or poised for activation.

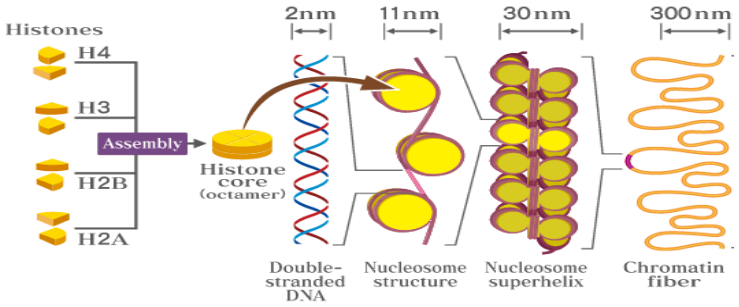


Figure 4: Construction of chromatin fiber (http://csls-text.c.u-tokyo.ac.jp/active/04_03.html)

1.3 p53 Protein

1.3.1 Overview

Thirty years ago, a nuclear phosphoprotein of 53 kDa that interacts with the simian virus 40 (SV 40) T antigens and is common detected at high levels in cancer cells was identified. This was the beginning of the explosive field of p53 (Brosh and Rotter, 2009). After 10 years, researchers detected that it was a missense mutation in the DNA-binding domain of the p53 Which leads to oncogenic functions of p53 protein, which was later called “gain of oncogenic function” (Bai and Zhu, 2006).P53 is a tetramer transcription factor which adjusts to a large number of genes, including DNA damage and oncogene activation. P53 is also a stress response protein and is activated by these signals through post translational modifications, which leads to a high transcription activity and therefore to a high protein level. The p53 protein has the ordinary characteristics of a transcription factor. It consists of a core DNA-binding domain, an amino-

terminal transactivation domain, a caboxy-terminal tetramerization domain and also regulatory domains. The activated p53 represses apoptosis, DNA repair, cellular transformation and differentiation in defected cells and leads to changes in metabolism (Brosh and Rotter, 2009). The occurrence of tumor protein p53 (TP53) mutations vary considerably among cancer types, for instance from 10% in haematopoietic malignancies to 50 -70% in cancers of colon, head, neck, lung and ovary (Bai and Zhu, 2006). Whereas somatic TP53 mutations play an important role in sporadic cancer, germline TP53 mutations cause unusual type of cancer predisposition also known as Li-Fraumeni syndrome. The Li-Fraumeni syndrome is a scarce autosomale-dominant highly shrill cancer predisposition syndrome (Brosh and Rotter, 2009). The results of somatic TP53 mutations are dysfunction or deficiency of p53 by which the p53 pathway is damaged during tumorigenesis. Germline TP53 mutations increase the risk of sarcomas, brain and breast cancers (Whibley, Pharoah and Hollstein, 2009). There are two key categories of TP53 mutations, conformable to their effect on the thermodynamic stability of the p53 protein. These two key categories are qualified as “DNA-contact” and “conformational” mutations. DNA-contact mutations contain changes in residues directly involved in DNA binding, for example R248Q and R273H. Conformational mutations are structured in two groups, first mutations with local reasons, such as R249S and G245S, and second mutations with global conformational distortions such as R175H and R282W (Brosh and Rotter, 2009 and Slee et al. 2004). The TP53 is under tumor suppressor genes inimitable a result of so many missense-mutations that can appear and this generates an area of mutant p53 proteins with different levels of residual activity. Depending on the mutation, divers’ elements of normal p53 mediated reply can be lost and which leads to new non-wild-type functions (Whibley et al. 2009).
The question is, if it is the loss of wild-type p53 function, the purchase of dominant-negative quality, the gain of new oncogenic function or maybe a combination of all (Brosh and Rotter, 2009).

1.3.2 Structure of p53

The p53 protein has a molecular weight of 53 kDa and is encoded by a gene containing 11 exons and 10 introns (Lamb and Crawford, 1986). This 20Kb gene is located on the small arm of

chromosome 17. P53 has three functional domains, a C-terminal tetramerization domain, N-therminal activation domain and a DNA-binding domain.

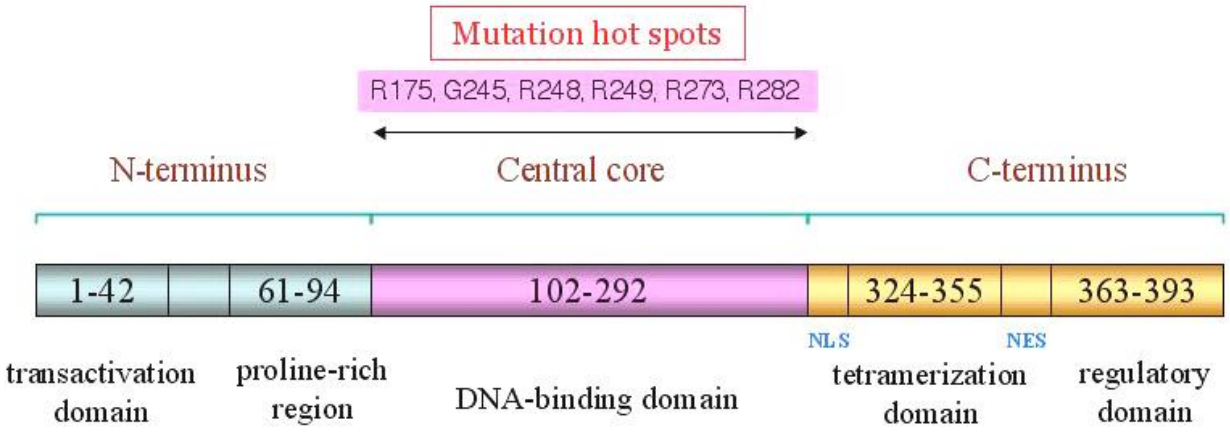


Figure 5: Schematic representation of the p53 structure. The protein bears 393 amino acids and has three functional domains. It contains an N-terminal activation domain, a DNA binding domain and a C-terminal tetramerization domain. The DNA binding domain is needed for sequence´specific DNA binding, the N-terminus domain contains the transactivation subdomain, and the C-terminus domain is considered to perform a regulatory function (Bai and Zhu, 2006).

The C-terminal region consists of a strong regulatory domain, an oligomerization domain, and a nuclear localization signal sequence (NLS) and a nuclear export signal sequence. This C-terminal region has also a regulatory function, which occurs due to C-terminal acetylation and phosphorylation (Slee et al. 2004). The N-terminal domain bears repeated copies of the PXXP region, which is the proline-rich region that also includes a transactivation subdomain. The central core region is essential for sequence-specific DNA binding and contains amino acid arrears. These amino acid arrears are hot spots in different human cancers. Representative are Arg175, Gly245, Arg248, Arg249, Arg273 and Arg282 (Bai and Zhu, 2006). The simple C-terminal of p53 has also the capacity as a negative regulatory domain and can induce cell death. Corresponding to the allosteric model, in which the C-terminal tail of p53 is a negative regulator it can regulate the skill of its core DNA binding domain to close the DNA binding domain as a covered conformation. In that case the interaction between the C-terminus and the core DNA binding domain is broken by posttranslational modification. The DNA binding domain becomes active and therefore generates an upgraded transcription activity. Many of p53 cancer studies

showed that the majority of the mutations in p53 are missense mutations and that they are basically located in the central DNA-binding domain. More than 80% of these studies have concentrated on residues between AS 126 –306 (Cho et al. 1994 and Bai and Zhu, 2006).

1.3.2 Mode of p53 regulation

Transcription of genes can be activated or suppressed by the p53 protein. Sequence-specific DNA binding is the main reason of transcriptional activation, whereas p53 is using direct and also indirect ways to repress the gene transcription. Almost all p53-activated genes exhibit at least one assumed DNA-binding site that temperately matches the consensus p53 response element. P53 can bind and then hire general transcription proteins (TATA-binding protein-associated factors) to the promoter and –enhancer region of p53-regulated genes to initiate transcription by protein-protein interactions. Latest studies showed that p53 can also hire histone acetyltransferases (HATs) CBP, PCAF and p300 to the promoter-enhancer region of genes. These happen by high-affinity protein-protein binding (Gu et all. 1997, Thut et al. 1995 and riley et al. 2008). Through these HATs acetylate Lys remain of histones in chromatin and enhance the transcriptional activity. In several genes, the binding of p53 to its response elements (REs) develops a direct repression of that gene. Actually, three common accepted forms of direct p53-mediated repression are known. The first one is the binding-site overlap (steric interference), the second is p53 squelching of transcriptional activators and the third is p53-mediated enlist of histone deacetylases (HDACs). P53-mediated repression by steric interference accompanies sequence-specific DNA binding by p53 which covers the binding site of another stronger transactivating protein. The p53 squelching starts by p53-mediated protein-protein interactions. Based on the observation that p53 binds the transcription machinery proteins TBP (TATA-box-binding protein), TAF6 (TBP-associated factor-6), TAF9 (TBP-associated factor-9) and different *in vitro*, in the beginning it was accepted that p53 repression was received by p53 binding and suppression of these TATA-box-bound basically factors *in vivo*. Experimental proof showed that the preferred *in vivo* method of p53-mediated squelching is gained by binding and inhibiting the transactivators of the CCAAT box (Riley et al. 2008). There are two approved forms of indirect p53-mediated repression. The first type appears by p53-mediated activation of CDKN1A, which

burkes the cyclin D-CDK4 complex by direct binding. The consequence is the absence of hyperphosphorylation of the retinoblastoma protein from G1 stage of the cell cycle (Loehr et al. 2003).The unphosphorylated retinoblastoma protein disables the function of the E2F transcription factor family by direct binding. In the second mode, p53 binds to different transcription factors and, combined, they repress a gene without a p53-specific response element (Riley et al. 2008).

1.4 Single molecule tracking

In the last 20 years, the discovery speed of respective to single-molecules in biological systems has been remarkable. The story starts with Arthur Ashkin in 1986 when he published his work using optical traps to analyze individual dielectric particles. In1993, Steven M. Block was using the optical trap to measure the 8-nm step size of kinesin. In 2005 he measured the single-base-pair step size of RNA polymerase moving on DNA. Also the story for optical detection sounds very similar. In 1989 W.E. Moerner and L. Kador published the detection of single-molecule contamination at liquid helium temperatures. By 2000, Toshio Yanagida and also Hansgeorg Schindler made the first single-molecule *in vivo* analysis. Today, most of the single-molecule fluorescence methods are done *in vitro*, whereas *in vivo* analysis is a growing field (Selvin and Ha, 2008).

The single-molecule analysis is an important method to detect biomolecules *in vitro*. This advantage has been adjusted to studies using living cells, in which it is possible to quantify the dynamic and kinetic characteristics of single-molecule reactions *in vivo*. With SMT it is possible to follow directly the motions of linear and rotational molecular motors, enzymatic reactions, structural dynamics of proteins and DNA-protein interactions *in vitro*.

In living cells proteins act as part of molecular networks that have specific features, such as gene expression, membrane transport or energy transduction. Dynamic and kinetic information's of the single processes within the networks, such as the translocation and movement of proteins, protein-protein interactions and enzymatic reactions, must be ascertained in living cells. Single-molecule analysis *in vivo* has the ultimate level of sensitivity and can be used to locate and monitor single reactions in living cells, whereas it has multiple other advantages. For example, concerning single-molecule measurements, the reactions must be synchronized. Normally each

molecule of a protein reacts several times and positions in a cell. To receive the kinetic and dynamic parameters of a reaction, specific techniques are necessary to synchronize the start point of every molecule. If it is not possible to synchronize the middling steps of a reaction, single-molecule measurements have the convenience to avoid these problems. Measurements of single molecules also deliver information about the distribution and fluctuation of kinetic and dynamic parameters. Distribution and fluctuation of each molecule of a reaction are belike important to understand the mechanisms of cellular events. The third point is that single-molecule analysis allows the correlation between input and output of single events of protein reactions to be quantified. In single-molecule experiments, it is possible to control each input and output event individually. For statistic analysis of the data, it is important to check the single-molecules manually. The automatic image processing is a challenge for experiments with single-molecules in living cells, due to the non homogeneous background and limited single to noise ratio. In spite of these drawbacks, single-molecule data are extremely important and useful to understand cell biology.

Single-molecule techniques in living cells allow to visualize the movements and locations of molecules, and to discover a number of molecules that are participating in cellular reactions. Activation of molecules has been identified and kinetics of intracellular molecules has been measured. This technique has also proven to be useful to quantify cellular reactions and it will be essential for the further understanding of the molecular reactions of cellular answer. In the future, the combination of single-molecule-manipulation, single-molecule-visualization and single-molecule-electrophysiology techniques will be fundamental to allow us to understand the nanobiology of living cells (Sako and Yanagida, 2003).

2 Materials

2.1 Cell culture of H1299 cells

The H1299 cells were stored in a 1.5 ml tube at liquid nitrogen and after the unfreezing; these cells were cultured in 250 ml flask and transfected in 1.5 ml Coverglas chambers. For the cultivation and transfection three different media were used. Medium I was based on RPMI – 1640 and included 10% Fetal Bovine Serum and 0.5% Penicillin/ Streptomycin. The medium II was an OPTI – MEM with reduced Serum, HEPES, L- Glutamine and 2.4 g/ L Sodium Bicarbonate. The Medium III was based on DMEM without Phenol red and contained 10% Fetal Bovine Serum, 4.5 g/ L D-Glucose and 0.5 % Penicillin/ Streptomycin.
After 1 – 2 days the medium was refreshed. In an interval from 2 – 3 days the cells were splitted 1: 3 so that the cells were in the logarithmic growth phase at the time of the experiment.

Following reagents were used in the cell culture:

Reagent	Company
Fetal Bovine Serum	Gemini Bio-Products
Penicillin/ Stretomycin	Gibco
Dulbecco's Phosphate Buffered Saline 1x	Gibco
Ethyl Alcohol	The Warner-Graham Company
Trypsin-EDTA	Gibco
250 ml Tissue culture flask	Becton Dickinson
1.5 ml Chambered Coverglass	Lab-Tek
5 ml Stripette	Corning
10 ml Stripette	Corning
50 ml Polypropylene Conical Tube	BD Biosciences
1.5 ml Polypropylene Flat Cap	Sarstedt
RPMI 1640	Gibco

Table 1: General reagents

Instrument	Company
Circulating water bath	Precision
Hood	BioRad

Table 2: Instruments used during cell culture

2.2 Cloning Polymerase Chain Reaction (PCR) Products into a Vector

2.2.1 Polymerase Chain Reaction (PCR) with the "Expand high fidelity PCR System"

The first step of the Cloning PCR Products into a Vector was a classical PCR reaction. For the PCR only 1 µl (100pg/ µl) of the DNA was used, to avoid poisoning of the PCR reaction. For the mater mix was used the "Expand high fidelity PCR System" with following reagents:

Reagent	Company
Expand High Fidelity Enzym Mix	Roche
Expand High Fidelity buffer with 15mM $MgCl_2$	Roche
Deoxynucleoside Triposhate Set	Roche

Table 3: Reagents used for Reaction Mix

For the working solution of the Oligonucleotides the stock solution with a concentration of 100 µM were diluted to 10 µM with water.

Primer	Primer Sequence
DM0031	5'-GACCGCGATCGCCCAGTCAGATCCTA-3'
A005	5'-TGCGGTTTAAACCTGGCTCCTTCCCA-3'
P53_fwd	5'-GGGTACGATTTAGGTGACACTATAG-3'
P53_rev	5'-GAGCCCGAATTCGTTTAAACGTCTGAGTCAGGCCCTTCTGTCT-3'
P53d30_rev	5'-GAGCCCGAATTCGTTTAAACCCTGCTCCCCCCTGGCTCC-3'

Table 4: Primer for the PCR reaction with the "Expand high fidelity PCR System"

Instrument	Company
Water bath Isotemp 202	Fisher Scientific
Touch Vortexer	Glas-Col
Mini Centrifuge	Fisher Scientific
C1000™Thermal Cycler	BioRad

Table 5: Instruments used during PCR

2.3 Analyzing of PCR- Products

The obtained PCR products from the "Expand high fidelity PCR System" and the" GoTaq® Green PCR" were analyzed with "Gel Electrophoresis".

2.3.1 Agarose Gel Electrophoresis

Reagent	Company
10x TAE	Quality Biological INC.
Sea Kem® GTG® Agarose	Lonza
Ethidium Bromide Solution (10mg/ ml)	Invitrogen
10x Loading dye	Fermentas
GeneRuler DNA Ladder Mix	Fermentas

Table 6: Reagents used for Electrophoresis

Instrument	Company
EC 105	E-C Apparatus Corporation
Owl Separation Systems (ClassII)	Thermo Scientific
Microwave	Profile
Gel Doc™XR Imaging System	BioRad
Scale Voyager ®Pro	OHAUS

Table 7: Instruments used for Electrophoresis

2.4 Clean up of PCR-Product

For purification, the "Wizard® SV Gel and PCR Clean-Up System" from Promega was used. The principle of the Wizard® SV Gel and PCR Clean-Up System was that the DNA can be purified used microcentrifugation to force the solution through the SV Minicolumn and washed the DNA. The Cleanup of the PCR product was performed with the Wizard®SV Gel and PCR Clean-up System from Promega.

