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MASTERARBEIT

Characterization of the Molecular Signal Transduction Pathway of the Tyrosine Kinase Abl1 in Response to Genotoxic Stress

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Tanja Plessl, BSc

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Abbreviations

ABI1	Abl interactor 1
ABI2	Abl interactor 2
Abl1	Abelson tyrosine-protein kinase 1
Akt	RAC-alpha serine/threonine-protein kinase
ALL	Acute lymphocytic leukaemia
ATM	Ataxia telangiectasia mutated
ATP	Adenosine-5'-triphosphate
ATR	Ataxia telangiectasia and Rad3-related protein
BRCA1	Breast cancer type 1 susceptibility protein
cDNA	Complementary DNA
Ck2	Casein kinase 2
CML	Chronic myelogenous leukaemia
CRKL	Crk-like protein
DKO	Double knock-out
DSB	Double strand breaks
DUB	Deubiquitinating enzyme
EV	Empty vector
GFP	Green fluorescent protein
HCT-116	Human Colorecatal Carcinoma cells
HECT	Homologous to the E6-AP Carboxyl Terminus
HEK293	Human Embryonic Kidney 293 cells
HSV	Herpes simplex virus
HUBL	HAUSP UBL-domain
IC50	Half maximal inhibitory concentration
IP	Immunoprecipitation
JAMM	JAB1/MPN/MOV34 metalloenzyme
Mdm2	Mouse double minute 2 homolog
NES	Nuclear export signal
NLS	Nuclear localization sites

OTU	Ovarian tumour protease
P53	Protein 53
PTEN	Phosphatase and tensin homolog
Rcc1	Regulator of chromosome condensation
RING	Really Interesting New Gene
SH2	Src-homology-2 domain
SH3	Src-homology-3 domain
shRNA	Short hairpin RNA
SSB	Single strand breaks
TRAF	Tumour-necrosisfactor-receptor-associated factor
U2OS	Human Bone Osteosarcoma cells
UBA	Ubiquitin associated domain
UBD	Ubiquitin binding domains
UBL	Ubiquitin-like domain
UCH	Ubiquitin C-terminal hydrolase
UIM	Ubiquitin-interacting motif
UPS	Ubiquitin proteasome pathway
USP	Ubiquitin-specific protease
WB	Western blot
WCE	Whole-cell extract

Abstract

The non-receptor tyrosine kinase Abl1 regulates its substrates through phosphorylation and is involved in diverse biological pathways such as cell proliferation, cell migration, apoptosis and DNA repair. The sub-cellular localization of the kinase is crucial for its biological function and in particular the activity of the nuclear form of Abl1 is known for triggering cell death in response to DNA damage. The goal of this work was to identify new substrates of Abl1 and further elucidate its underlying pathways in response to DNA damage.

Performing affinity purification coupled to mass spectrometry analysis for cytoplasmic and nuclear Abl1, USP7 was identified as a new interactor of the nuclear form of this tyrosine kinase. USP7 is an ubiquitin specific protease responsible for the deubiquitination and stabilization of proteins levels like in the case of the tumor suppressor p53 in response to DNA damage. In our studies USP7 was found to be a substrate of Abl1, which was also predicted by the presence of a specific consensus sequence in USP7 for this tyrosine kinase. Moreover, it was possible to show that Abl1 is phosphorylating USP7 in response to DNA damage. In particular, the treatment of cells with doxorubicin, a chemotherapeutic drug provoking DNA double strand breaks, induced the activation of nuclear Abl1 and the consequent tyrosine phosphorylation of USP7. This event was positively correlating with increasing levels of p53 and its Ser15 phosphorylated form. As p53 is a substrate of USP7 and its stabilization was only evident in the presence of an active Abl1, we hypothesized that p53 levels are positively regulated by Abl1 through USP7 in response to genotoxic stress. Indeed, cells silenced for USP7 were more resistant to cell death triggered by genotoxic stress compared to wild type cells.

Overall, this study described USP7 as a new substrate and player of Abl1 signal transduction pathway in the DNA damage response. Nevertheless, additional experiments are necessary to further clarify the role of USP7 in the signaling pathway connecting Abl1 with p53 after genotoxic stress.

Zusammenfassung

Abl1 gehört zur Proteinfamilie der nicht membrangebundenen Tyrosinkinasen und ist durch die Phosphorylierung verschiedener Substrate verantwortlich für die Regulation diverser biologischer Prozesse wie Zellproliferation, Zellmigration, Apoptosis und DNA Reparatur. In diesem Zusammenhang ist die subzelluläre Lokalisation der Kinase von großer Bedeutung, die nukleare Form von Abl1 wird zum Beispiel nach Beschädigung der DNA aktiviert. Ziel dieser Masterarbeit war die Identifizierung neuer Substrate von Abl1 um den zugrundeliegenden Signaltransduktionsweg bei DNA Schäden aufzuklären.

Zuerst wurde eine Affinity Purification für die cytoplamatische und nucleare Form von Abl1 durchgeführt und die gewonnenen Eluate anschließend mit einem Massenspektrometer analysiert. Dieser Ansatz identifizierte die Interaktion von nuclearem Abl1 mit USP7, einer Ubiquitin-spezifische Protease verantwortlich für die Deubiquitinylierung von Proteinen wie dem Tumor-Suppressor Protein p53. Die bioinformatische Analyse der USP7 Proteinsequenz mit dem Programm Scansite identifizierte eine mögliche Phosphorylierungsstelle für Abl1. Die Phosphorylierung von USP7 durch Abl1 wurde mittels biochemischer Methoden bestätigt. Um die physiologische Funktion dieser Interaktion zu analysieren wurde das Medikament Doxorubicin verwendet um DNA-Schäden zu induzieren und Abl1 im Nucleus zu aktivieren. Diese post-translationale Modifikation korrelierte mit erhöhten Levels des USP7 Substrates p53 und dessen Ser15 phosphorylierte Form. Die Stabilisierung von p53 wurde in Anwesenheit von USP7 und aktiviertem Abl1 beobachtet, was auf eine positive Regulierung von USP7 durch Abl1 nach DNA Schäden schließen lässt. Dies wurde durch eine geringere Induktion der Apoptose nach genotoxischem Stress in Abwesenheit von USP7 in Zellen bestätigt.