Kit components	Company
Membran Binding Solution	Promega
Membran Wash Solution	Promega
Nuclease free Water	Promega
Collection Tube	Promega
Wizard® SV Minicolumns	Promega

Table 8: Components of the Wizard® SV Gel and PCR Clean-Up System

2.5 Cloning

2.5.1 Digestion

For the optimized reactions for the PCR product and the Flexi® Vector pFN22A (HALO-Tag®7) CMV d1 following reagents were used:

Reagent	Company
5x Flexi®Digest Buffer	Promega
Flexi®Enzym Blend (SgfI & PmeI)	Promega
Nuclease-Free Water	Promega
pFN22A (HALO-Tag ®7) CMV d1	Promega

Table 9: Reagents used for the Digestion

Restiction Enzyms	Company
SgfI recognition site: 5'...GCGATCGC...3' 3'...CGCTAGCG...5'	Promega
PmeI recognition site: 5'...GTTTAAAC...3' 3'...CAAATTTG...5'	Promega

Table 10: Restriction enzymes for the Digestion

Instrument	Company
Water bath Isotemp 202	Fisher Scientific
Touch Vortexer	Glas-Col
Mini Centrifuge	Fisher Scientific
Centrifuge 5415D	Eppendorf

Table 11: Instruments used for Digestion

2.5.2 Ligation of PCR Product and Flexi® Vector pFN22A *(HALO-Tag®7) CMV d1*

The ligation of the PCR Product HALO – R273 H and Flexi® Vector pFN22A (HALO-Tag®7 CMV d1 was performed with the Flexi®System Entry/ Transfer kit from Promega.

Reagent	Company
2x Flexi® Ligastion Buffer	Promega
DNA Ligase	Promega
Nuclease-Free Water	Promega

Table 12: Reagents used during the Ligation

2.5.3 Transformation

For transformation, the "Subcloning Efficiency™ DH5α™ Competent Cells kit" from Invitrogen was used.

In addition, Luria- Bertoni (LB) medium and Ampicillin plates, for transforamtion, were prepared.

Kit components	Company
DH5α™ Competent Cells	Invitrogen
pUC19 Control DNA (100 pg/ µl)	Invitrogen

Table 13: Components of the Subcloning Efficiency™ DH5α™ Competent Cells kit

Reagent	Company
Luria- Bertoni (LB) medium	BLF – 7030
Ampicillin	Sigma
Bacto ™Agar	Becton, Dickinson and Company

Table 14: Additional reagents used for Transformation

Instrument	Company
Mini Centrifuge	Glas-Col
Multi-Blok®Heater	Lab Line
VIP™Series	SANYO Scientific
MAXQ 4000	Thermo scientific
Digital Incubator	Boekel Scientific

Table 15: Instruments used for Transformation

2.5.4 Control of the Insert

For the control of the clones from the transformation, the "GoTaq® Green Master Mix" was used.

Reagent	Company
GoTaq® Green Master Mix	Promega
H_2O	-

Table 16: Reagents used for the GoTaq® Green PCR

For the working solution of the Oligonucleotides the stock solution with a concentration of 100 µM were diluted to 10 µM with water.

Name	Primer Sequence
DM0031	5'-GACCGCGATCGCCCAGTCAGATCCTA-3'
A005	5'-TGCGGTTTAAACCTGGCTCCTTCCCA-3'
N074_pFN	5'-ATTTCCGGCGAGCCAACC-3'
DM006	5'-CGGATCAGCTTGCATGCCTGC-3'

Table 17: Oligonucleotides for the control of the insert

Instrument	Company
Water bath Isotemp 202	Fisher Scientific
Touch Vortexer	Glas-Col
Mini Centrifuge	Fisher Scientific
C1000™Thermal Cycler	BioRad

Table 18: Instruments used for the GoTaq® Green PCR

2.5.5 Big dye sequencing

For the sequencing of the probes the Applied Biosystems 3130*xl* Genetic Analyzers was used.

Name	Primer Sequence
DM0031	5'-GACCGCGATCGCCCAGTCAGATCCTA-3'
A005	5'-TGCGGTTTAAACCTGGCTCCTTCCCA-3'
N074_pFN	5'-ATTTCCGGCGAGCCAACC-3'
Dm063	5'-CCATCCTCACCATCATCACA-3'
DM066	5'-CTTCCTTTCGGGCTTTGTTAG-3'
DM036	5'-ACACGCAAATTTCCTTCCAC-3'

Table 19: Primer for Big dye sequencing

Instrument	Compeny
Touch Vortexer	Glas-Col
Mini Centrifuge	Fisher Scientific
Applied Biosystems 3130*xl* Genetic Analyzers	Applied Biosystems

Table 20: Instruments used for the big dye sequencing

2.6 Plasmid preparation

2.6.1 Miniprep preparation

For plasmid preparation, the "QIAprep® Spin Miniprep Kit" of Quiagen was used. The concentration of the clones was measured with the "Nano Drop; ND Spectrophotometer" of ISOGEN LIFE SCIENCE.

Kit components	Company
QIAprep® Spin Columns	Quiagen
Buffer P1 (Resuspension Buffer)	Quiagen
Buffer P2 (Lysis Buffer)	Quiagen
Buffer N3 (Neutralization Buffer)	Quiagen
Buffer PB (Binding Buffer)	Quiagen
Buffer PE (Wash Buffer)	Quiagen
H_2O	-

Table 21: Components of the Miniprep Kit

2.7 Single molecule tracking

2.7.1 Transfection with Lipofectamin LTX

For the transfection with Lipofectamine LTX, medium II and III were used with following reagents:

Reagent	Company
DMEM with 4.5 g/ L D-Glucose	Gibco
Fetal Bovine Serum	Gemini Bio-Products
Penicillin/ Stretomycin	Gibco

Table 22: Reagents for Medium II

The medium III was based on DMEM without Phenol red and contained 10% Fetal Bovine Serum, HEPES and 4.5 g/ L D-Glucose and 0.5 % Penicillin/ Streptomycin.

For the optimized transfection of the H 1299 cells, Lipofectamine LTX from Invitrogen was used with following reagents:

Reagent	Company
DMEM with 4.5 g/ L D-Glucose	Gibco
OPTI – MEM with reduced Serum	Gibco
Fetal Bovine Serum	Gemini Bio-Products
Penicillin/ Stretomycin	Gibco
Dulbecco's Phosphate Buffered Saline 1x	Gibco
(DPBS)	Gibco
Lipofectamine	Invitrogen

Table 23: Reagents used for Transfection

2.7.2 SMT Microscopy

For the SMT microscopy the following instruments were used:

Instrument	Company
Olympus IX-81	Olympus Corp.
Piezoelectric stage	Physik Instrumente
EM-CCD camera (Cascade 512B)	Photometrics
Pulse generator (BNC-575)	Berkeley Nucleonics
Air-stream stage incubator	Nevtek
Matlab	Mathworks

Table 24: Instruments used for single molecule tracking

3 Methods

3.1 Cell Culture

3.1.1 Cell Culture of H1299 cells

Lung carcinoma H1299 cells, which are p53 null, were thawed in the water bath at 37 °C. The cells were pipette into a 10 ml falcon tube with proliferation medium (RPMI – 1640 medium with 10% fetal bovine serum (FBS) and 0.5% penicillin-streptomycin) and centrifuged at 1000 rpm for 10 minutes. The supernatant was rejected and the cell pellet was resuspended with 1 ml fresh proliferation medium. The cells were incubated into a 50 ml flask at 37 °C and 5% carbon dioxide. The medium was changed every 2 – 3 days with warmed RPMI – 1640 medium with 10% FBS and 0.5% penicillin-streptomycin. After 2 – 3 days the flasks were passaged into a fresh 50 ml flask.

3.2 Polymerase Chain Reaction (PCR)

3.2.1 Overview

“Polymerase Chain Reaction” (PCR) is an often used technique, to amplify a specific DNA sequence. It is an extremely efficient and sensitive method. The method is performing on thermal cycling, which consists out of cycles of repeated heating and cooling steps. The first step is a temperature increase, the DNA double helix is separated into two strands, and this step is called denaturation. After this, short specific DNA fragments (primers) anneal to their complementary sequence in the template DNA, at a lower temperature. The enzyme, DNA Taq Polymerase adds single nucleotides to the 3’ ends of the annealed primers. The result of this method is that each single template strand has a new complementary strand (http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechPCR.shtml).

3.2.2 Polymerase Chain Reaction (PCR) with the "Expand high fidelity PCR System"

The "Expand high fidelity PCR System" was especially optimized to efficiently amplify DNA fragments up to 5 kb. It was composed of a unique enzyme mix containing thermostable Taq DNA polymerase and Tgo DNA polymerase, a thermostable DNA polymerase with proofreading activity. This mixture was designed to generate PCR products of high yield, high fidelity and high specificity from all types of DNA.

The first step was to prepare the working solution of the forward and reverse primer. Therefore working solutions of the primer stock with a concentration of 100 µM were diluted to 10 µM with nuclease free water. The PCR for of the delta30 mutation was done with the forward primer DM0031 and the reverse primer A005.For the PCR of the mutation mSB and mTAD were used the forward primer P53_fwd and the reverse primer P53_rev. For the combination mSB_delta30 and mTAD_delta30 the forward primer P53_fwd and the reverse primer P53d30_rev were used. The Mix 1 and Mix 2 were prepared in sterile microfuge tubes on ice.

Mix 1 (for one reaction):

Componet	Volume	Final concentration
Deoxynucleotide mix, 10 mM of each dNTP	1 µl	200 µM of each dNTP
Forward Primer	1 µl	300 nM
Reverse Primer	1 µl	300 nM
Template DNA	1 µl	100 pg/ µl
Nuclease free water	21 µl	
Final Volume	**25 µl**	

Table 25: Final concentration of Mix 1

Mix 2 (for one reaction):

Componet	Volume	Final concentration
Expand High Fidelity buffer 10x with 15 mM	5 µl	1x (1.5 mM MgCl2)
MgCl2	0.75 µl	2.6 U/ reaction
Expand High Fidelity enzyme mix		
Nuclease free water	21 µl	
Final Volume	**25 µl**	

Table 26: Final concentration of Mix 2

After adding all components of Mix 1 and Mix 2 combined both mixes in a 0.2 ml PCR tube on ice. It was important to gently vortex the mixture to produce a homogeneous reaction and then centrifuged briefly to collect sample at the bottom of the tube. The last step was to place samples in the thermal block cycler, and start cycling used the amplification program in table 27.

Steps	Temperature	Time
Initial Denaturation	95 °C	45 seconds
Denaturation	95 °C	45 seconds
Annealing	57 °C	45 seconds
Elongation	72 °C	3 minutes
Final Elongation	68 °C	10 minutes
Cooling	4 °C	Unlimited time

Table 27: Final Amplification Program

3.3 Analyzing of PCR Products

3.3.1 Agarose Gel Electrophoresis

Electrophoresis is a widely used technique in molecular biology, to separate DNA or RNA molecules by size, as they move through a gel matrix in an electric field (Robinson et al., 2003b). At the agarose gel electrophoresis is agarose the matrix. The DNA fragments were visualized with ethidium bromide. Ethidium bromide is a fluorescent nucleic acid dye, which fluorescence under ultraviolet light. The amplicons from the PCR with the "Expand high fidelity PCR System" were analyzed on an agarose gel as a quality control.

In a first step, 100 ml of 10x TAE buffer was diluted with H_2O, to a 1x TAE buffer. For the gel a 1% agarose in 1xTAE were used.
The next step was to weigh in the 0.5 g of agarose and added 50 ml of 1xTAE and heat the liquid in the microwave until the agarose was fully dissolved. After 10 minutes cool down step, to the gel mixture was added 2.5 µl from the ethidium bromide stock (10mg/ ml). The mixture was poured into the appropriate gel chamber in the vertical position and the comb was put into the gel chamber. After a 30 minute polymerization phase, the comb of the gel was removed and the transfer chamber was filled with 1x TAE buffer in such a way that the gel slots were filled with buffer. The amplicons were diluted with a 10x loading dye from Fermentas, 5 µl from the 10x loading dye and 50 µl from each amplicon. To control the fragment size, a standard the "GeneRuler DNA ladder mix", with a concentration of 0.1 µg/ µl was used. The prepared amplicons were loaded on the gel. In the first slot was always 10 µl of the "GeneRuler DNA ladder mix" from Fermentas. The transfer was realized for 45 minute at 70 volt (V). Finally, the DNA fragments on the gel were evaluated and photographed with the Molecular Imager GelDoc XR from BioRad and the right band was sliced out of the gel with a scalpel and put into a 1.5 ml tube for the Cleanup of the PCR product.

3.4 Cleanup of the PCR product

After amplification, the resulting PCR products were purified with the "Wizard®SV Gel and PCR Clean-up System" of Promega. During this procedure, PCR products were isolated from primers, salts, unincorporated nucleotides and Taq DNA Polymerase.
The principle of the Wizard® SV Gel and PCR Clean-Up System was that the DNA can be purified used microcentrifugation to force the solution through the SV Minicolumn and washed the DNA (www.Promega.com).
Following the electrophoresis, the DNA band was sliced from the gel and placed the gel slice in a 1.5 ml microcentrifuge tube. Primarily added 10 µl of the "Membrane Binding Solution" per 10 mg of gel slice and incubated at 65 °C until the gel slice was completely dissolved, but not longer than 10 minutes. For the DNA binding it was required to insert the "SV Minicolumn" into a Collection tube. The next step was to transfer the dissolved gel mixture to the Minicolumn assembly. After incubation for 1 minute the Minicolumn assembly was centrifuged at 16.000 x g for 1 minute. The flow through was discard and the Minicolumn was reinsert into the collection tube. Next, 700 µl of " Membrane wash solution" with ethanol was added to the Minicolumn and centrifuged at 16.000 x g for 1 minute, the flow through was discard and the Minicolumn was reinsert into a collection tube. This washing step were repeated with 500 µl of "Membrane wash solution" with ethanol and centrifuged at 16.000 x g for 1 minute, the flow through was discard and the Minicolumn was reinsert into a collection tube and centrifuged again at 16.000 x g for 1 minute to allowed evaporation of any residual ethanol. The Minicolumn was carefully transferred to a clean 1.5 ml microcentrifuge tube and added 50 µl of nuclease-free water to the Minicolumn for the elution. After incubation at room temperature of 1 minute, the column was centrifuged at 16.000 x g for 1 minute. The Minicolumn was discarded and the DNA was stored at -20 °C.

3.5 Cloning

3.5.1 Overview

The cloning was done with the Flexi®System, Entry/ Transfer Kit of Promega. The Flexi® Vector Systems used a flexible, directional cloning method to create plasmids to express protein-coding regions with or without peptide fusion tags. The features necessary for expression and the

protein-coding region can be shuttled between vectors using two rare-cutting restriction endonucleases, SgfI and PmeI. The Flexi® Vectors contain a lethal gene, barnase, for positive selection of the protein-coding sequence and an antibiotic resistance marker for selection of colonies containing the Flexi® Vector.

All Flexi® Vector can operate as an acceptor of a protein-coding region flanked by SgfI and PmeI sites. The SgfI site is upstream of the start codon of the proteincodingregion, and depending upon the Flexi® Vector used for cloning, this allows the expression of an amino- (N-) terminal-tagged protein by readthrough of the SgfI site. The PmeI site bears the stop codon for the protein-coding region and appends a single valine residue to the carboxy (C)-terminus of the protein. Protein-coding regions can be cloned into Flexi® Vectors containing SgfI and PmeI sites (Technical Manual, Flexi® Vector Systems, 2010).

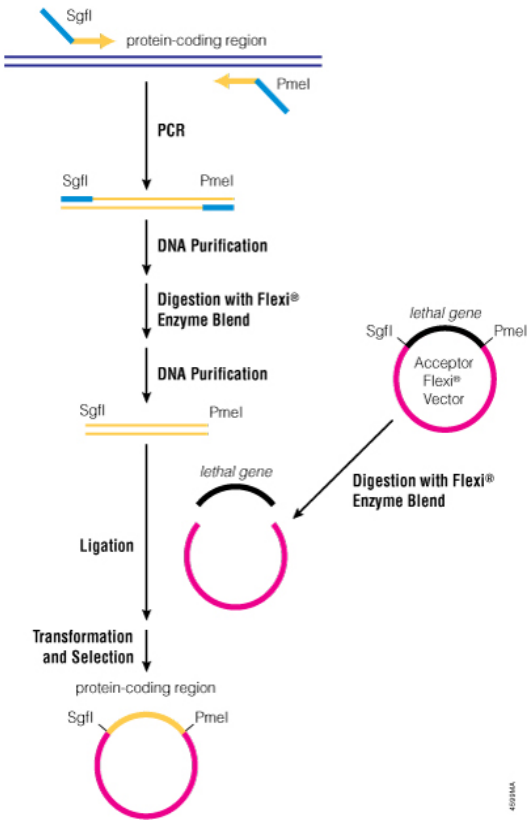


Figure 6: Overview of cloning with the Flexi®System, Entry/ Transfer Kit (Promega). (http://www.promega.com/products/cloning-and-dna-markers/cloning-tools-and-competent-cells/flexi-cloning-system/)

3.5.2 Digestion

The digestion reactions for the PCR product and the Flexi® Vector pFN22A (HALO-Tag®7) CMV d1 were performed concurrently. For digestion of the samples from the PCR and also for the vector pFN22A (HALO-Tag®7) CMV d1, the restriction enzymes SgfI & PmeI were used. The first step was to thawed the 5X Flexi® Digest Buffer, the Flexi® *Vector* pFN22A *(HALO-Tag ®7) CMV d1* and nuclease-free water, and stored it on ice. It was important that the 5X Flexi® Digest Buffer and the Flexi® Vector (HALO-Tag®7) CMV d1 was mixed well before they used. The final volume of the digestion reaction was 20 µl and was prepared in a new tube. The final concentration of the digestion mix for the PCR product and the Vector pFN22A you can see in table 28 and 29. The mixture was vortexed, centrifuged for 30 seconds at 14 000 rpm and incubated in a water-bath for 30 minutes at 37°C.

Componet	Volume
5X Flexi® Digest Buffer	4 µl
Purified PCR product	12 µl (204.4 ng)
Flexi® Enzyme Blend (SgfI & PmeI)	4µl
Final Volume	**20 µl**

Table 28: Final concentration of the digestion mix for the PCR product

Componet	Volume
5X Flexi® Digest Buffer	2 µl
Flexi® Vector pFN22A (HALO-Tag®7) CMV d1	1 µl (100 ng)
Flexi® Enzyme Blend (SgfI & PmeI)	1 µl
Nuclease free water	6 µl
Final Volume	**10 µl**

Table 29: Final concentration of the digestion mix for the Vector pFN22A

After this incubation, the mixture of the Flexi® Vector pFN22A *(HALO-Tag®7) CMV d1* was heated at 65°C for 20 minutes to inactivate the restriction enzymes and stored on ice until the PCR product was purified.

In the meantime of the waiting period, 20µl of "Membrane Binding Solution" was pipetted to the reaction with the PCR product and started with the cleanup of the PCR products. For the DNA binding it was required to insert the SV Minicolumn into a Collection tube. The next step was to transfer the PCR products with Membrane Binding Solution mixture to the Minicolumn assembly. After incubation for 1 minute the Minicolumn assembly was centrifuged at 16.000 x g for 1 minute. The flow through was discarded and the Minicolumn was reinsert into a collection tube. Next, 700 µl of "Membrane wash solution" with ethanol was added to the Minicolumn and centrifuged at 16.000 x g for 1 minute, the flow through was discard and the Minicolumn was reinsert into the collection tube. This washing step was repeated with 500 µl of "Membrane wash solution" with ethanol and centrifuged at 16.000 x g for 1 minute, the flow through was discard and the Minicolumn was reinsert into a collection tube and centrifuged again at 16.000 x g for 1 minute to allowed evaporation of any residual ethanol. The Minicolumn was carefully transferred to a clean 1.5 ml microcentrifuge tube and added 30 µl of nuclease-free water to the Minicolumn. After incubation at room temperature of 1 minute, the column was centrifuged at 16.000 x g for 1 minute. The Minicolumn was discarded and the DNA in the 1.5 ml tube was stored at -20 °C.

3.5.3 Ligation

After the cleanup of the PCR Product the following reaction components (see table 30) assembled in a sterile 1.5 ml tube for the ligation.

Componet	Volume
2X Flexi® Ligase Buffer	15 µl
Purified PCR product	11.5 µl (80.5 ng)
Flexi® Vector pFN22A *(HALO-Tag®7) CMVd1*	2 µl (63.1 ng)
T4 DNA Ligase (HC) (20u/µl)	1.5 µl
Final Volume	**30 µl**

Table 30: Final concentration of the Ligation Mix

The incubation time for this reaction was 1 hour at room temperature. After this incubation, the reaction was used for transformation into high-efficiency *E. coli* competent cells.

3.5.4 Transformation

The transformation was performed with the Subcloning Efficiency™ DH5*α*™ Competent Cells. They were recommended for routine subcloning into plasmid vectors. The Subcloning Efficiency™ DH5*α*™ Competent Cells were tested for transformation efficiency used th pUC19 control DNA supplied with the kit from Invitrogen. For using competent *E. coli* cells, it was important that they handled gently as they were highly sensitive to changed in temperature or mechanical lysis caused by pipetting. Thawed competent cells on ice, and transformed cells immediately following thawed.

The DH5*α*™ cells were thawed on ice, for each transformation reaction 50 µl of cells were used. The unused cells were refreezing in a dry ice/ethanol bath for 5 minutes before returned to the -80°C freezer. For this reaction, 5 µl of the ligation DNA was added to the cells and mixed gently. For the pUC19 control 2.5 µl (250pg) of DNA was added to the cells and mixed gently. After 30 minutes incubation on ice, the cells got a heat shock for 20 seconds in a 42°C water bath without shaking. The next step after the heat shock was to place the tubes on ice for 2 minutes. Then added 950 µl of pre-warmed LB - medium to each tube.The tubes were capped tightly and shacked horizontally (shaker, 225 rpm) for one hour. From each transformation were spread 100 µl on pre-warmed 100 µg/ ml ampicillin plates. Also for the pUC19 control was spread 100 µl on an LB plate that contained 100 µg/ ml ampicillin. The plates were incubated overnight at 37°C.

3.5.5 Control of the insert

After isolation of purified colonies, the obtained cells were analyzed for the presence of the mutant insert by PCR. For this the GoTaq® Green Master Mix was used.The GoTaq® Green Master Mix was a ready to use solution contained *Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. It also contained two dyes (a blue and a yellow) that allowed monitoring of progress during electrophoresis. Reactions assembled with The GoTaq® Green Master Mix had sufficient density

for directed loading onto agarose gels. The Mix was recommended for any amplification reaction that will be visualized by agarose gel electrophoresis and ethidium bromide stained.
The colonies were picked with a toothpick and dissolved in 10 µl GoTaq® Green Master Mix with the right primers and the rest of the DNA on the toothpick was spread on a fresh LB plate that contained 100 µg/ ml ampicillin. The plates were incubated overnight at 37°C.The following reaction components assembled to the GoTaq® Green Master Mix in a sterile 1.5 ml tube.

Componet	Volume
GoTaq® Green Master Mix	5 µl
Reverse Primer (10µM)	1 µl
Forward Primer (10µM)	1 µl
Nuclease free water	3 µl
Final Volume	**10 µl**

Table 31: Reaction Mix for the GoTaq® Green PCR

After adding all components of the Mix (see table 31), it was important to gently vortex the mixture to produce a homogeneous reaction and then centrifuged briefly to collect sample at the bottom of the tube. The final step was to place samples in the thermal block cycler, and start cycling used the thermal profile in table 32.

Steps	Temperature	Time
Initial Denaturation	95 °C	2 minutes
Denaturation	95 °C	30 seconds
Annealing	58 °C	30 seconds
Elongation	72 °C	30 seconds
Final Elongation	72 °C	5 minutes
Cooling	4 °C	Unlimited time

Table 32: PCR Program for GoTaq® Green PCR

After the cycling the DNA template was stored at -20 °C.

3.5.6 Analyzing of the insert

The amplicons from the PCR with the "GoTaq® Green Master Mix" were analyzed on an agarose gel as a quality control. For the gel a 1% agarose in 1xTAE with 10 wells were used. The first step was to weigh in the 0.5 g of agarose and added 50 ml of 1xTAE and heat the liquid until it was dissolved completely. Then cooled it down for 10 min. and added 2.5 µl from the ethidium bromide stock (10mg/ ml).

After this the liquid gel was filled into the gel chamber in the vertical position and the comb was put into the gel chamber. After a 30 minute polymerization phase, the comb of the gel was removed and the transfer chamber was filled with 1x TAE buffer in such a way that the gel slots were filled with buffer. The amplicons must not diluted with a 10x loading Dye from Fermentas, the GoTaq® Green Master Mix contained two dyes (a blue and a yellow) that allowed monitoring of progress during electrophoresis. The yellow dye used a 488 nm excitation wavelength. For the ladder, "GeneRuler DNA ladder mix" with a concentration of 0.1 µg/ µl was used. The prepared amplicons were loaded on the gel. In the first slot was always 10 µl from the "GeneRuler DNA ladder mix" from Fermentas. The transfer was realized for 45 minute at 70 Volt. After the Transfer the gel was evaluated and photographed with the Molecular Imager GelDoc XR from BioRad.

3.5.7 Big dye sequencing

3.5.7.1 Overview

In 1977, Frederick Sanger developed the chain -termination or dideoxy sequencing method, which is adapted on using dideoxynucleotides (ddNTP's) in accessorily to the normal nucleotides (NTP's) in the DNA. The dideoxynucleotides are equal as nucleotides but they have a hydrogen group on the 3' carbon in place than a hydroxyl group (OH). This modification of the nucleotides is preventing the adsorption of the further nucleotides and a phosphodiester bond cannot develop between dideoxynucleotide and the next incoming nucleotide, and the DNA chain is terminated.

For this method the primer or one of the nucleotides should be radioactively or fluorescently labeled so that the final product can be detected on a gel or with software.
For the sequencing of the probes the Applied Biosystems 3130*xl* Genetic Analyzers was used. This capillary electrophoresis offers several performance advantages for DNA analysis, compared with slab-gel techniques. The fast heat dissipation of capillaries, in combination with a detection cell heater, afford enhanced thermal control, which results in more accordant runs and faster run times. The samples are at once injected into the 4- or 16-capillary array in less than 30 seconds and this capillary electrophoresis also afford the use of electrokinetic injection to load samples into the capillaries. This technology allows using less DNA per sample than with slab-gel technology. The most important advantage of capillary electrophoresis is the elimination of manual operations, resulting in improved run-to-run composition and dependability.
The analysis of the nucleic acid is homogeneous maximization of the amount of signal per sample. The DNA passes by the detection cell, and a laser beam simultaneously illuminates the capillaries from both sides of the array. To arrange this, the light from a single laser font is dissociation, using optical elements to form a dual pathway. The emitted light is capture, separated by wavelength, and focused onto a charge coupled device (CCD). After collecting the fluorescent light over the CCD, the data are conferring to the instrument computer where they are converted by chemometric algorithmic processing into 4- or 5-dye electropherograms (www.appliedbiosystems.com).

For the sequencing preparations were used four different primers. The end concentration of each primer should be 6.4 pmol; the stock of the primer had a concentration of 100 µM this means 6.4 µl of a 1: 1000 dilution of the stock. The following reaction components assembled in a sterile 1.5 ml tube.

Componet	Volume
DNA of each clone	2 µl (300 ng)
Primer	6.4 µl (6.4 pmol)
Nuclease free water	6.6 µl
Final Volume	**15 µl**

Table 33: Final concentration for the DNA sequencing

The mixture for the sequencing was vortexed and centrifuged for 20 sec. The tubes with the mixture were analyzed with the 3130XL Genetic Analyzer.

3.6 Plasmid preparation

3.6.1 Overview

The Miniprep was performed with the QIAprep Spin Miniprep Kit from Qiagen. The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. The protocol simply consists of three basic steps, the first one is the preparation and clearing of a bacterial lysate followed by absorption of DNA onto the QIAprep membrane and the last step is washing and elution of plasmid DNA. This system also works without an alcohol precipitation (QIAprep Miniprep Handbook, 2006).

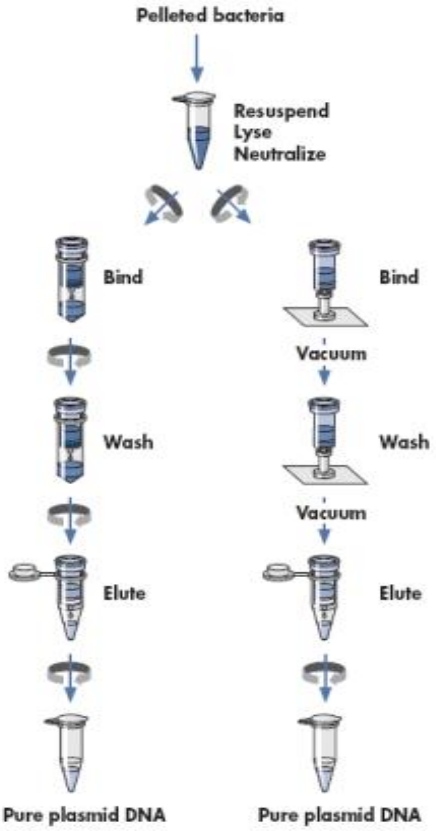


Figure 7: Different steps during Plasmid Preparation (QIAGEN® Plasmid Purification Handbook, 2006)

3.6.2 Miniprep

For the Miniprep five positive clones from the PCR were used. The first step was to made overnight cultures from each clone. For the overnight cultures were used 2 ml LB medium with 100 µg/ ml ampicillin and one colony of the clone and shacked them (shaker, 225 rpm) over night at 37 °C.

On the next day the first step was to pellet the 2 ml bacterial overnight culture by centrifugation at 3500 rpm for 3 minute at room temperature (15 – 25 °C). The supernatant was discarded and the pelleted bacterial cells were resuspended in 250 µl of Buffer P1 and transferred to a 1.5 ml microcentrifuge tube. For the lysis 250 µl of Buffer P2 was added and mixed thoroughly by inverting the tube 4-6 times until the solution became clear. Then added 350 µl of the Buffer N3 for the neutralization and mixed immediately and thoroughly by inverting the tube 4-6 times and centrifuged for 10 min at 13 000 rpm in a table top microcentrifuge. The supernatant from the centrifugation was pipetted into the QIAprep spin colum and centrifuged for 1 minute and the flow through was discarded. After this the QIAprep spin colum was washed with 500 µl of the Buffer PB and centrifuged for 1 minute. The flow through was discarded and the QIAprep spin colum was washed again with 750 µl of the Buffer PE for the washing. After the centrifugation of 1 minute the flow through was discarded and the QIAprep spin colum was transferred to the collection tube and centrifuged for 1 minute to removed residual wash buffer. The QIAprep spin colum was placed in a clean 1.5 ml tube. To eluted the DNA, 50 µl of nuclease free water was added to the center of the QIAprep spin colum and let stand for 1 minute at room temperature and centrifuged for 1 minute. The DNA was stored at -20 °C.

3.7 Concentration Determination of Plasmid DNA

3.7.1 Overview

The DNA concentration was measured with the NanoDrop ND-1000 spectrophotometer. The principle of the NanoDrop is, DNA absorbs ultraviolet (UV) light, with an absorption peak at 260 nm wavelength. The DNA exposed to UV light at a wave length of 260 nm, this light absorbance is measured by a photo-detector. The NanoDrop shows a 260/280 nm and a 260/230 nm ratio, which give information about the purity of the DNA.

3.7.2 Procedure

First the NanoDrop Software was started. Continuing was initiated by measuring 1 µl of H_2O. Following, a blank of 1 µl H_2O was measured. For the process of measuring the plasmid DNA, 1 µl of the sample was pipette onto the lower measurement pedestal. The sampling arm was closed the measurement was started. After few seconds the DNA appeared in ng/µl. The final step was to clean the Nanodrop, the DNA sample was wiped from the upper and lower pedestal and also cleaned with H_2O.

3.8 Single molecule tracking (SMT)

3.8.1 Overview

Single molecule tracking (SMT) allowed a real-time molecular look of physiological processes in living cells and also to gain insights into cellular events that are else covered. With the single molecule analysis reams of problems can be controlled. Prior conditions for intracellular single molecule detection are improvement of the light transmission in the microscope, fluorescence approval of the background and also a high-speed CCD camera system for the detection and digital imaging. Also an important point is to use fluorescence marker of the maximum photo stability, specific autofluorescent proteins, lipids or other probes are used. The first effective

monitoring of single proteins inside cells was realized with large model proteins as samples. In the last 10 years the awareness of the nuclear architecture has gain dramatically and now it is approved that the cell nucleus is an extreme organized organelle. Single molecule analysis acknowledges the very high restrictions on the mobility of large proteins inside the nucleus, which are often binding-unbinding events to immobile or slowly moving supramolecular structures and indicative of structural barriers. The molecules showing specific functions within the cell nucleus demonstrate a modulated mobility reflecting the specific interactions; provide more specific information on intranuclear dynamics and interactions. From the samples high-speed movies of the tasks of single molecules within living cells are recording, this admissible the detection and extraction of single particle with special image processing tools (Siebrasse et al. 2006) (Sako and Yanagida, 2003).

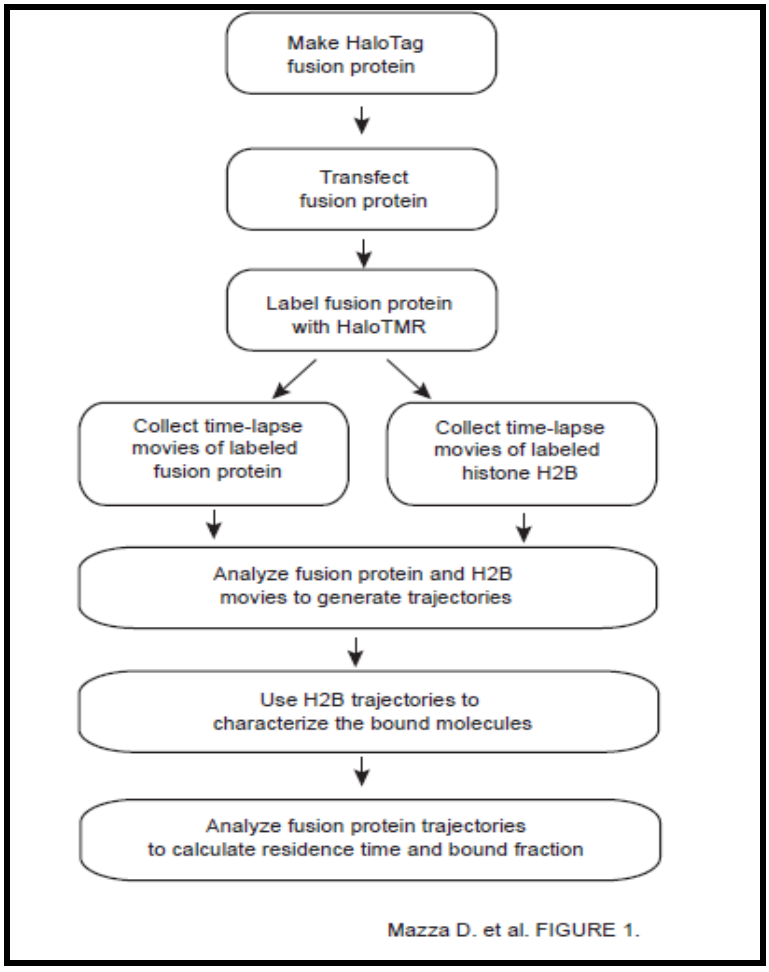


Figure 8: Flowchart of the protocol for single molecule tacking (Mazza et al., 2012).