Diese Arbeit beschreibt mit USP7 ein neues Substrat der Tyrosinkinase Abl1 und zeigt eine neue Verbindung dieser Proteine nach genotoxischem Stress. Jedoch sind weitere Experimente notwendig um die Rolle von USP7 im Signalweg von Abl1 und p53 aufzuklären.

1. Introduction

1.1 Functions of Protein Tyrosine Kinases

The non-receptor tyrosine kinase Abl1 (Abelson tyrosine-protein kinase 1) regulates its substrates through phosphorylation. Phosphorylation is an important post-translational modification defined by the covalent attachment of one or multiple phosphate groups to the specific amino acid of a substrate protein. The process is catalyzed by protein kinases which belong to the enzymatic class of transferases that add a phosphate group from ATP (Adenosine-5'-triphosphate) to the OH group of tyrosine, threonine or serine residues of their substrates (Fig. 1). The attachment of a phosphate group can alter the conformation and the charge of a protein and eventually its enzymatic activity. Phosphorylation is a covalent modification that can be reverted by specific enzymes called phosphatases which belong to the class of hydrolases. The antagonistic actions of phosphorylation and dephosphorylation can regulate protein localization, enzymatic activity and protein-protein interactions (Lehninger et al., 2008).

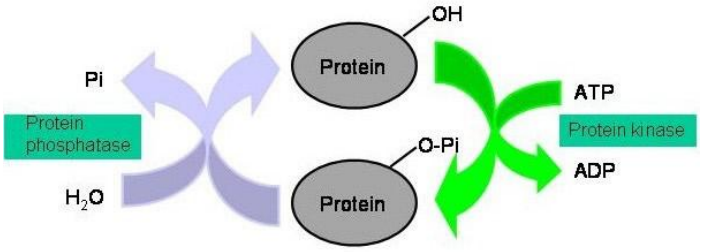


Fig.1: Phosphorylation and dephosphorylation (http://kinasephos.mbc.nctu.edu.tw/document.html)

So far 140 phosphatases and more than 600 protein kinases were identified in the human genome (Hunter, 2007). Kinases can be divided into two big branches: Serine/Threonine kinases are phosphorylating serine or threonine residues of their substrates whereas tyrosine kinases phosphorylate tyrosine residues. Phosphorylation takes place on specific amino acids of a substrate usually located within common structural motifs, known as consensus sequences. These sequences

are then recognized by kinases and phosphorylation can occur. Consensus sequences display high variety - some kinases prefer to phosphorylate within basic residues, others may prefer residues next to proline residues (Ubersax and Ferrell, 2007).

The catalytic function of kinases is executed by the catalytic domain which shows high sequence conservation among kinase family members. This domain binds ATP and covalently transfers phosphate groups on the specific residues of proteins. In addition, kinases can also display additional variable domains important for the regulation of their kinase activity as well as their subcellular localization (Berg et al., 2002).

1.1.1 Cellular Tyrosine Kinases

Tyrosine kinases are implicated in various cellular processes such as cell proliferation, survival and in a wide range of signal transduction cascades (Pendergast, 2002; Smith and Mayer, 2002). The activities of these kinases are tightly regulated and cycling cells show very low levels of tyrosine phosphorylated proteins. Tyrosine kinases can be further separated into two classes: receptor and non-receptor tyrosine kinases. Receptor tyrosine kinases are transmembrane proteins characterized by an extracellular domain for ligand-binding and an intracellular domain with catalytic kinase activity. These receptors are especially important in response to extracellular growth signals (Hubbard and Miller, 2007). On the other hand, non-receptor tyrosine kinases do not have a transmembrane domain and are located in cytosol, nucleus and other subcellular compartments (Neet and Hunter, 1996). They are characterized by their tyrosine kinase domain and additional domains mediating protein-protein and protein-DNA interactions as well as enzymatic regulation (Miller, 2003). The non-receptor tyrosine kinases are locked in an inactive state by inhibitory proteins or through intramolecular autoinhibiton (Krause and Van Etten, 2005) and are usually activated through phosphorylation of tyrosine residues located in the activation loop by either autophosphorylation or phosphorylation through another kinase (Tonks and Neel, 1996).

1.1.2 Non-receptor Tyrosine Kinases as Targets in Cancer Therapy

The treatment of cancers using conventional cytotoxic chemotherapy often leads to severe toxic side effects due to a lack of effective discrimination between rapidly dividing normal cells and tumor cells. Therefore there is a lot of effort going on in the development of targeted therapies, which interfere with molecular targets important for tumor growth (Krause and Van Etten, 2005). Among the most important targets for these therapies are non-receptor tyrosine kinases responsible for cancer development (Arora and Scholar, 2005). Indeed, these proteins are involved in various cellular processes and therefore are deregulated in several types of cancer such as hematologic cancers, lung cancer, breast cancers and many more (Arora and Scholar, 2005). Deregulated non-receptor tyrosine kinase activity can lead to the increase of cell survival, proliferation, antiapoptotic effects, angiogenesis and metastasis in tumor cells (Vlahovic and Crawford, 2003). That is the reason why these enzymes are of high medical relevance and have become important targets for therapeutic intervention (Krause and Van Etten, 2005).

Several different ways leading to aberrant kinase functions were described for patients and thereby inducing the development of cancer. One mechanism is the fusion of kinases with other proteins as a consequence of chromosomal translocation. Frequently, this leads to an alteration of the kinase folding and disruption of its regulated state, for example in the case of the oncoprotein Bcr-Abl (Smith and Mayer, 2002). The presence of mutations can also alter the folding of the kinase, thereby disrupting their autoregulated state and promoting their constitutive activation (Lynch et al., 2004). Furthermore increased tyrosine kinase activity can also be caused by elevated kinase expression or the decrease of tyrosine phosphatase activity (Watanabe et al., 2004).