3.8.2 Transfection

For the transfection of the lung carcinoma H1299 cells, Lipofectamine LTX Reagent was used. The Lipofectamine LTX Reagent is a perfect, animal-origin free cationic lipid formulation for the transfection of DNA into eukaryotic cells. This reagent had highest transfection expression performance with low cytotoxicity in many cell types (www.Invitrogen.com).
One day before the transfection, the H1299 cells were seeded on cover slides, for this 40 µl from the cell suspension and 1 ml of DMEM with 10% FBS and penicillin-streptomycin were used. For the transfection 100 ng of DNA was used and mixed with 1.2 µl of Lipofectamin reagent and incubated for 25 minutes at room temperature. After the incubation time, 900 µl of DMEM with 10% FBS and penicillin-streptomycin was pipetted to the transfection mix. The next step was to remove the old medium from the cells and replaced the transfection mix and incubated over night at 37 °C and 5% carbon dioxide.
At the next day, the cell permeable fluorescent ligands HaloTag-TMR (Tetramethylrhodamine) was added to the wells at concentration of 5nM and incubated for 30 minutes at 37 °C. Next the medium was removed and the cells were washed 3 times with 1 ml DPBS. After this 1 ml of Phenol-red-free DMEM with 10% FBS, L-Glutamine and penicillin-streptomycin was pipette to the cells and incubated for 15 minutes at 37 °C. After the incubation, the medium was removed and the cells were washed 3 times with 1 ml DPBS and after this 1 ml of DMEM with 10% FBS, L-Glutamine and penicillin-streptomycin were pipette to the cells and incubated for 15 minutes at 37 °C, to remove the unliganded fluorescent molecules. According the incubation the old medium was removed from the cells and replaced it to a fresh medium and the cells were then mounted on the microscope.

3.8.3 SMT Microscopy

SMT were performed on a p53 construct fused with a HaloTag receptor transiently transfected in the human H1299 p53-null cell line. The ligand tetramethylrhodamine (TMR) is membrane permeable and binds covalently to the Halo Tag fusion protein.
For the collection of the single molecule tracking data a custom-built widefield microscope with a highly inclined illumination schema was used. The incitation ray, a 25mW 561 nm laser, was

supplied to the back port of an Olympus IX-81 inverted microscope and focused on the rear aperture of the objective (150x, NA 1.445 oil immersion objective). An area stop was used to bind the excitation to a field of about 15 μm in diameter. The angle of the beam was selected to be about 60° from the optical axis at the oil-coverslip interface. The sample was fixed on a piezoelectric stage approval selection on the focal plane without changing the position of the objective. The focal level was elect to lie in a middle section of the cell nucleus, at deepness of 3 to 4 μm from the area of the coverslip.

The EM-CCD camera and the laser spring were synchronized by averages of a pulse generator in order to beware photobleaching when the camera shutter was closed. The probe was kept at 37 °C with an air-stream platform incubator and the acquisition rate of 25 Hz was used.

The collected movies were analyzed by custom-written Matlab routines to determine and track single molecules. The first step was to process the images with a band-pass filter, to constant the diffraction limited spots appropriating to single molecules and to disable pixel noise. For the identification of the single molecules a peak identification algorithm was used and a lower intensity threshold (set to 300) was used to disallow dim peak poignancy corresponding to out-of-focus molecules. The sub-pixel localization was the defined by fitting two-dimensional Gaussians to the raw images using the before identified peak coordinates as starting guesses. The trajectories were manually audited to minimize mistracking (Mazza et al. 2012).

4 Results

4.1 Polymerase Chain Reaction (PCR) with the "Expand high fidelity PCR System"

The PCR of each mutant was performed with the "Expand high fidelity PCR System". It was composed of a unique enzyme mix containing thermostable Taq DNA polymerase and Tgo DNA polymerase, a thermostable DNA polymerase with proofreading activity. This mixture was designed to generate PCR products of high yield, high fidelity and high specificity from all types of DNA. The PCR was performed with an input of 100 pg/ µl of DNA.
The aim of the PCR step was, to generate a delta 30 mutation in some of the mutants and also to get a larger amount of DNA for the further steps. The PCR products were analyzed by agarose gel electrophoresis in presence of a GeneRuler DNA ladder mix, to determine the size of the amplicons. For the further, the PCR products were used for the cloning reaction with the Flexi®System, Entry/ Transfer Kit from Promega.

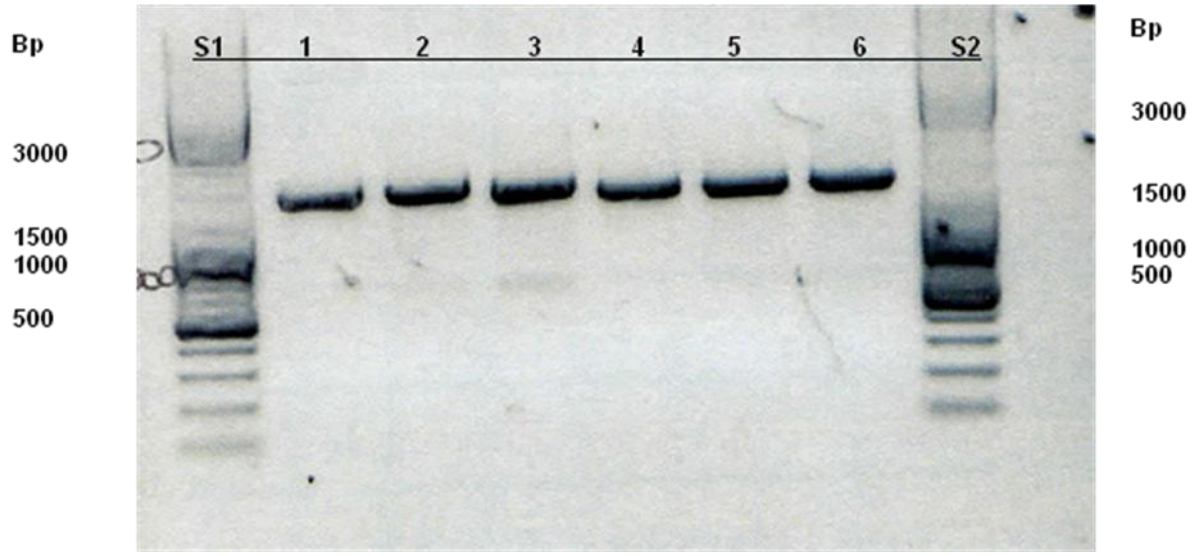


Figure 9: Gel with 1% agarose in TAE-buffer was done at 110V for 30 minutes. S1 and S2 stands for 10 µl GeneRuler DNA ladder mix (Fermentas), the line shows 5 µl from the 10x loading dye and 50 µl from each

amplicon. 1 shows the mutant R273H, 2 the R280K mutant, the number 3 the R248W mutant, 4 shows the R273H delta30 mutant, number 5 the R280K delta30 and 6 the PCR product of the R248W delta30 mutant.

In this experiment the DNA-amplification products are visible on the agarose gel. The quality of the DNA was good, because the amplification products showed DNA-fragments leading between 2000 and 2500 bp.

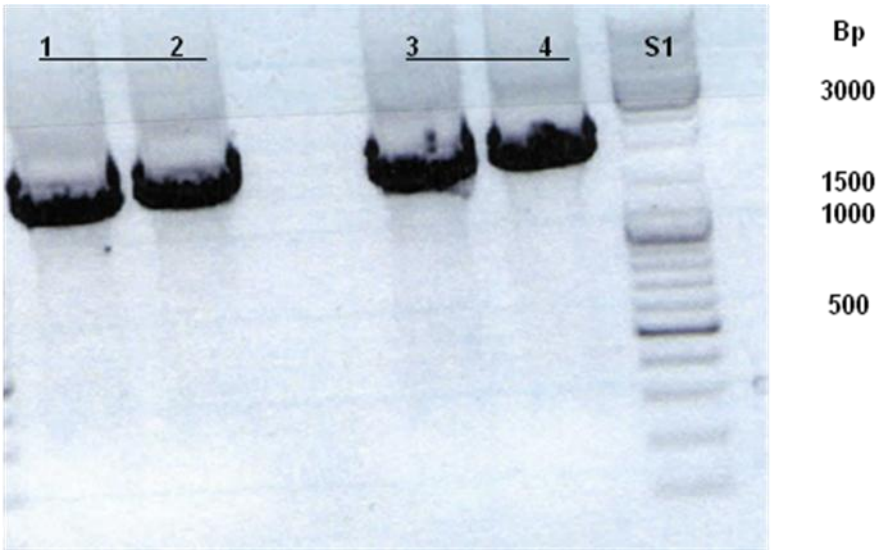


Figure 10: Gel with 1% agarose in TAE-buffer was done at 110V for 30 minutes. S1 stands for 10 µl GeneRuler DNA ladder mix (Fermentas), the line shows 5 µl from the 10x loading dye and 50 µl from each amplicon. 1 and 2 are showing the PCR product of mTAD mutant, the numbers 3 and 4 are showing the mSB mutant.

In this experiment the DNA-amplification products are visible on the agarose gel. The quality of the DNA was good, because the amplification products showed DNA-fragments leading between 2000 and 2500 bp.

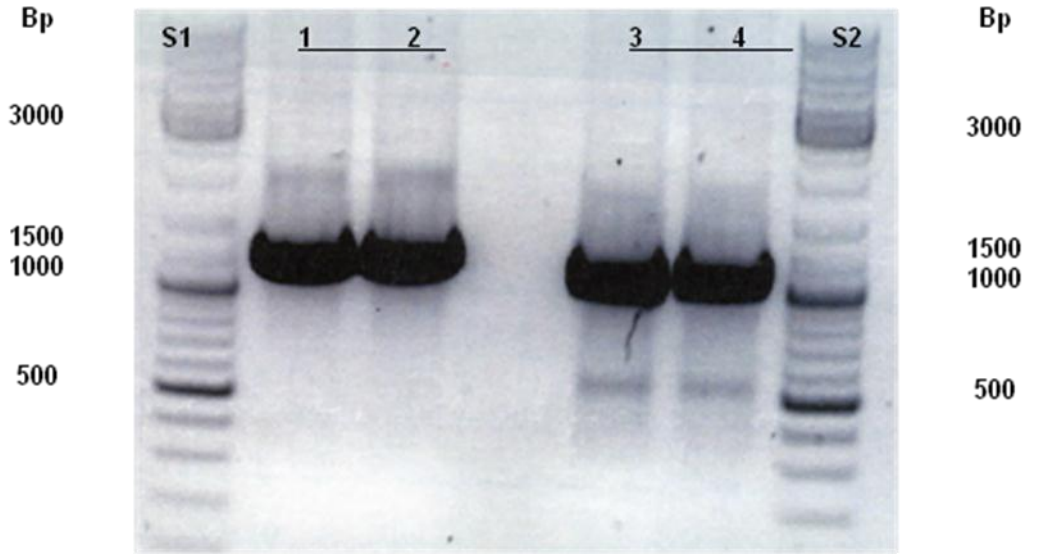


Figure 11: Gel with 1% agarose in TAE-buffer was done at 110V for 30 minutes. S1 and S2 stands for 10 µl GeneRuler DNA ladder mix (Fermentas), the line shows 5 µl from the 10x loading dye and 50 µl from each amplicon. 1 and 2 are showing the PCR product of the L22Q+W23S mutant, the numbers 3 and 4 are showing the L22Q+W23S delta30 mutant.

In this experiment the DNA-amplification products are visible on the agarose gel. The DNA bands of the L22Q+W23S delta30 mutant showing some unspecific binding, it could be the result of unspecific binding of primers.The quality of the DNA was good, because the amplification products showed DNA-fragments leading between 1500 and 2000 bp.

4.2 Cloning

4.2.1 Digestion

For the further procedures, it was necessary to digest the PCR amplicons. The PCR products were digested with two different restriction enzymes, SgfI and PmeI. Also the vector pFN-21A

was digested with the restriction enzymes SgfI and PmeI. After digestion, the products were analyzed by agarose gel electrophoresis.

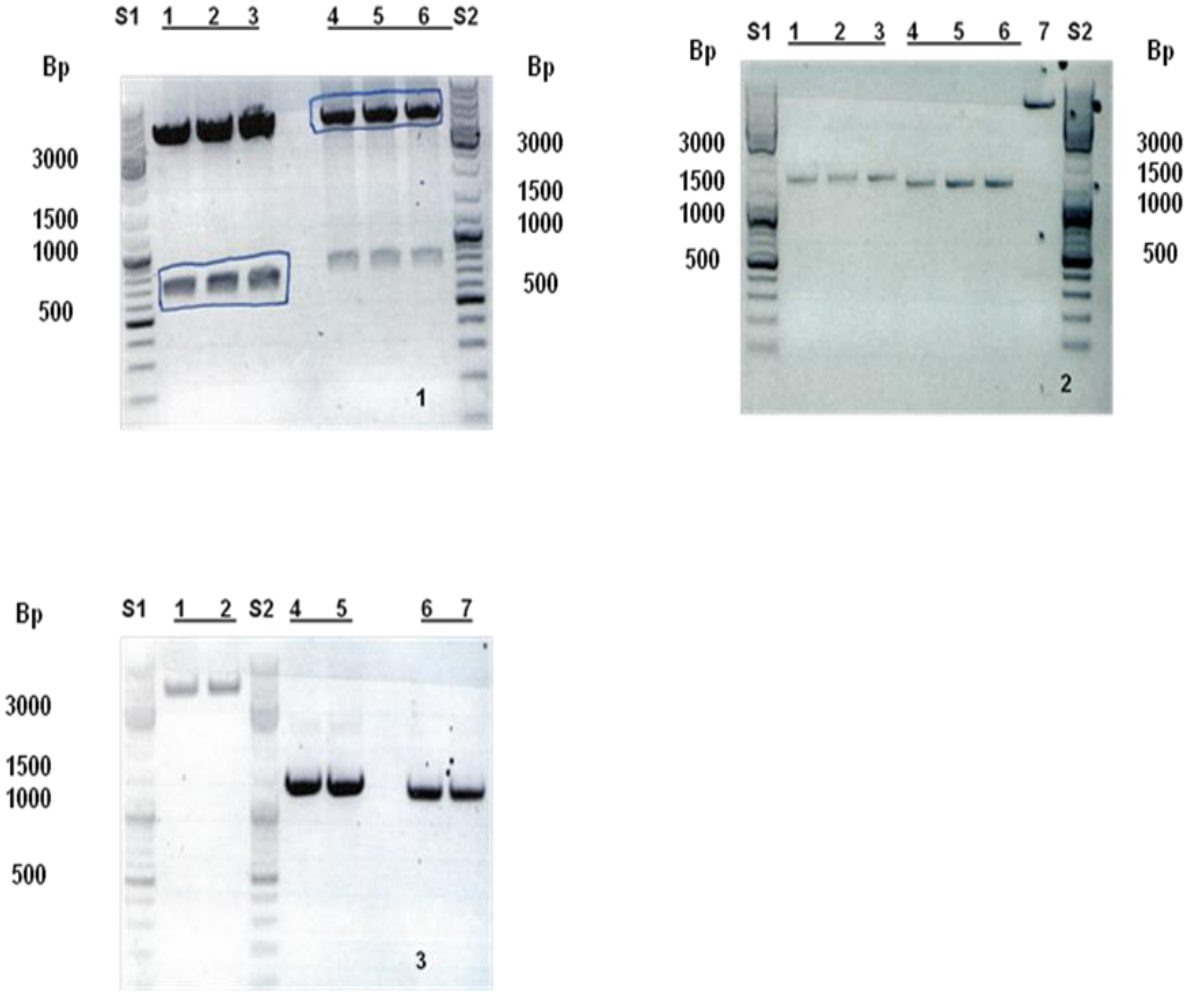


Figure 12: Gel with 1% agarose in TAE-buffer was done at 110V for 30 minutes. S1 and S2 stands in gel 1 – 3 for 10 µl GeneRuler DNA ladder mix (Fermentas), the line shows 5 µl from the 10x loading dye and 50 µl from each amplicon. In gel 1, the numbers 1 – 3 are showing the mSB_delta 30 mutant (700 bp) and the mTAD mutant is showing at 4 – 6 in gel1.

1 in gel 2 is showing the R273H mutant, number 2 shows the R280K mutant, number 3 the R248W mutant. The number 4 in gel 2 is the R273H_delta 30 mutant, 5 is the R280K_delta 30 mutant and number 6 is the R248W_delta 30 mutant.

In gel 3 the numbers 1 and 2 are showing the vector pFN22A *(HALO-Tag®7) CMVd1* as control. Number 4 and 5 are showing the L22Q+W23S mutant, 6 and 7 are showing the L22Q+W23S_delta 30 mutant.

The gel picture of gel 1with the mSB_delta 30 mutant and the mTAD mutant is showing 2 different DNA fragments for each of the mutant on the gel. It could be the result of not completed digestion of the DNA with the enzymes SgfI and PmeI, maybe the time with 1h was too short or the temperature was not at 37 °C. But the right fragment size was bride enough to us it for the ligation. The electrophoreses showed that the expected fragments, which had a fragment size of 700 bp for the mSB_delta 30 mutant and 4225 bp for the mTAD mutant, were amplified.
The picture of gel 2 with the mutants R273H, R280K and R248W showed the right fragment size with 2100 bp. Also the fragment size of the R273H_delta 30, R280K_delta 30 and R248W_delta 30 mutants were right.
The result for the gel 3, showed also the right fragment size with 2100 bp for the L22Q+W23S mutant and 2070 bp for the L22Q+W23S_ delta 30 mutant.

4.2.2 Transformation of all mutants after the ligation

The PCR amplicons were cloned into the pFN-21A vector. These pFN-21A constructs, including the insert, were transformed to Subcloning Efficiency™ DH5α™ compenent cells, and cultured on LB plates that contained 100 µg/ ml ampicillin. After an over-night incubation at 37°C (see figure 13), white colonies were generated on the plates. From this plate 6 – 18 colonies were used for the insert check.

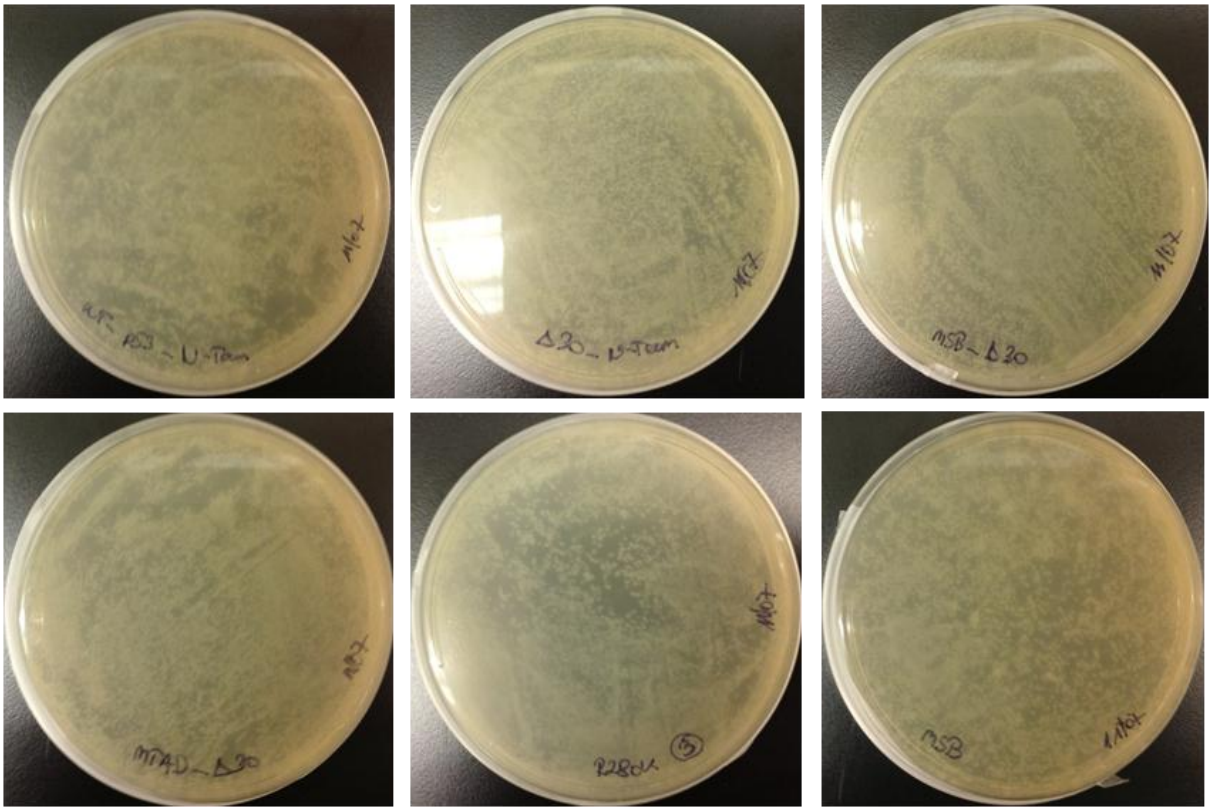


Figure 13: Transformation on LB plates with 100 µg/ ml ampicillin, after an over-night incubation at 37°C. 1 shows WT_p53, 2 the delta_30 mutant, 3 is the mSB_delta 30 mutant, the number 4 shows the mTAD_delta 30, 5 is R280K and in number 6 is the mSB mutant. The colonies showing good quality and were used for the insert check.

The result of the transformation was really good for all of the mutants. In the pictures of figures 13 and 14 there are a lot of colonies on the plates. The quality of the transformation with the "Subcloning Efficiency™ DH5α™ competent cells" was good.

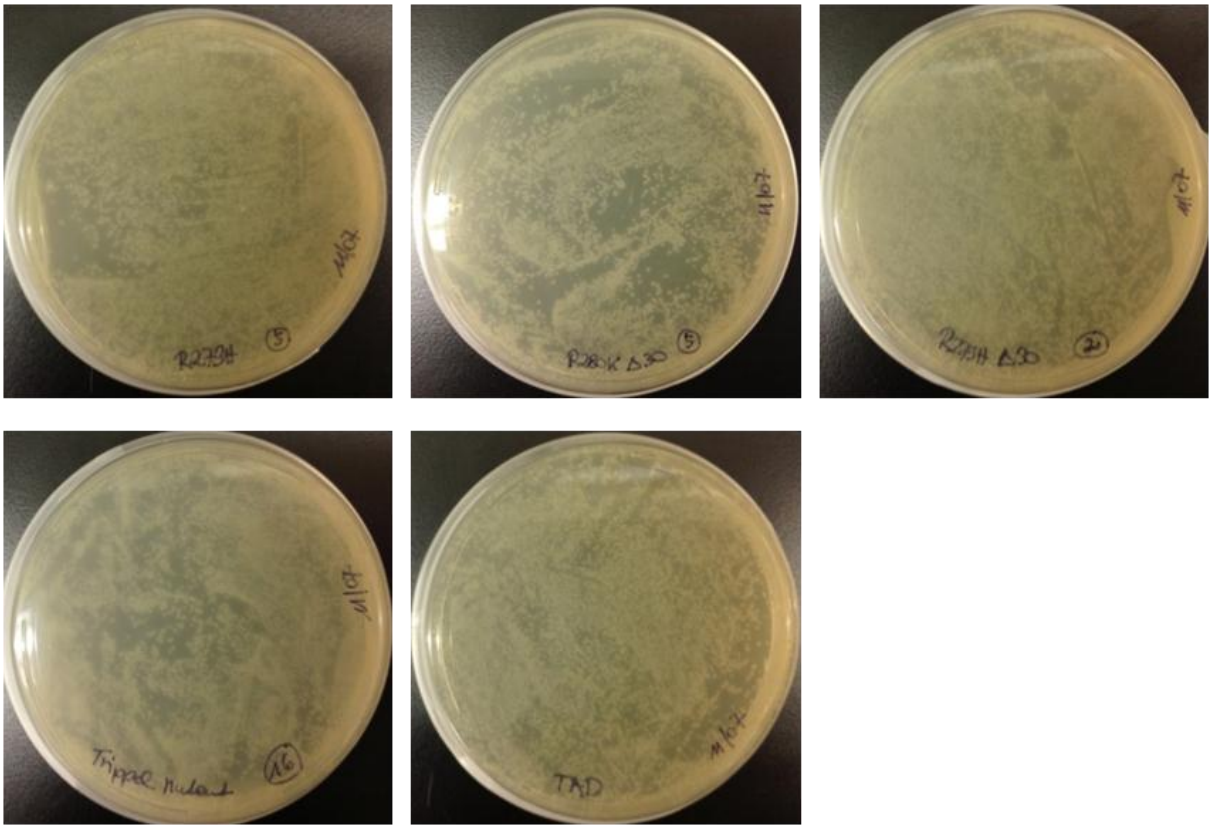


Figure 14: Transformation on LB plates with 100 µg/ ml ampicillin, after an over-night incubation at 37°C. 7 shows R273H, 8 the R280K_delta 30 mutant, 9 is the R273H_delta 30 mutant, the number 10 shows the Triple mutant and in number 11 is the mTAD mutant. The colonies showing good quality and were used for the insert check.

4.2.3 Control of the insert

For the control of the insert, a PCR with the "GoTaq® Green Master Mix" were realized and analyzed on an agarose gel as a quality control.

For the gel a 1% agarose gel in 1xTAE with 2.5 µl from the ethidium bromide stock (10mg/ ml) were used. To control the fragment size, a standard ladder "GeneRuler DNA ladder mix" (Fermentas), with a concentration of 0.1 µg/ µl was used.

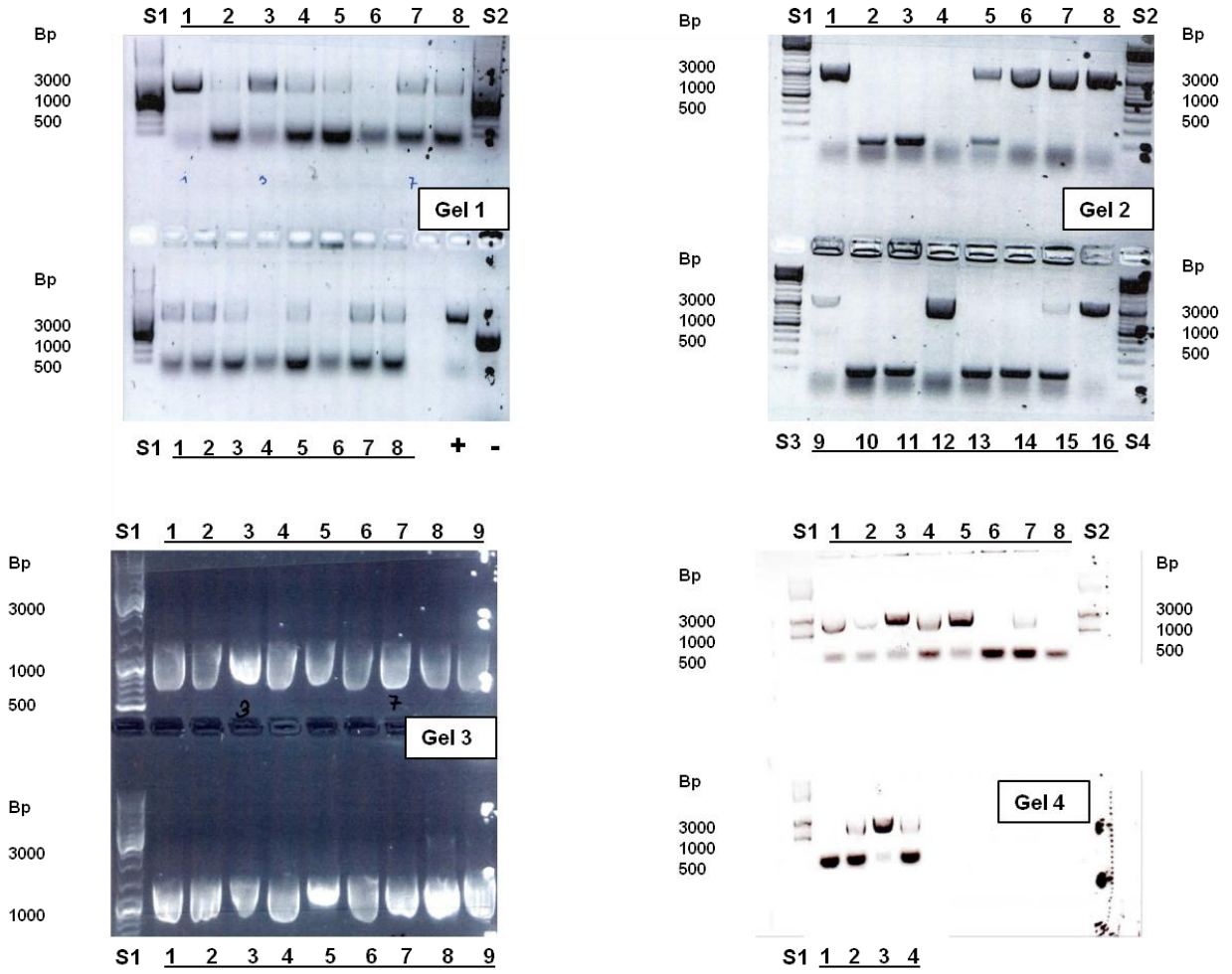


Figure15: Gel with 1% agarose in TAE-buffer was done at 110V for 30 minutes. S1, S2, S3 and S4 stands in gel 1, 2, 3 and 4 for 10 µl GeneRuler DNA ladder mix (Fermentas). In gel 1 the upper line and lower line shows the mSB mutant after the PCR for the insert check. In gel 2 the upper and lower line shows the mTAD mutant after the PCR for the insert check. The gel 3 the upper and lower line shows the Triple mutant after the PCR for the insert check. In gel 4 the upper line from 1 – 6 shows the mSB_delta 30 mutant and 7 – 8 the mTAD_delta 30 mutant after the PCR for the insert check and in the lower line from 1 – 4 are also the mTAD_delta 30 mutant after the PCR for the insert check.

The agarose gel electrophoresis showed the expected fragments. The positive looking PCR products were used for the sequencing reaction.

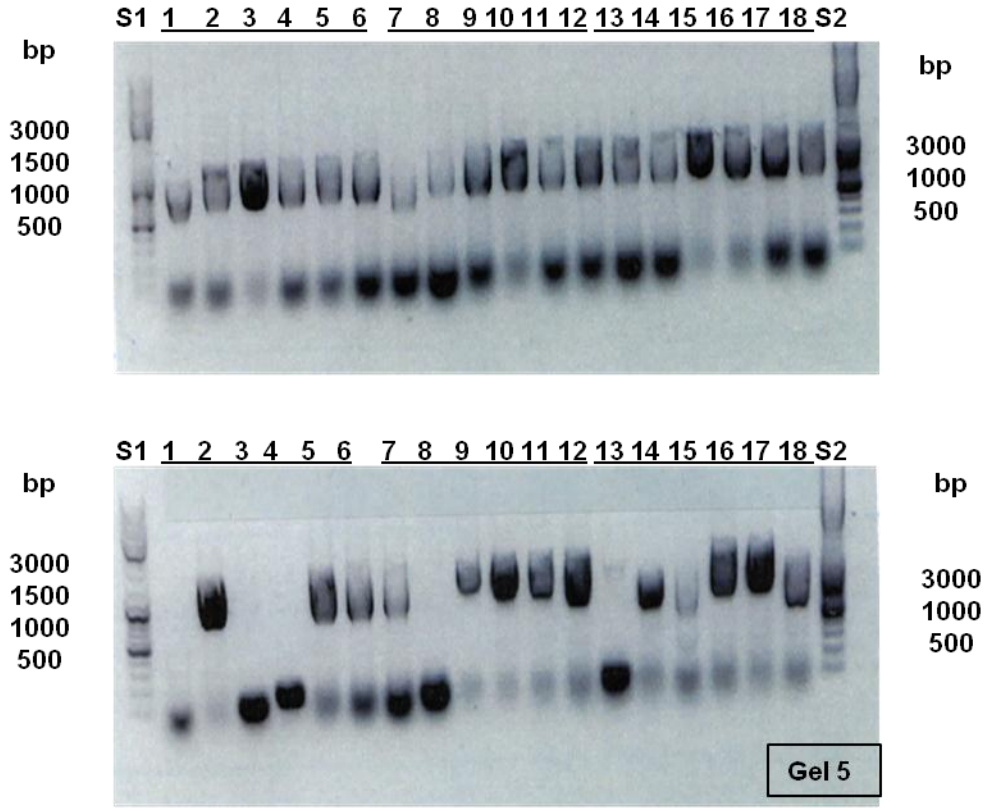


Figure 16: Gel with 1% agarose in TAE-buffer was done at 110V for 30 minutes. S1 and S2 stands in gel 5 for 10 µl GeneRuler DNA ladder mix (Fermentas). The upper line shows from 1 – 6 the R273H_delta 30 mutant, from 7 – 12 the R280K_delta 30 mutant and from 13 – 18 the R248W_delta 30 mutant after the PCR for the insert check. The lower line shows from 1 – 6 the R273H mutant, from 7 – 12 the R280K mutant and the numbers 13 – 18 showing the R248W mutant after PCR for insert check.

The agarose gel electrophoresis showed the expected fragments. The positive looking PCR products were used for the sequencing reaction.