Non-receptor tyrosine kinases can be pharmacologically targeted through several successful strategies. The first and most prominent approach is to inhibit them through the use of small molecules inhibiting the catalytic domain by interfering with the binding of ATP or the substrates (Schindler et al., 2000). Another strategy is the development of non-ATP competitive inhibitors targeting other domains and regulating the kinase activity allosterically (Converso et al., 2009). Targeting additional sites of

kinases, for example the myristil-binding site of Abl1, also leads to changes in the dynamics of the ATP-binding site and inhibits kinase activity (Zhang et al., 2010). Furthermore monobodies were shown to disrupt the SH2-kinase domain interface critical for Bcr-Abl catalytic activity, thereby inhibiting the kinase activity and inducing apoptosis in CML (chronic myelogenous leukaemia) primary cells, demonstrating another druggable target (Grebien et al., 2011).

Nevertheless, increasing resistance to tyrosine kinase targeted therapies is a severe problem leading to patient relapse and the risk of disease progression in cancer (Shah and Sawyers, 2003). The evolving resistances to tyrosine kinase inhibitors are often caused by mutations in the kinase domain. Most of them are point mutations reducing the binding of the small molecule inhibitor to the ATP-binding site (Gorre et al., 2001). There are some approaches to overcome resistance to kinase targeted therapies. First of all, combining tyrosine kinase inhibitors with conventional chemotherapy or with drugs targeting other signaling cascades in tumor cells is a common treatment (Krause and Van Etten, 2005). Another strategy is the generation of new inhibitors with increased potency and activity against the tyrosine kinases (Weisberg et al., 2006). Combination of kinase inhibitors with drugs targeting downstream signaling components are also under investigation, but the elevated kinase activity by acquired resistance mutations is often dominant and therefore following up this approach remains challenging (Deininger et al., 2005). Another therapeutic option may be the combination of drugs targeting additional sites in kinases with commonly used ATP-binding sites inhibitors (Zhang et al., 2010).

So far, lots of deregulated non-receptor kinases were shown to cause cancer or other diseases and the development of inhibitors resulted in a promising clinical outcome. However, more knowledge about their effects and targets has to be gained to improve current and future therapies (Hantschel and Superti-Furga, 2004; Weisberg et al., 2006).

1.1.3 The Non-receptor Tyrosine Kinase Abl1: Mechanisms of Regulation and Signaling

Abl1 (Abelson tyrosine-protein kinase 1) is a non-receptor tyrosine kinase originally identified as the cellular homolog of the *v-Abl* retroviral oncogene product of Abelson murine leukemia virus (A-MuLV) (Abelson and Rabstein, 1970). Abl1 is implicated in various cellular processes such as cell proliferation, survival and regulation of oxidative stress and DNA damage response (Pendergast, 2002; Smith and Mayer, 2002).

The Abl1 gene is localized on chromosome 9 and encodes alternatively spliced first-exon sequences resulting in two different proteins known as type 1a and 1b (Fig. 2) (Hantschel and Superti-Furga, 2004; Shtivelman et al., 1986). While the 1b isoform is myristoylated at the amino terminus, the 1a variant does not show this modification (Hantschel et al., 2003). Myristoylation is a posttranslational modification targeting proteins to membranes. However, additional acylations, such as palmitoylation, are necessary for stable membrane association therefore most myristoylated proteins are only partly or transiently localized to membranes (Resh, 1994).

Abl1 is a multidomain protein belonging to the Src kinase family. As the other proteins present in this family, Abl1 has an SH3 domain (Src-homology-3 domain), an SH2 (Src-homology-2 domain) domain, a catalytic domain and a long, non-structured carboxyterminal part called the last exon region (Nagar et al., 2003). The Abl1 SH3 domain binds proteins displaying proline-rich sequences such as PXXP whereas the SH2 domain binds tyrosine-phosphorylated sequences, and both domains are important for the activation of cellular signal transduction pathways (Sirvent et al., 2008; Songyang et al., 1993). On the other hand, the last exon region is important to determine the subcellular locations of Abl1, as it contains three nuclear localization sites (NLS) as well as a nuclear export signal (NES) (Taagepera et al., 1998). Furthermore it is also necessary for protein-protein interactions by having binding sites for the Ser/Thr kinase ATM (Ataxia telangiectasia mutated) (Shafman et al., 1997), p53 (Goga et al., 1995) and several other proteins. The kinase domain is the most important part for the enzymatic function of Abl1. As mentioned before, this domain is highly conserved in members of the tyrosine kinase family. It contains the

activation loop which is a structural motif that has to be phosphorylated to fully activate the kinase (Hantschel and Superti-Furga, 2004).

Abl1 is involved in different forms of human leukemia by formation of the oncoprotein Bcr-Abl (Konopka et al., 1984). The Bcr-Abl fusion results form a translocation between chromosome 22 and 9 (Philadelphia chromosome) which replaces sequences from the first exon of *Abl1* with those of the *BCR* gene. In cells, this leads to high proliferation rates and transformation due to the high tyrosine kinase activity of Bcr-Abl (Daley et al., 1990; Hantschel and Superti-Furga, 2004).

In cycling cells, Abl1 is present in an autoinhibited state; the activation loop of the kinase domain is folded into the active site, therefore neither substrates nor ATP can bind (Schindler et al., 2000). While this autoinhibited form of Abl1 is not phosphorylated, the kinase gets highly phosphorylated in tyrosine residues upon activation. Therefore there is a positive correlation between the tyrosine phosphorylation state and activity of this non-receptor kinase (Brasher and Van Etten, 2000).

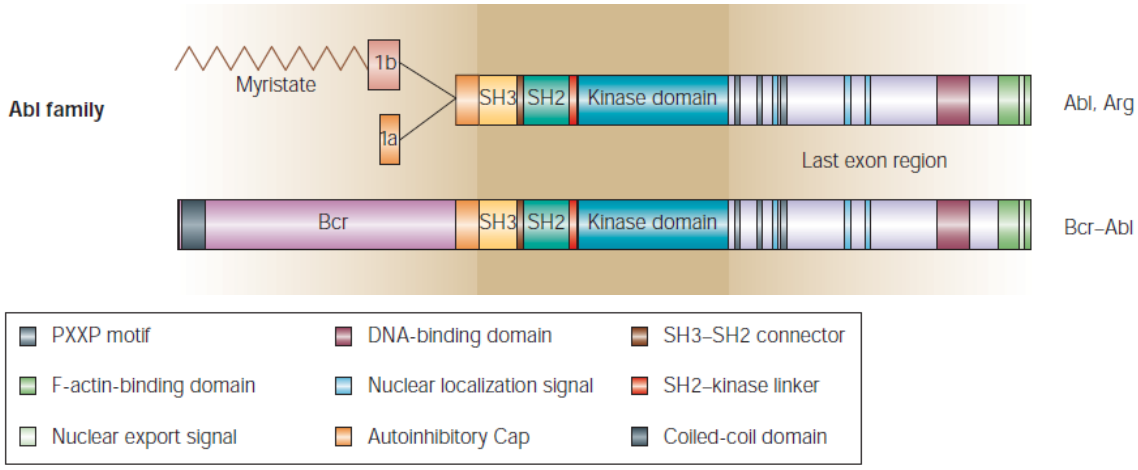


Fig.2: Structural organization of the Abl family of tyrosine kinases (Hantschel and Superti-Furga, 2004)