4.3 Sequencing from the Inserts of the Constructs

For the sequencing of the mutations, plasmid DNA was extracted from a 2 ml culture from each candidate. The inserts were sequenced with the primer see in table 34. For the sequencing 300 ng of DNA was used. The end concentration of each primer was 6.4 pmol. For the analyzing, the 3130XL Genetic Analyzer was used.

Name	Primer Sequence
DM0031	5'-GACCGCGATCGCCCAGTCAGATCCTA-3'
A005	5'-TGCGGTTTAAACCTGGCTCCTTCCCA-3'
N074_pFN	5'-ATTTCCGGCGAGCCAACC-3'
Dm063	5'-CCATCCTCACCATCATCACA-3'
DM066	5'-CTTCCTTTCGGGCTTTGTTAG-3'
DM036	5'-ACACGCAAATTTCCTTCCAC-3'

Table 34: Primer for Big dye sequencing

Mutant	Sequencing Result
WT_p53_N-terminus	Sequence confirmed with the 3130XL Genetic Analyzer
Delta 30_N- terminus	Sequence confirmed with the 3130XL Genetic Analyzer
mSB_N- terminus	Sequence confirmed with the 3130XL Genetic Analyzer
mSB_delta 30_N-terminus	Sequence confirmed with the 3130XL Genetic Analyzer
mTAD_N- terminus	Sequence confirmed with the 3130XL Genetic Analyzer
mTAD_delta 30_N- terminus	Sequence confirmed with the 3130XL Genetic Analyzer
R280K_N- terminus	Sequence confirmed with the 3130XL Genetic Analyzer
R280K_delta 30_N- terminus	Sequence confirmed with the 3130XL Genetic Analyzer
R273H_N- terminus	Sequence confirmed with the 3130XL Genetic Analyzer
R273H_delta 30_N- terminus	Sequence confirmed with the 3130XL Genetic Analyzer
Triple_N- terminus	Sequence confirmed with the 3130XL Genetic Analyzer

Table 35: Results of the sequencing with the 3130XL Genetic Analyzer

The table 35 shows the results of the Big dye sequencing with the 3130XL Genetic Analyzer. The sequencing confirmed that the sequence of each mutant was right. Therefore we can say that the cloning was successful and the mutants were used for single molecule experiments.

4.3.1 Concentration Determination of Plasmid

The DNA concentration was measured with the NanoDrop ND-1000 spectrophotometer after the Miniprep. The NanoDrop shows a 260/280 nm and a 260/230 nm ratio, which give information about the purity of the DNA.

Mutants	Concentration (ng/ µl)	260/230	260/280
WT_p53_N-terminus	153.93	2.18	1.85
Delta 30_N- terminus	123.55	1.96	1.86
mSB_N- terminus	102.28	2.07	1.93
mSB_delta 30_N-terminus	100.46	2.01	1.87
mTAD_N- terminus	129.22	2.52	1.87
mTAD_delta 30_N- terminus	125.64	2.70	1.86
R280K_N- terminus	102.55	2.84	1.82
R280K_delta 30_N- terminus	107.83	2.67	1.81
R273H_N- terminus	119.28	2.79	1.82
R273H_delta 30_N- terminus	107.00	2.76	1.80
Triple_N- terminus	134.13	2.27	1.87

Table 36: Concentration of the mutants

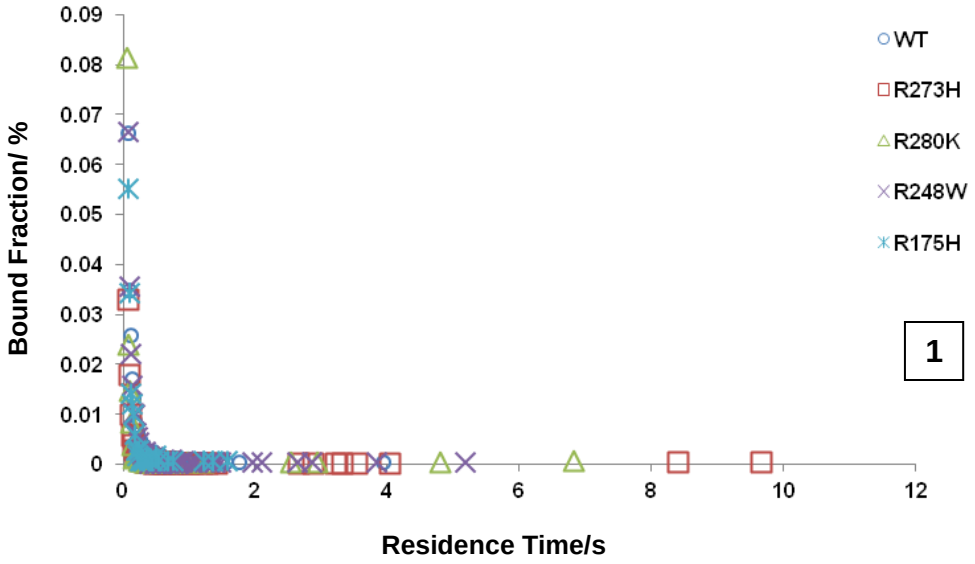
The DNA concentration was a little too low but it was enough for the transfection and the single molecule experiments.

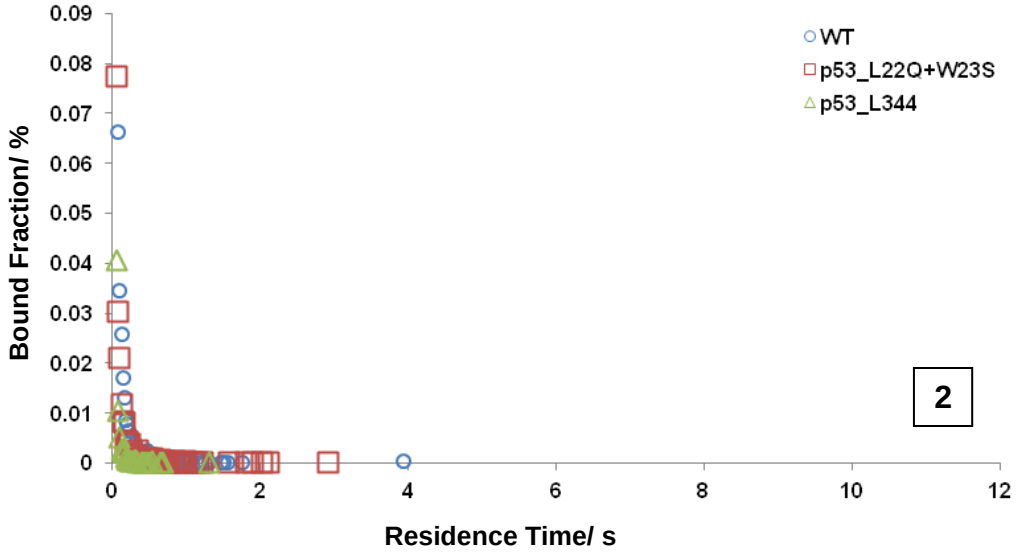
4.4 Single molecule tracking

With single molecule tacking it is possible to quantify binding of nuclear proteins to DNA. T he first step is to create a HaloTag fusion to the protein of interest. This allows subsequent in vivo labeling of the protein with a derivative of tetramethylrhodamine, which is more photostable fluorophore than GFP. Next we detect the single molecules using a microscope that minimizes out-of-focus light by means of a slanted excitation light beam known as HILO illumination. The next step was to collect images of single molecules in the cell nucleus as a function of time to produce movies of single molecule motion. Then track the motion of every molecule in the movie to generate trajectories and then analyze the trajectory to identify the segments that correspond to chromatin binding events. With the analyzing of the trajectories it allows to determine what fraction of molecules are bound, and how long this molecules are bound. This produces an estimate of the total bound fraction and the distribution of residence times on chromatin for the protein of interest.

4.4.1 Change of the C-terminus to N-Terminus

Previous experiments with ChIP-Seq showed that the N-terminus is much better than the C-terminus for p53. The first step was to change the HALO-tag from the C-terminus to the N-terminus at the proteins of interest. The protocol of the change is showed in chapter 3.5.
The H1299 cells were transfected with the different p53 mutants and after an incubation of 15h; movies of the cells with the molecules were collected.
After the analyzing of the trajectories, I got an estimate of the total bound fraction and the distribution of residence times on chromatin for the protein of interest.


Bound Fraction/ %
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
2
4
6
8
10
12
Residence Time/s
WT
R273H
R280K
R248W
R175H
1


Bound Fraction/ %
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
2
4
6
8
10
12
Residence Time/ s
WT
p53_L22Q+W23S
p53_L344
2

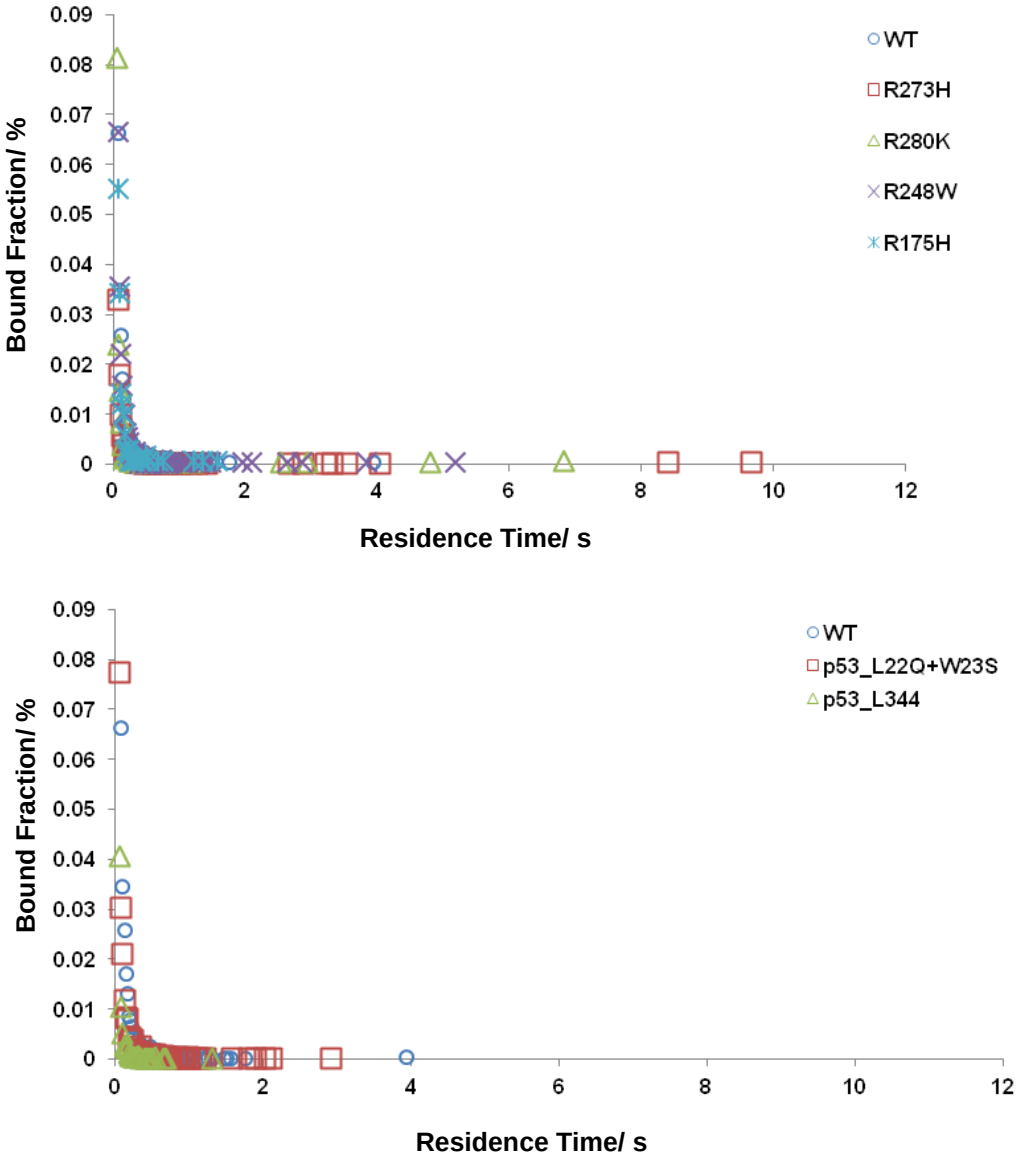


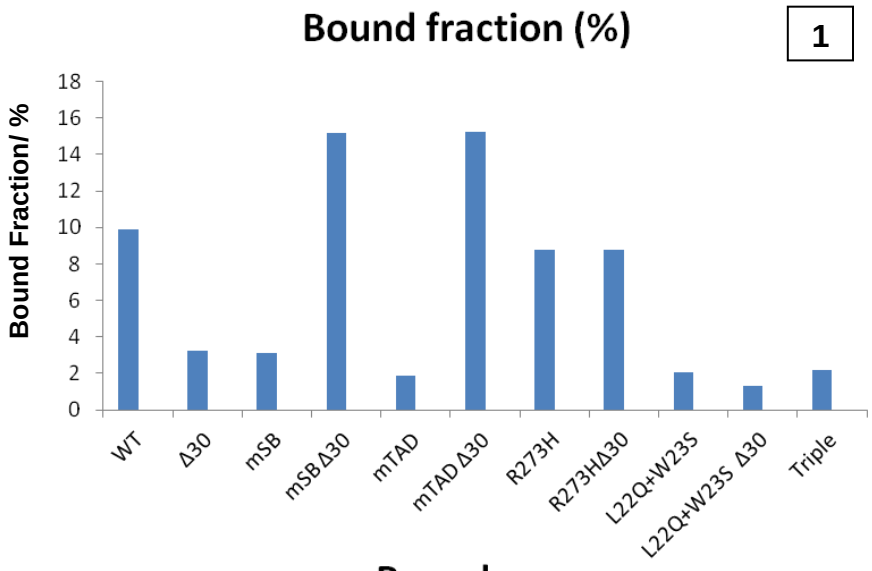
Figure 17: The SMT was perfomed with 25 Hz and 500 fames. The histogram 1 shows four different mutants and WT as control. R271H, R280K, R248W and R175H are mutations in the specific binding domain of p53. The histogram 2 shows two different Mutants and WT as control. The mutants L22Q+W23S and L344 are mutations in the transactivation domain.

This SMT did not show so much difference in the binding between the p53 WT and the specific binding mutants and also the transactivation mutants. Further it is necessary to generate some mutants in the tail domain delta 30 which has been hypothesized to mediate non-specific DNA

binding.

4.4.2 Single molecule tracking after cloning of delta 30 mutants

The delta 30 mutation was generating with a PCR step. After the cloning the H1299 cells were transfected with the new mutations and analyzed with Single molecule tracking. In this experiment the difference between mutants with a delta 30 mutation and without this mutation were analyzed with single molecule tracking.



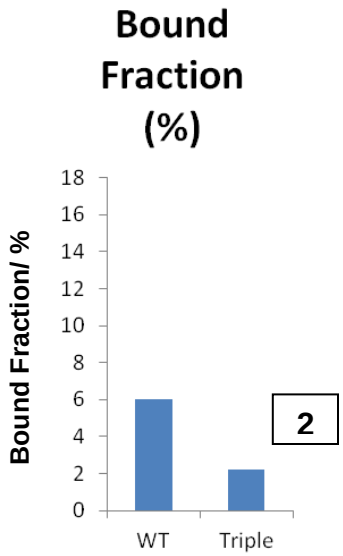


Figure 18: The SMT was performed with 25 Hz and 500 fames. Histogram 1 shows the bound fraction in %, for the different combinations of the p53 mutants. The histogram 2 shows the comparison of p53 WT and the Triple mutant. You can see the big drop of the bound fraction of the Triple mutant compared to the p53 WT.

After the analyzing of the trajectories, I got an estimate of the total bound fraction and the distribution of residence times on chromatin for the protein of interest.
These results of the SMT from the p53 mutants in combination with delta 30 showed some difficult data. The mSB delta 30 and the mTAD delta 30 have a higher bound fraction than WT and also the R273H and the R273H delta 30 mutant show similar bound fractions than the WT.

The used H1299 cells for this experiment were 2 months in culture and it seems this period of culturing is too long for single molecule tracking. The experiment must be repeated with fresh cultured cells.

4.4.3 Single molecule tracking after cloning of delta 30 mutants (second round)

The whole experiment was repeated investigating the influence of the delta 30 mutation.
After the cloning the H1299 cells were transfected with the new mutations and analyzed with Single molecule tracking.
After collecting the movies of single molecule motion, the motion of every molecule in the movie were track and then analyze the trajectory to identify the segments that correspond to chromatin binding events.