For the activation of Abl1, the protein has to be phosphorylated on the tyrosine residue 412 located in the activation loop of the kinase domain (Nagar et al., 2003). In the inactive state, this tyrosine forms a hydrogen bond with an aspartic acid that is crucial for the regulation of the catalytic activity. The presence of negative charges in the phosphate group disrupts this interaction and therefore activates Abl1 kinase activity (Schindler et al., 2000). In agreement with this result it was shown that mutation of the tyrosine 412 to phenylalanine prevents Abl1 activation. Furthermore, other tyrosine residues, like the tyrosine 245 located in the SH2-kinase-domain linker were shown to be crucial for the full activation of this kinase (Brasher and Van Etten, 2000).

Once Abl1 is activated it can phosphorylate its substrates on tyrosine residues. The tyrosine kinase has a high substrate specificity which is mediated by the SH2 domain that contributes to target site specificity and catalytic activity (Yadav and Miller, 2008). Substitution of the SH2 domain of Abl1 with the SH2 domain of another protein, results in a change in substrate specificity (Mayer and Baltimore, 1994). It was shown that Abl1 preferably phosphorylates peptides with the consensus sequence VYxxP where V is valine, Y is tyrosine, X is any amino acid, and P is proline (Songyang et al., 1993).

1.1.4 Inhibition of Abl1 and Bcr-Abl Tyrosine Kinase Activity

The activated Abl1 kinase can be downregulated by several mechanisms. Recruitment of tyrosine phosphatases through adaptor proteins present in the SH3 domain can dephosphorylate tyrosine 412 and thereby inactivate the kinase (Wen and Van Etten, 1997). In addition, there are many proteins inhibiting Abl1 kinase activity through binding to the SH3 domain of Abl1 (Wen and Van Etten, 1997) or other sequences as the ATP-binding lobe (Welch and Wang, 1993). Moreover, activated Abl1 can be downregulated by ubiquitin-dependent proteasomal degradation (Echarri and Pendergast, 2001).

Apart from cellular mechanisms for Abl1 inhibition there were several successful approaches to pharmacologically inhibit the kinase. The search for small molecule

inhibitors of tyrosine kinases resulted in the discovery of imatinib (STI-541), that shows a high affinity and specificity for Abl1. This inhibitor targets only a few kinases including Abl1, c-Kit (stem-cell-factor receptor) and PDGF-R (platelet-derived-growth-factor receptor) (Nagar et al., 2003). Imatinib acts as a competitive inhibitor of the ATP-binding pocket and effectively blocks tyrosine kinase activity. In detail, it was shown that imatinib binds to the highly conserved ATP-binding pocket, which forces the activation loop into an inactive, not phosphorylated state (Schindler et al., 2000). Consequently imatinib is used very successfully in clinics for the treatment of Bcr-Abl positive leukemias, including chronic myelogenous leukaemia (CML) and a subset of acute lymphocytic leukaemias (ALL). Compared to cytotoxic chemotherapy, this treatment is well tolerated and fewer side effects are observed (Arora and Scholar, 2005). As mentioned before, resistance to tyrosine kinase inhibitors is in general an increasing problem and this is also the case for the treatment with imatinib (Gorre et al., 2001). To counteract the increasing amount of resistant patients to imanitib therapy, new, more specific inhibitors of Abl1 kinase activity were developed. Among those, nilotinib was shown to be one of the most powerful drugs and therefore it was approved 2007 for the treatment of chronic myelogenous leukemia in patients resistant or intolerant to prior therapy that included imatinib (Jabbour et al., 2007; Weisberg et al., 2006). The drug was designed to target the ATP-binding pocket with higher affinity than imatinib. Nilotinib was shown to be more potent against wild-type Abl1 than imatinib and was also active against imatinib-resistant forms of Bcr-Abl. The drug binds to the inactive conformation of Abl1 and like imatinib it disrupts the ATP-phosphate-binding site, thereby inhibiting the catalytic activity of the enzyme (Weisberg et al., 2006).

1.1.5 Role of Nuclear Abl1 in DNA Damage Response

Abl1 is implicated in various cellular processes such as cell proliferation, survival, regulation of oxidative stress and DNA damage response by phosphorylating its target proteins (Pendergast, 2002; Smith and Mayer, 2002). Abl1 can be located at various subcellular sites including cell membrane, mitochondria, endoplasmatic reticulum, cytosol and nucleus (Ito et al., 2001; Taagepera et al., 1998; Van Etten et al., 1989). Moreover, nucleo-cytoplasmic shuttling of the kinase is mediated by its NLS and NES located in the carboxyl terminal region of the protein and thus allows the execution of functions in both cellular compartments (Yoshida et al., 2005).

While the Abl1 cytoplasmic form was mainly associated with cell proliferation and cytoskeleton reorganization signaling pathways (Sirvent et al., 2008), the nuclear form of Abl1 was shown to be essential in the DNA damage response (Shaul, 2000). DNA damage can occur due to exogenous factors such as ionizing radiation (IR) or chemotherapeutic drugs or because of endogenous factors such as errors during DNA replication or metabolic by-products. This can lead to either single-stranded DNA breaks (SSBs) or to double-stranded breaks (DSBs). For SSBs, only one of the two strands has a defect whereas DNA double-strand breaks are formed when both strands of the DNA duplex are broken. Both forms of strand breaks are dangerous for cells and can cause genomic rearrangements and cell death. Therefore, cells have established several DNA repair mechanisms to overcome these risks. Nuclear Abl1 plays an important role in induction of these DNA repair processes. It is activated through ionizing radiation and various genotoxic drugs, which generate DNA double strand breaks (Kharbanda et al., 1995). The activation is executed by the serine/threonine kinase ATM (Ataxia telangiectasia mutated) which is an important sensor of DNA damage. ATM gets activated after DNA damage and phosphorylates various key proteins involved in initiation of DNA damage response and DNA repair pathways (Banin et al., 1998). One target of ATM is Abl1, which gets phosphorylated on its Ser 465 residue leading to the activation of the tyrosine kinase (Baskaran et al., 1997).

Depending on the severity of the DNA damage induced by genotoxic stress there are differences in the activity of Abl1. The presence of mild damage leads to low activation of Abl1, which induces cell cycle arrest and DNA repair. On the other hand, severe DNA damage induces high tyrosine kinase activity of Abl1, which triggers Abl1-mediated cell death (Shaul, 2000).

In case of mild damage, Abl1 regulates several substrates such as ATM, Rad51 and BRCA1 (Fig. 3) (Shafman et al., 1997; Sun et al., 2000; Yuan et al., 1998). These proteins are involved in the DNA damage repair process. Rad51 for example, functions in the homologous recombination of DNA double strand break repair by catalyzing ATP-dependent pairing and strand exchange between homologous DNA molecules and is regulated through Abl1 dependent phosphorylation (Yuan et al., 1998).

In the presence of severe DNA damage, the pro-apoptotic functions of Abl1 are triggered by phosphorylating different substrates involved in apoptotic pathways. Among them are p63, p73 and Mdm2 (Mouse double minute 2 homolog) (Fig. 3). This function is extremely important as it has been shown that Abl1 deficient cells do not undergo apoptosis in response to DNA damage (Yuan et al., 1997). After DNA damage, nuclear Abl1 is activated and phosphorylates its substrates, for example the E3 ubiquitin ligase Mdm2 on tyrosine residues 276 and 394 (Goldberg et al., 2002) (Gong et al., 1999). These events inhibit Mdm2 activity with the consequent stabilization of the tumor suppressor p53 and the induction of apoptosis (Li et al., 2003). p53 is continuously ubiquitinated by Mdm2 to ensure its continuous degradation in unstressed cells. When Mdm2 is phosphorylated by Abl1, the E3 ligase activity is inhibited and p53, that is stabilized, and can induce apoptosis. (Goldberg et al., 2002). p53 is a key player of the DNA damage response, regulating the induction of apoptosis, DNA repair and cell cycle arrest. Therefore its correct regulation is essential for cells. Furthermore, this tumor suppressor protein is not only regulated through Mdm2, but there are several other proteins involved in this mechanism. They can work together, have opposite functions or are being involved in feedback mechanisms to always ensure correct activation or downregulation of p53. Apart from Mdm2, the DNA damage-dependent activity of p53 is also regulated through

phosphorylation by ATM on serine 15 of p53 (Banin et al., 1998). Nevertheless, Abl1 indirectly regulates p53 activation through inhibition of Mdm2 as described above.

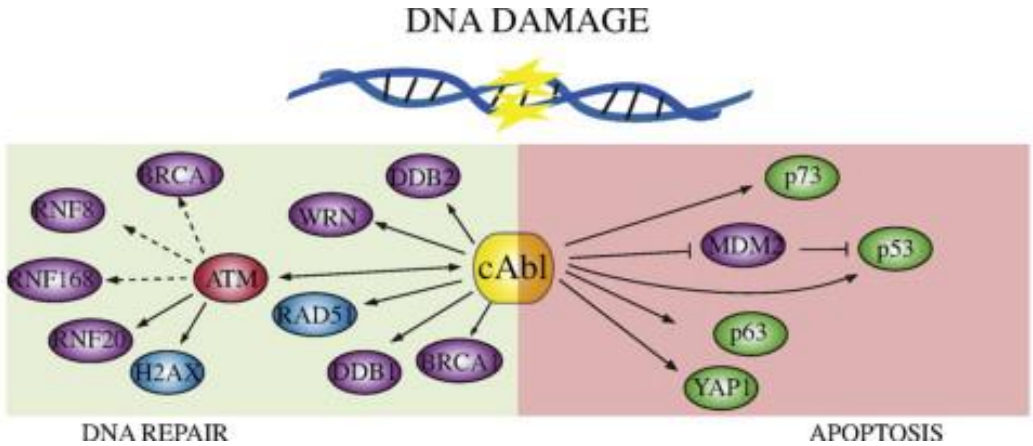


Fig. 3: Substrates of Abl1 activated after DNA damage. In the presence of mild DNA damage Abl1 induces proteins involved in the DNA repair mechanisms whereas severe damage leads to activation of pro-apoptotic proteins (Maiani et al., 2011).

1.2 Another Post-translational Modification: Ubiquitination

Ubiquitination is, as well as phosphorylation, a covalent posttranslational modification process with a crucial role in a lot of biological processes by regulating the fate of proteins. Ubiquitin is a small protein (76 amino acids) which is attached to a substrate through generation of an isopeptide bond between its carboxyl-terminal Gly76 and the ε-amino group of a substrate lysine. A protein can be modified by the addition of only one single ubiquitin molecule, which is known as monoubiquitination event, or by the addition of several ubiquitin molecules to generate so-called polyubiquitin chains. These are formed by conjugation of the additional ubiquitin molecules to the lysine of the previous one (Hershko and Ciechanover, 1998). As ubiquitin itself has seven lysine residues and a free N terminus which are available, different chain types can be formed. All of these linkage types are present in mammalian cells which points out the importance of biological information encoded by the different topologies. The type of ubiquitin chain determines the fate of the ubiquitinated protein (Vucic et al., 2011).