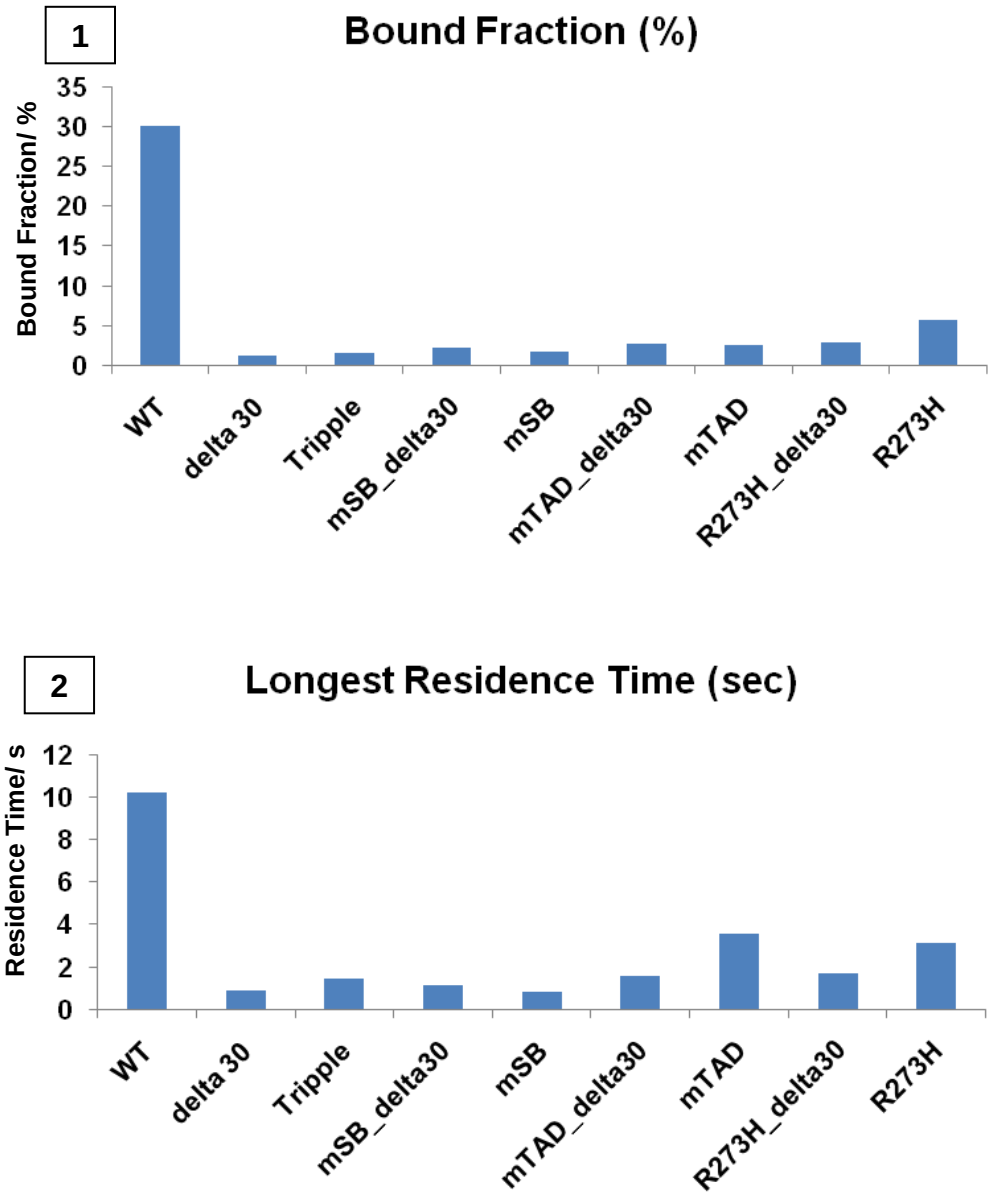


Figure 19: The SMT was performed with 25 Hz and 500 fames. Histogram 1 shows the bound fraction in %, for the different combinations of the p53 mutants and the combination of the delta 30 mutation. The bound fraction of the WT is 30% and much higher than of the other mutants.

The histogram 2 shows the longest residence time in sec. of the different p53 mutants and the combinations with delta 30. The residence time of the WT p53 is the longest compared to the other mutants.

Mutants	Longest Residence Time (sec)	Bound Fraction (%)
WT p53	**10.2**	**30**
delta 30	**0.88**	**1.2**
Triple	**1.44**	**1.6**
mSB	**0.8**	**1.77**
mSB_delta 30	**1.12**	**2.2**
mTAD	**3.56**	**2.5**
mTAD_delta 30	**1.6**	**2.8**
R273H	**3.12**	**5.8**
R273H_delta 30	**1.72**	**2.9**

Table 37: Values of the bound fraction in % and the longest residence time in sec. of the p53 mutants

The repeated experiment showed another result compared to the first run, because this time fresh cultures H1299 cells were used for the transfection and the subsequent single molecule tracking. After the analyzing of each trajectory the result showed a big difference from the mutants in their bound fraction and residence time compared to the WT p53 (see table 37).
This result confirmed my hypothesis that the bound fractions of the mutants are less compared to the WT p53 based on the mutations in the specific binding domain and also transactivation domain.

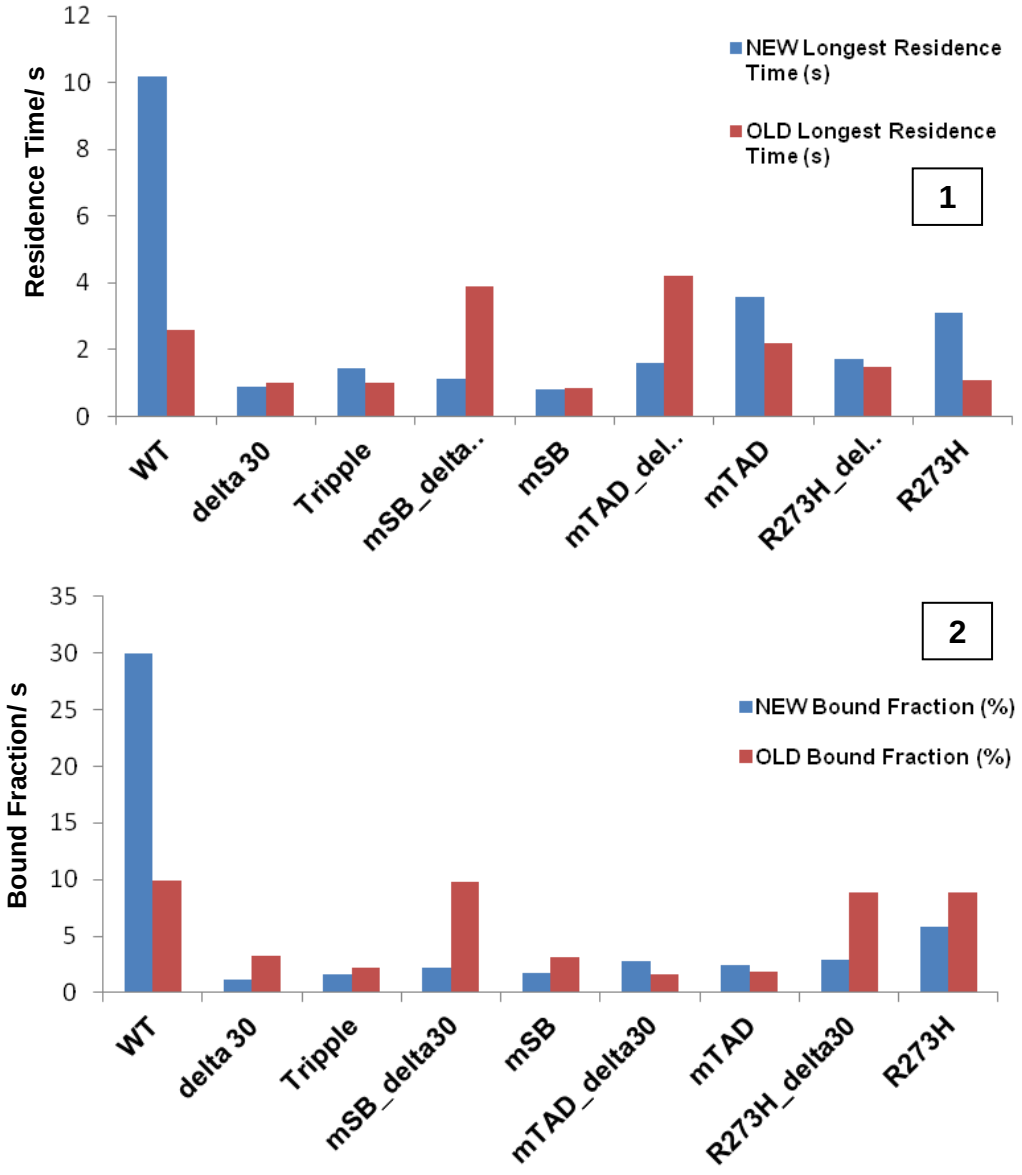


Figure 20: The histogram 1 shows the comparison of the longest residence time from the first and the second round of the Single molecule tracking after cloning of delta 30 mutants. The red bars show the new data from the second round and the blue bars the old data from the first experiment. The histogram 2 shows the comparison of the bound fraction from the first and the second round of the Single molecule tracking after cloning of delta 30 mutants. The red bars show the new data from the second round and the blue bars the old data from the first experiment.

The repeated experiment showed different data in bound fraction and residence time, because this time fresh H1299 cells were used for the single molecule tracking. It looks that way that the age of the cells affects the result of the single molecule tracking.
For the results from the second round of this experiment it is necessary to confirm this data with an experiment on different days, to see how the mutants are behavior on different days.

4.4.4 Time series over some days

The following single molecule tracking were performed on three different days to confirm the results from the cloning of delta 30 mutants. For this experiment WT p53 and mTAD_delta 30 were used for the SMT. The H1299 cells were fresh cultured for this SMT.

Mutants	Bound Fraction (%) 1	Bound Fraction (%) 2	Bound Fraction (%) 3
WT p53	**9.2**	**8.9**	**8.8**
mTAD_delta30	**2.2**	**2.3**	**2.5**

Table 38: This table shows the bound fraction in % from three different SMT experiments

Mutants	Longest Residence Time (Sec) 1	Longest Residence Time (Sec) 2	Longest Residence Time (Sec) 3
WT p53	**2.8**	**2.0**	**2.56**
mTAD_delta30	**1.4**	**1.6**	**1.68**

Table 39: This table shows the longest residence time in sec. from three different SMT experiments

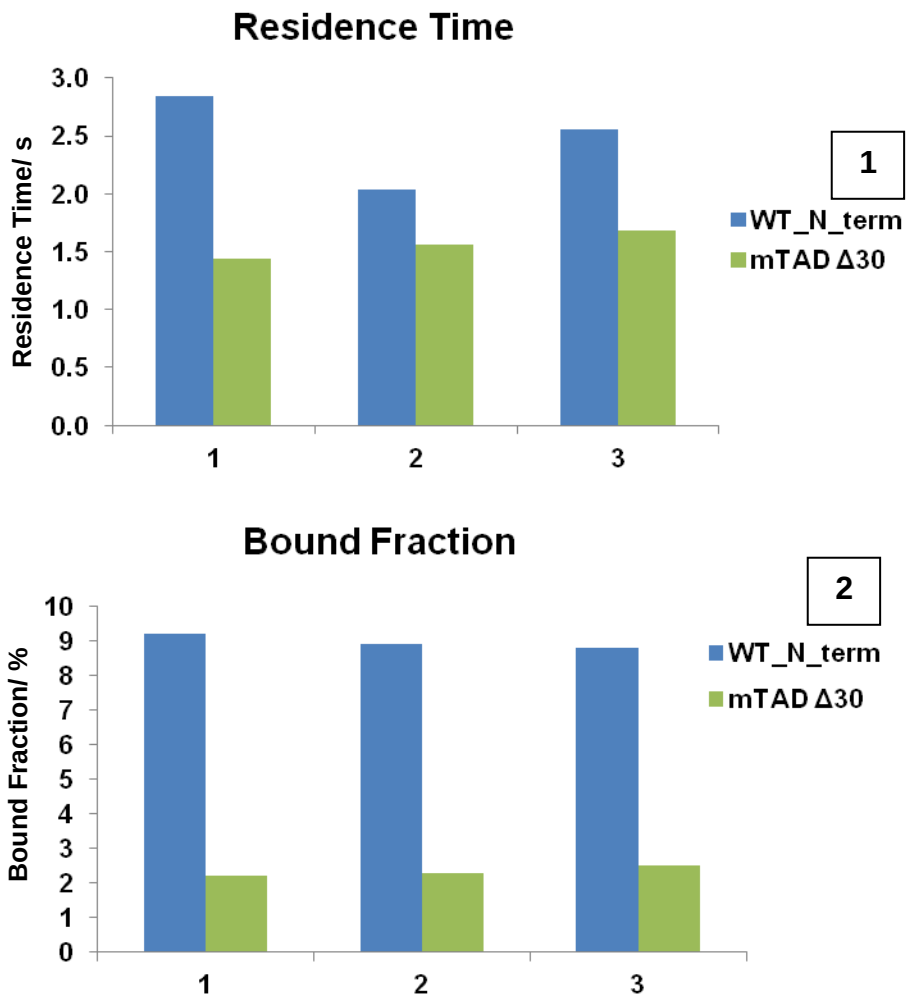


Figure 21: The graph 1 shows the comparison of residence time from three different days. The data from the SMT of this three days show similar results for Wt p53 and also for mTAD_delta30. The graph 2 shows the comparison of the bound fraction from three different days. The data from the SMT of this three days show similar results for Wt p53 and also for mTAD_delta30.

The comparison of WT p53 and mTAD_delta 30 on three different days with fresh cultured H1299 cells showed similar results. In the future it is important to use fresh cultured H1299 cells for single molecule tracking.

4.5 Analyzing of single molecule tracking

The tracked trajectories can be composed of various segments during which the molecule undergoes either diffusion or binding. The key is to identify the segments of the trajectory that reflect binding. The procedure accomplishes this by characterizing the behavior of a molecule that is known to be stably bound to chromatin, such as H2B.
The binding events for the protein of interest are extracted from the trajectory data by identifying segments of the trajectory for which the distance moved by the molecule between consecutive frames is less than or equal to the maximum displacement (r_{max}) measured for H2B. Also the number of time points in the bound segment is larger than some minimum value N_{min}. This value removes short segments where the molecule is loose diffusing but by chances does not move very far. The value of N_{min} at which residence times saturate can be used as the final value of N_{min} for calculating the residence time distribution and bound fraction.
The software realizes bound segments of the based on the input values of r_{max} and $N_{min.}$ The term of each bound segment is calculated generating a distribution of residence times on chromatin. This can be plotted in two different types, as a cumulative histogram or as a conventional histogram. The second type, the cumulative histogram is also known as the survival time distribution S (t), it accords to the probability of having molecules bound longer than a time t. I was using for my experiments the survival time distribution because it allows more direct comparison of different experiments.

4.5.1 Analyzing of single molecule tracking with different N_{min}

The hand checked movies of all my mutants (30 movies for each mutant) were analyzed with three different $N_{min.}$ For the maximum displacement r_{max} the value of 0.3 were used, this value was measured for H2B.
To find the appropriate $N_{min,}$ the hand checked tracks were analyzed with N_{min} 2, N_{min} 10 and N_{min} 16.

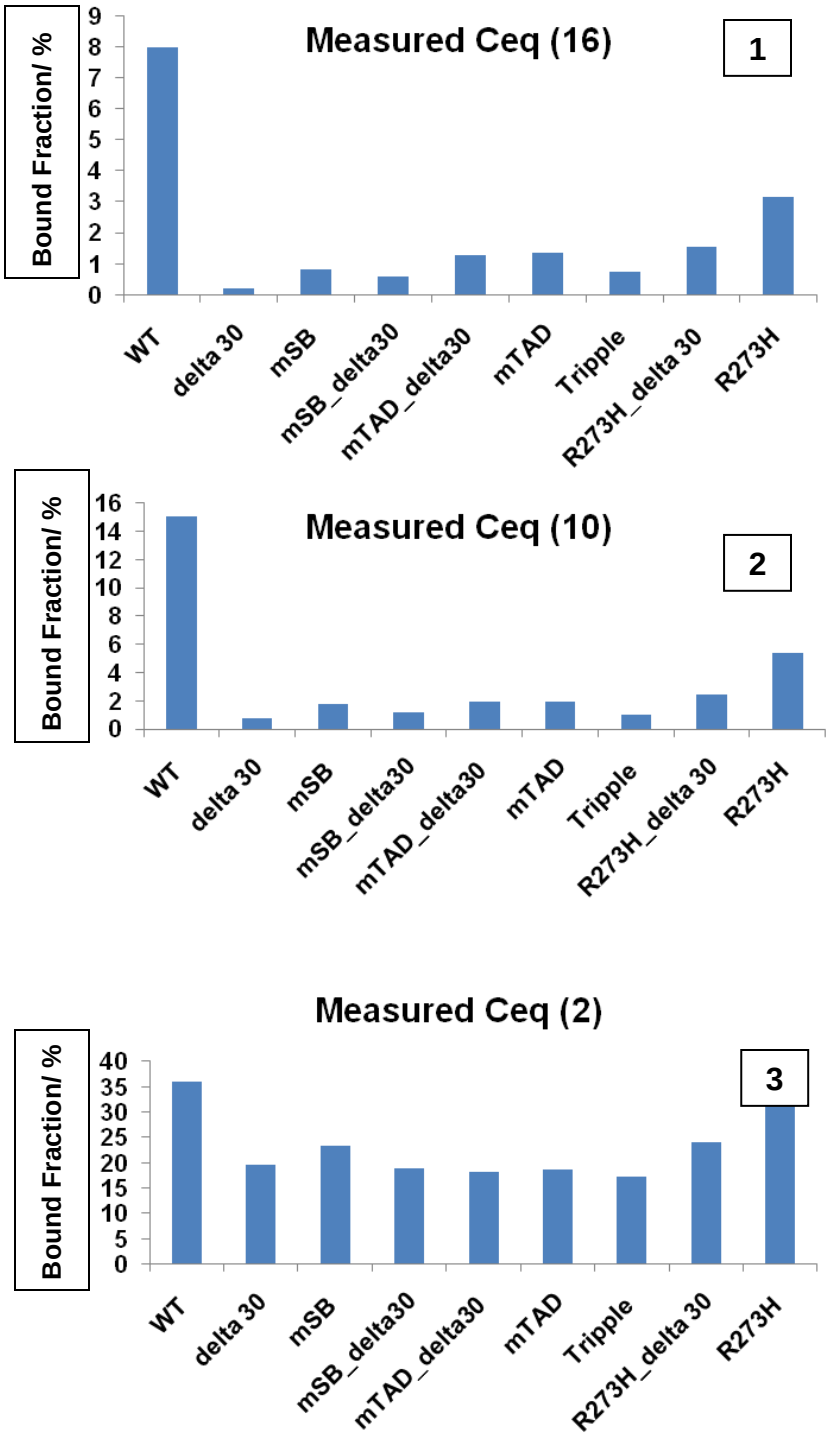


Figure 22: The graph 1 shows the results with N_{min} 16. With N_{min} 16 the bound fraction of each mutant is very low. The graph 2 shows the results with N_{min} 10, the results with N_{min} 10 are really close to N_{min}16. The graph 3 shows the results with N_{min} 2. The difference to N_{min} 10 and N_{min} 16 is large compared to N_{min} 2, because a bigger area was analyzed.