Lys48-linked and Lys11-linked polyubiquitin chains target proteins to the proteasome, where they are degraded into small peptides (Pickart, 2001). Additionally, ubiquitination is involved in the regulation of protein activity and localization: Indeed, Lys63-linked polyubiquitin chains are important for protein-protein interaction, protein trafficking and activation of downstream molecules such as kinases (Yang et al., 2010). These ubiquitin chains display a high difference in their three-dimensional structure compared to Lys48-linked chains, which may account for the distinct functions (Varadan et al., 2004). Linear ubiquitin chains are generated through the free N terminus of ubiquitin and, similar to Lys63-linked chains, are important for the recruitment and assembly of protein complexes (Kirisako et al., 2006). Apart from the different roles described for polyubiquitin chains, it was also shown that monoubiquitination regulates the location of cellular proteins (Hicke, 2001). For example the protein NEMO, which plays a role in NF-κB signaling, is exported from the nucleus to the cytosol after monoubiquitination (Janssens and Tschopp, 2006).

The process of ubiquitination is performed by an enzymatic cascade with a set of three enzymes: The E1 (ubiquitin activating enzyme) protein is activating ubiquitin molecules by using ATP and transfers them to a specific cysteine of the E2 protein

(ubiquitin-conjugating enzyme). The E2 enzyme is then associated with an E3 protein (ubiquitin ligase enzyme) that is responsible of the recognition of the substrate. The ubiquitin is therefore transferred from the E2 enzyme and covalently attached to the substrate due to the E3 ligase activity. (Yang et al., 2010) (Fig. 4).

There are only two E1 proteins described in the human proteome which are necessary for the initiation of the ubiqutination process by activating ubiquitin in an ATP-dependent manner. The next step is the conjugation reaction, where the ubiquitin molecule gets transferred from an E1 to an E2 enzyme. E2 enzymes display a greater diversity than E1's, around 10 of E2 enzymes are known in the human proteome and except for UBE2N (Ubiquitin-conjugating enzyme E2 N) most of them trigger Lys48-linked ubiquitination (Bhoj and Chen, 2009). Once conjugated to ubiquitin, the E2 enzyme carries the molecule to the E3 enzyme, the ubiquitin ligase. The group of E3 enzymes displays the largest diversity, because they mediate substrate specificity. Around 600 different enzymes are known in the human proteome (Bhoj and Chen, 2009).

In the last step of the ubiquitination process, E3s catalyze the formation of the isopeptide bond between the activated carboxy-terminal glycine of ubiquitin and the lysine of the substrate. After the attachment of the first ubiquitin molecule to the substrate, this ligation can be repeated with one of seven internal lysine residues of the ubiquitin molecule itself (Husnjak and Dikic, 2012). In the end, this leads to the formation of different polyubiquitin chains whereas in the case of monoubiquitination no further ubiquitin molecules are added (Vucic et al., 2011; Yang et al., 2010).

E3 ubiquitin ligases contain one or more domains promoting ubiquitination. They can be divided in RING domain or HECT domain E3 ligases (Pickart, 2001). The RING domain containing ligases are characterized by a catalytic zinc-finger-like module that is coordinated by two zinc ions whereas the HECT domains are defined by a catalytic cysteine residue responsible for accepting the charged ubiquitin form the E2 (Pickart, 2001).

As mentioned before, substrates with conjugated Lys48-linked polyubiquitin chains are targeted to the proteasome. This process of ubiquitination followed by degradation is known as ubiquitin proteasome pathway (UPS). The ubiquitin-conjugated substrates bind to the proteasome, either directly or through shuttling proteins and

then they are degraded by the 26S proteasome into short peptides (Zwickl et al., 2000).

The deregulation of the UPS is associated with the pathogenesis of various human diseases, including cancer. Nevertheless, only a small part of genes with possible links to the pathway were studied in detail. Furthermore, no comprehensive analysis of mutations in the UPS involved in human diseases exists (Nalepa et al., 2006). Additionally, also targets of the UPS can be mutated in disease: When a substrate is mutated in residues important for the recognition by the UPS, this protein cannot be degraded anymore, resulting in altered cellular properties (Vucic et al., 2011).

Therefore there is a lot of research going on in targeting this pathway by either directly inhibiting the proteasome or enzymes modifying the ubiquitination status of proteins. The targeting of ubiquitinating enzymes is thought to generate more specific and less toxic antitumor agents (Chauhan et al., 2012). One of the pharmacologically most attractive points in the ubiquitination process is the ubiquitin activation step. This process uses ATP and is therefore a classical target where the binding of ATP is inhibited through a drug. A major problem of this target is that it is involved in lots of different processes in the cell and inhibition would interrupt many pathways including some which are not involved in the disease. Nevertheless, the finding that defects in protein degradation are involved in tumorigenesis and other diseases, targeting the UPS pathway could create new therapeutical strategies (Nalepa et al., 2006).

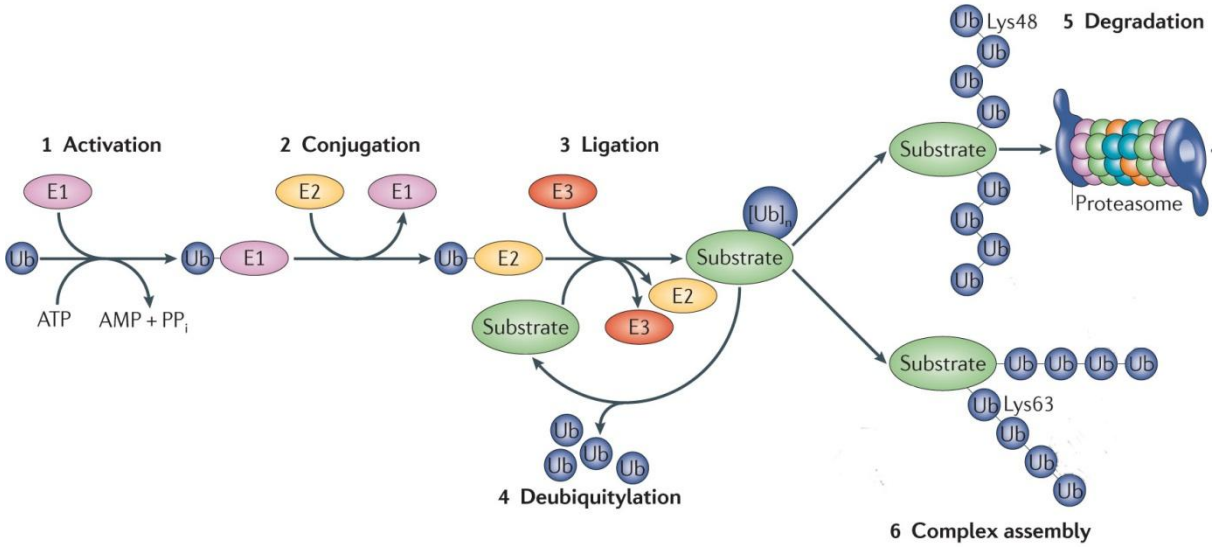


Fig. 4: The enzymes and reactions of the Ubqiuitin proteasome pathway. Ubiquitination of proteins is mediated by the E1 ubiquitin-activating enzyme (step 1), E2 ubiquitin-conjugating enzyme (step 2) and E3 ubiquitin ligase (step 3). The different polyubiquitin chains have variable consequences for the recipient protein – Lys 48 linked chains mark the protein for degradation (step 5) whereas Lys63 linked chains promote assembly of signaling complexes (step 6). Deubiquitylating enzymes can reverse substrate protein ubiquitylation (step 4). (Vucic et al., 2011)

1.2.1 Deubiquitinating Enzymes

Ubiquitination is a reversible modification and the dynamic process of ubiquitination and deubiquitination is important for the correct function of cells. The human genome encodes for more than 100 deubiquitinating enzymes (DUBs), which can remove ubiquitin chains from proteins and thereby oppose the function of E3 ligases (Finley et al., 2004). They are catalyzing the proteolytic reaction between the lysine ε-amino group and a carboxyl group from the C terminus of ubiquitin (Komander et al., 2009).
Deubiquitinating enzymes have distinct major roles in the cell: They are important for the regulation of proteins by eliminating the ubiquitin chains from proteins being post-translationally modified. This leads to protein stabilization through rescue from proteasomal degradation or to reversal of the ubiquitin signaling (Li et al., 2002). Furthermore, DUBs are necessary for ubiquitin homeostasis by recycling ubiquitin. DUBs can also trim polyubiquitin chains and then another type of polyubiquitin chain can be generated within the substrate, a process known as ubiquitin chain editing

(Newton et al., 2008). They are also able to shorten multi-ubiquitin chains bound to proteins by sequentially removing the terminal ubiquitin molecule (Lam et al., 1997).

DUBs are assigned to the classes of cysteine proteases and metalloproteases. Based on their catalytic mechanism they can be further classified into five different families: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumour proteases (OTUs), Josephins and JAB1/MPN/MOV34 metalloenzymes (JAMMs) (Komander et al., 2009).

The JAMMs are zinc metalloproteases, whereas the other families are cysteine proteases. In general, cysteine proteases have divergent folds but the catalytic centers overlay when the ubiquitin molecule is bound (Komander and Barford, 2008).

The organization of the family of ubiquitin-specific proteases (USP) will be discussed in more detail. Most of the USPs contain multiple domains for protein-protein interactions: One of the most abundant feature is the presence of ubiquitin binding domains (UBDs) - including the zinc finger ubiquitin-specific protease domain (ZnF-UBP domain), the ubiquitin-interacting motif (UIM) and the ubiquitin associated domain (UBA domain), which binds ubiquitin with low affinity (Hurley et al., 2006).

Deubiquitinating enzymes show specificity to both substrate and ubiquitin chain type by distinguishing between ubiquitin-like molecules, linear peptides and isopeptides as well as between different chain types and linkages (Komander et al., 2009). The exact mechanism for conferring this specificity is still unknown but there are some common principles which were shown. First, there are DUBs which show linkage specificity and only shorten a specific type of ubiquitin chain (Komander et al., 2008). Furthermore, the cleavage of ubiquitin chains can be done at the ends (exo) or in the middle of a chain (endo) differing between exo-DUBs, which only have to bind a single ubiquitin molecule and endo-DUBs with binding of ubiquitin on both sides of the cleavage site (Hu et al., 2005) (Fig. 5).

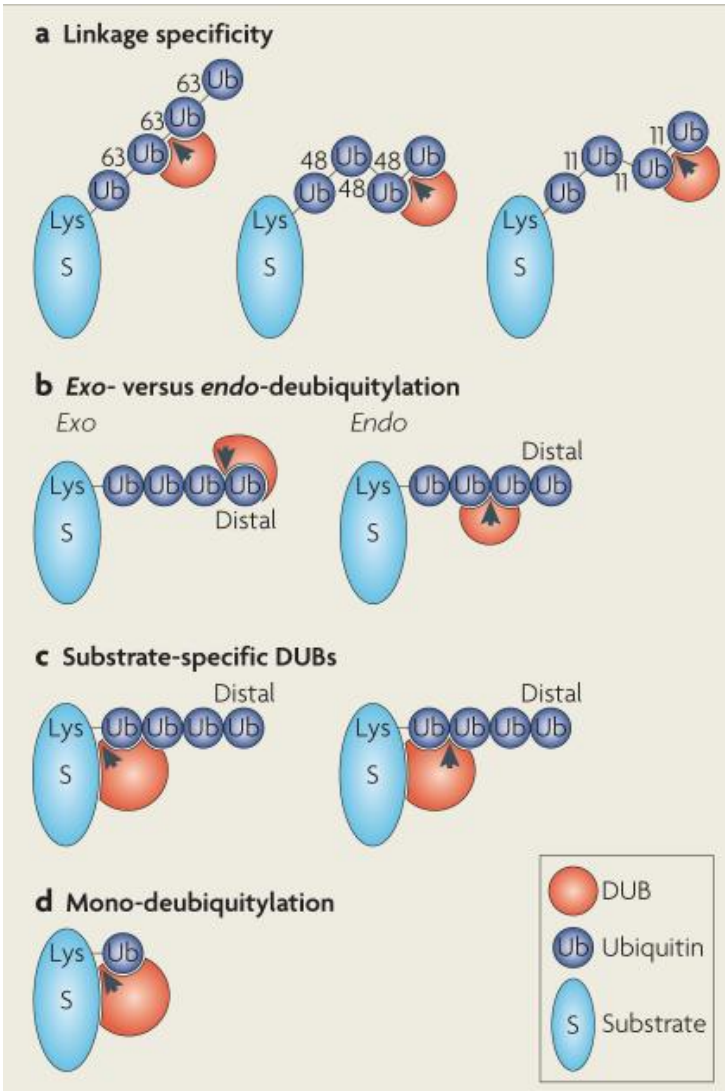


Fig. 5: Substrate specificity of deubiquitinases (DUBs). a) Linkage specifc DUBs discriminate between different types of lys-linked polyubiquitin chains. **b)** Deubiquitination can occur from the ends (exo) or within a chain (endo).**c)** DUBs can also target their substrate specifically. **d)** DUBs can also remove only parts of the ubiquitin-chain leaving the substrate monoubiquitinated. Then the monoubiquitin may be extended again with a different type of linkage. (Komander et al., 2009)