Analyzing of single molecule tracking with different N_{min} showed different results in the bound fraction. The bound fraction of $N_{min}16$ of each mutant was very low. Also the bound fraction of $N_{min}10$ was similar to the results of $N_{min}16$. Through the results of $N_{min}2$ indicate a bigger bound fraction because a bigger area was analyzed.

The bound fraction of the mutant's mSB_delta 30, R273H_delta 30 and the Triple was less compared to the mutant's mSB and R273H. Also the bound fraction of delta 30 mutant was less compared to the WT. In summary the results showed that all mutants with the delta 30 combination showed less binding compared to there mutants without the delta 30 combination. With the exception of the bound fraction of mTAD and mTAD_delta 30, these mutants showed a similar bound fraction.

For the near future it is important to repeat these experiments and analyze the SMT with $N_{min}2$ and compare these results by ChIP-Sep experiments. The comparison of single molecule tracking to ChIP Seq is also interesting because ChIP Seq is known to measure specific site binding, whereas single molecule tracking might be measuring some non-specific binding to chromatin and/ or some specific-site binding to chromatin.

5 Discussion

The "Expand high fidelity PCR System" was the first method which was used to generate PCR products of high yield, high fidelity and high specificity from p53 C-terminus DNA to generate a mutation in the tail domain delta 30 which is mediate to non specific DNA binding. The advantages of this technique are the high efficiency, the thermostable Taq DNA polymerase with proofreading activity and the Tgo DNA polymerase. The PCR step itself was easy and fast and did not raise any problems so far. The handling of PCR product analysis with agarose gel electrophoresis was also very simple and the separation of DNA by size showed clear bands on the gel. Specific DNA fragments were visualized with ethidium bromide as quality controls. The next step was the clean up of the PCR products obtained from gel electrophoresis with the "Wizard®SV Gel and PCR Clean-up System". The principle of this technique was DNA purification via microcentrifugation. During this procedure, PCR products were isolated from primers, salts, unincorporated nucleotides and Taq DNA Polymerase. The clean up of PCR products was efficient and DNA could further be used for cloning with the Flexi®Vector System.

The Flexi®System, Entry/ Transfer system used a flexible and directional cloning method to create plasmids to express protein-coding regions with peptide fusion tags. These features are necessary for expression and the protein-coding region can be shuttle between vectors using two rare-cutting restriction endonucleases, SgfI and PmeI. Flexi® Vectors contain the lethal gene barnase for positive selection of the protein-coding sequence, and an antibiotic resistance marker for selection of colonies containing the Flexi® Vector. All Flexi® Vectors can operate as an acceptor of a protein-coding region flanked by SgfI and PmeI sites. The SgfI site is upstream of the start codon of the protein coding region, and depending on the Flexi® Vector used for cloning, this allows the expression of an amino-(N)-terminal-tagged protein by readthrough of the SgfI site. The PmeI site contains the stop codon for the protein-coding region and appends a single valine residue to the carboxy-(C)-terminus of the protein. Protein-coding regions can be cloned into Flexi® Vectors containing SgfI and PmeI sites (Technical Manual, Flexi® Vector Systems, 2010). The digestion reactions for the PCR product and the Flexi® Vector pFN22A (HALO-Tag®7) CMV d1 were performed concurrently with the restriction enzymes SgfI & PmeI. To check the quality of the digestion reaction the fragments were analyzed with agarose

gel electrophoresis (see chapter 4.2.1). The results were often not satisfying, because mSB delta 30 mutants and the mTAD mutants showed two different DNA fragments for each of the mutant on the gel. It could be the result of not completed digestion of the DNA with the enzymes SgfI and PmeI, maybe the time with 1h was too short or the temperature was not at 37 °C. Because of the fact that right fragment size was bride enough to it was used for ligation. The fragment size for the R273H, R280K, L22Q+W23S and R248W mutants and also for the R273H_delta 30, R280K_delta 30, L22Q+W23S_ delta 30 and R248W_delta 30 mutants were correct.
The next step was the ligation of the different p53 mutants into the pFN-21A vector. This step was simple and high efficient.

The ligation reaction was used for transformation into Subcloning Efficiency™ DH5α™ Competent Cells. To check the quality and efficiency of the transformation pUC19 DNA was used as a control DNA. For selection of the colonies the antibiotic resistance marker ampicillin was used. The results were white colonies on the LB plates with ampicilin. For the classification 6 – 18 colonies were used for the insert check. The transformation was the first challenge deal in this experiment. The transformation efficiency was not that good in the first experiment and was repeated. It was possible in the second round, to get a much higher transformation efficiency compared to the first round.
To control the insert, a PCR with the "GoTaq® Green Master Mix" was done and analyzed on an agarose gel as a quality control. In the agarose gel electrophoresis the expected fragments of all p53 mutants were shown. These positive PCR products were used for the sequencing reaction with the 3130XL Genetic Analyzer. For the sequencing of the p53 mutants, plasmid DNA was extracted from a 2 ml culture for each of the candidates. For the sequencing reaction 6 different primers (see table 34) were used. These measurements with the 3130XL Genetic Analyzer confirmed that the sequence of each mutant was right (see table 35). Therefore it was confirmed that the cloning was successful and the mutants were further used for single molecule experiments.

The plasmid preparation was performed with the QIAprep Spin Miniprep Kit (Qiagen). This procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. The protocol consists of three basic steps, the first one is the

preparation and clearing of a bacterial lysate, followed by absorption of DNA onto the QIAprep membrane and the last step is washing and elution of plasmid DNA. This system works without an alcohol precipitation (QIAprep Miniprep Handbook, 2006). For the Miniprep, the positive clones (see chapter 4.3; table 35) from the sequencing were used. First overnight cultures from each clone were made and the plasmids were isolated with the Miniprep Kit.
The optimal concentrations of these mutants for single molecule tracking were checked (see chapter 4.3.1) with the NanoDrop ND-1000 spectrophotometer. This procedure shows a 260/280 nm and a 260/230 nm ratio, which gives information about the purity of the DNA. The DNA concentration was a little too low (see table 36 in chapter 4.3.1) but it was enough for the transfection and the single molecule experiments.

H1299 cells are from Human non-small cell lung carcinoma cell line derived from the lymph node. These cells have a homozygous deletion of the TP53 gene and do not express the tumor suppressor p53 protein which in part accounts for their proliferative affinity.
While for transfection of the H1299 cells, Lipofectamine LTX Reagent was used, for transfection of the lung carcinoma H1299 cells, Lipofectamine LTX Reagent was selected. The Lipofectamine LTX reagent is a perfect animal-origin free cationic lipid formulation for the transfection of DNA into eukaryotic cells. The transfection steps itself are easy and fast and do not raise any problems so far and gave a transfection rate of 80%.
The next day after transfection (15h), the cell permeable fluorescent ligand HaloTag-TMR (Tetramethylrhodamine) was added to the cells with the different mutants. After the treatment with TMR the cells were washed to remove the unliganded fluorescent molecules and were now ready for the microscope.

With the single molecule tacking it is possible to quantify binding of nuclear proteins to DNA. The Halo Tag fusion with the different p53 mutants allows subsequent in vivo labeling of the protein with a derivative of tetramethylrhodamine, which is a more photostable fluorophore than GFP. Previous experiments with ChIP-Seq showed that the N-terminus is much better than the C-terminus for p53. The goal was to change the HALO-tag from the C-terminus to the N-terminus of the proteins of interest (p53, see chapter 3.5). After transfection of the H1299 cells with the different p53 mutants' movies of the cells including the molecules were collected.

The single molecules were detected using a microscope that minimizes out-of-focus light by mean of a slanted excitation light beam known as HILO illumination. The next step was to collect images of single molecules in the cell nucleus as a function of time to produce movies of single molecule motion. Then the motion of every molecule in the movie was tracked to generate trajectories which were further analyzed to identify the segments that correspond to chromatin binding events. With the analyzing of the trajectories it could be determined which fraction of the molecules were bound, and how long this molecules were bound. This produced an estimate of the total bound fraction and the distribution of residence times on chromatin for the protein of interest.

The first SMT experiment did not show much difference in the binding between the p53 WT and the specific binding mutants and also the transactivation mutants. The method was performed with 25 Hz and 500 frames. For further experiments it was necessary to generate mutants with delta 30 to get less binding of the molecules. These delta 30 mutations were generated with a PCR step (see chapter 3.2.2). In SMT, the difference between mutants with a delta 30 mutation and without this mutation was analyzed by single molecule tracking. After trajectories analyzing, the estimate of the total bound fraction and the distribution of residence times on chromatin for the protein of interest. The results of the p53 mutants in combination with delta 30 showed some difficult data. The mSB delta 30 and the mTAD delta 30 have a higher bound fraction than WT and also the R273H and the R273H delta 30 mutants show similar bound fractions the WT. The used H1299 cells for this experiment were 2 months in culture and it seems that this period of culturing is too long for single molecule tracking. To compare the data of this experiment to the data of the first one it had to be repeated with fresh cultured cells.
After collecting the movies of single molecule motion with 25 Hz and 500 frames, the motions of each molecule in the movie were tracked and then the trajectory was analyzed to identify the segments that correspond to chromatin binding events. The repeated experiment showed a different result compared to the first run, because this time fresh cultures H1299 cells were used for transfection. After analysis of each trajectory, the results showed a significant difference concerning mutants in their bound fraction and their residence times, compared to the WT p53 (see chapter 4.4.3; table 37). It seems that the age of the cells affects the results of single

molecule tracking. This result confirmed my hypothesis that bound fractions of mutants are less often present compared to the WT p53 based on the mutations in the specific binding domain and also transactivation domain. To confirm the results from the second round of this experiment it was necessary to do time series experiments, to see how the mutants behave on different days. Within this time series experiments, the single molecule tracking was performed on three different days to confirm the results of the cloning of delta 30 mutants. The mutants WT p53 and mTAD_delta 30 were used for the SMT. The comparison of WT p53 and mTAD_delta 30 on three different days with fresh cultured H1299 cells showed similar results (see chapter 4.4.4; table 38 and 39). In the future it is important to use fresh cultured H1299 cells for single molecule tracking.

In chapter 4.5 the hand checked movies of all my mutants (30 movies for each mutant) were analyzed with three different $N_{min.}$ For the maximum displacement , $r_{\max}$, the value of 0.3 was used and measured for H2B. To find the appropriate $N_{min,}$ the hand checked tracks were analyzed with N_{min} 2, N_{min} 10 and N_{min} 16. This value removes short segments where the molecule is loose diffusing but by chances does not move very far. The value of N_{min} at which residence times saturation can be used as the final value of N_{min} for calculating the residence time distribution and bound fraction. The software realizes bound segments of the based on the input values of $r_{\max}$ and $N_{min.}$ The term of each bound segment is calculated by generating a distribution of residence times on chromatin. This can be plotted in two different types, as a cumulative histogram or as a conventional histogram. The second type, the cumulative histogram is also known as the survival time distribution S (t); it accords to the probability of having molecules bound longer than a time t. For all SMT, the survival time distribution was used because it allowed more direct comparison of different experiments.
The N_{min} 16 showed a very rare bound fraction of each mutant. The results of N_{min} 10 are very close to the N_{min} 16. The difference to N_{min} 10 and N_{min} 16 is larger compared to N_{min} 2, because a larger area is analyzed.

The results from the single molecule tracking should now be compared to ChIP Seq analysis. We would expect to find that we should lose all ChIP Seq peaks with just a delta 30 mutation. We should see similar results with the Core mutant or with the mTAD mutant.
The comparison of single molecule to ChIP Seq is also interesting because ChIP Seq is known to measure specific site binding, whereas single molecule tracking might be measuring some non-specific binding to chromatin and/ or some specific binding to chromatin.

Generally, it was possible to generate mutants from p53 that showed less binding within the single molecule tracking. To confirm these data it will be necessary to verify this data by ChIP Sep experiments.

6 Conclusion

The explosive field of p53 research started thirty years ago. P53 is a tetramer transcription factor which affects a large number of genes, including genes encoding for proteins leading to DNA damage and oncogene activation. P53 is also a stress response protein and is activated by signals through post translational modifications effects in high protein level and transactivation activity (Brosh and Rotter, 2009).

The accurate regulation of gene expression depends many cellular processes. An important factor in eukaryotic cells is the initiation of transcription, which is regulated by chromatin structure within promoter and enhancer regions and also by large factors that interact with these regions. This interaction factors are transcription factors (TF), which are thought to be the first to bind to genes specific regulatory sites and help to hire other regulatory factors for that gene.

Therefore, with single molecule tacking (SMT) it is possible to quantify the binding of nuclear proteins to DNA. Protein binding to chromatin can be measured in living cells by detecting the retardation of a fluorescently tagged protein as it interacts with comparatively immobile scaffold. The collected images of the single- molecule in the cell nucleus are a function of time to produce movies of single molecule motion. The motions of the molecules in the movie generate trajectories to identify the segments that correspond to chromatin binding events. The trajectories allow determining what fraction of the molecules bind and how long these molecules are bound. This produces an estimate of the total bound fraction and the distribution of residence times on chromatin for the protein of interest.

In conclusion, single molecule tacking is a new technology, may be a crucial goal in the understanding and quantifying the binding of nuclear proteins to DNA and, more importantly, improve the quality of a patient’s life (Voldborg et al. 1997)

7 List of abbreviations

Abbreviation	Meaning
TP53	Tumor protein p53
WT	Wild-type
NLS	Nuclear localization signal sequence
PXXP region	Proline-rich region
Arg	Arginin
Gly	Glysin
AS	Amino acid
HATs	Histone acetyltransferases
CBP	cAMP response element-binding protein
PCAF	P300/CBP-associated factor
REs	Response elements
HDACs	Histone deacetylases
TBP	TATA-box-binding protein
TAF6	TBP-associated factor-6
TAF9	TBP-associated factor-9
CDKN1A	Cyclin dependent kinase 1A
D-CDK4	cyclin-dependent kinase 4
E2F	Transcription factor E2F
ml	Milliliter
RPMI – 1640 medium	Roswell Park Memorial Institute – 1640 medium
DMEM	Dulbecco's Modified Eagle's Medium
PCR	Polymerase Chain Reaction
MgCl2	Magnesium chloride
fwd	Forward
rev	Reverse
LB medium	Luria- Bertoni medium
H2O	Water

DPBS	Dulbecco's Phosphate Buffered Saline 1x
FBS	Fetal bovine serum
dNTP	Desoxyribonukleosidtriphosphate
°C	Degree Celsius
TAE	Tris-Acetat-EDTA-Puffer
V	Volt
C-terminus	Carboxy- terminus
N-terminus	Amino- terminal
ddNTP	Dideoxynucleotides
OH	Hydroxyl group
CCD	Charge coupled device
UV	Ultraviolet light
EM-CCD	Electron multiplying charge-coupled device
Hz	Hertz
GFP	Green fluorescent protein
%	Percent
sec	Second
H2B	Histon H2B
r max	Maximum displacement
N min	Minimum value
t	Time
CHIp-seq	Chromatin immunoprecipitation sequencing
TF	Transcription factors
SMT	Single molecule tracking
DNA	Deoxyribonucleic acid
TMR	Tetramethylrhodamine
RNA plo	RNA polymerase
rRNA	Ribosomal ribonucleic acid
TFIID	Transcription factor IID
poly (A) tail	Polyadenylation tail
POU	Hypophysenspecific octamer transcription factor

nt	Nucleotide
FRAP	Fluorescence recovery after photobleaching
µm	Micrometer
DHS	DNase I hypersensitive sites
kb	Kilo base
CTCF	11-zinc finger protein
bp	Base pairs
H2A	Histon 2A
H3	Histon 3

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Fugure 19: The SMT was performed with 25 Hz and 500 fames. Histogram 1 shows the bound fraction in %, for the different combinations of the p53 mutants and the combination of the delta 30 mutation. The bound fraction of the WT is 30% and much higher than of the other mutants.

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