Deubiquitinating enzymes are highly regulated in cells through several different mechanisms. They can be post-translationally modified and there is an intensive crosstalk between phoshorylation and ubiquitin signaling networks (Hunter, 2007). There are also DUBs which are incorporated into larger protein complexes, which serve as scaffolds or are regulated through allosteric activation (Cohn et al., 2009).

Nevertheless, the processes of ubiquitination and deubiquitination are not fully understood yet and need to be further investigated to elucidate ubiquitin network players and physiological mechanisms governing them. Furthermore, ubiquitin ligases

as well as deubiquitinating enzymes are interesting therapeutic targets as they influence protein stability.

1.2.2 USP7: An Important Deubiquitinating Enzyme

USP7 (Ubiquitin carboxyl-terminal hydrolase 7) is a deubiquitinating enzyme encoded in humans by the *USP7* gene. The enzyme was originally identified in association with the ICP0 protein of herpes simplex virus (HSV), therefore it is also known as HAUSP (herpesvirus-associated USP). USP7 has been shown to stabilize ICP0, an E3 ubiquitin ligase, by preventing its degradation which leads to the efficient initiation of the viral lytic cycle (Everett et al., 1997).

USP7 is a deubiquitinating enzyme which belongs to the USP family. As other cysteine proteases, it has a highly reactive cysteine necessary for the hydrolysis of the peptide bound. This residue belongs to a so called catalytic triad in the active center of the enzyme which is composed of histidine, aspartic acid and cysteine residues (Lehninger et al., 2008).

The catalytic domain of USP7 shows the typical conserved fold of USP family members (Sheng et al., 2006). In addition, USP7 contains an N-terminal TRAF (Tumour-necrosis factor-receptor-associated factor) domain, that plays a role in recruiting target proteins such as p53 and Mdm2 (Hu et al., 2002).

The C-terminal region of USP7 contains five ubiquitin-like domains (UBL), therefore this domain is known as the HUBL domain (HAUSP UBL-domain). It has been shown that this HUBL domain is necessary for the full activity of USP7 (Faesen et al., 2011; Sheng et al., 2006). The UBL domains are called ubiquitin-like because their folding is similar to ubiquitin, although they are lacking the terminal glycine residue necessary for conjugation to a substrate lysine residue (Komander et al., 2009).

In detail, the two C-terminal UBL domains present in the enzyme are sufficient to bind and fully activate the USP7 catalytic domain. This process facilitates the binding of ubiquitin and improves the deubiquitinase activity by helping to rearrange the catalytic triad through association with a switching loop next to the active site (Faesen et al.,

2011). In summary, the activation of USP7 depends on the function of UBL domains (Faesen et al., 2011).

The catalytic domain of USP7 consists of three subdomains, which were compared with the palm, thumb and fingers of a hand. In this model, the catalytic center is located between the palm and thumb subdomains, whereas the fingers grab the ubiquitin molecule (Fig. 6) (Hu et al., 2002; Komander et al., 2009). The catalytic center of USP7 is defined by two well-conserved motifs: the cysteine and the histidine boxes. When the ubiquitin molecule is binding, the enzyme undergoes conformational change to bring the catalytic cysteine in proximity to the histidine residue. The active site cysteine then acts as a nucleophile to attack lysine-glycine isopeptide bonds present between substrate and ubiquitin molecule (Komander et al., 2009; Lehninger et al., 2008).

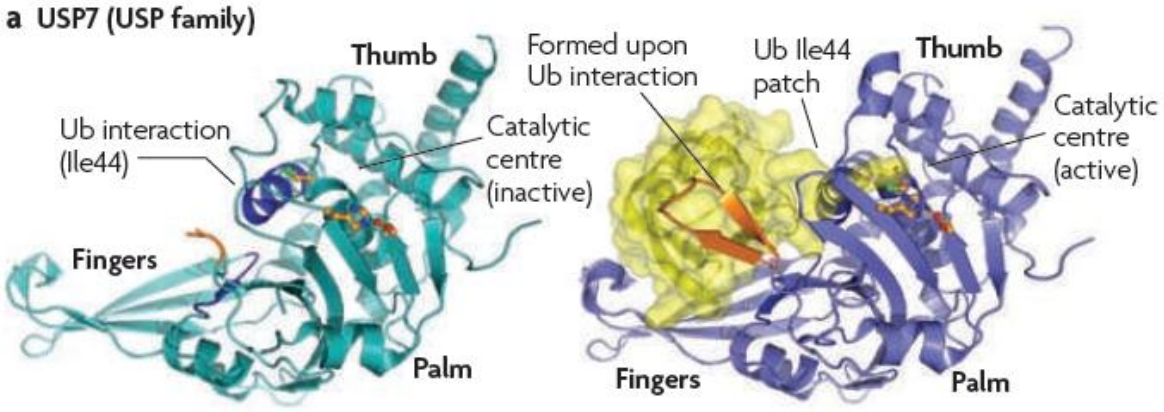


Fig. 6: Catalytic domain of USP7. Inactive (green) and active (blue) structure of the catalytic domain of USP7 with Ubiquitin bound to the catalytic domain is shown in yellow. The thumb, palm and fingers subdomains of the USP domain are indicated (Komander et al., 2009).

USP7 is itself a target for ubiquitination. It has been shown that it is targeted to proteasomal degradation by the ICP0 protein of herpes simplex virus (HSV) (Everett et al., 1997).

USP7 plays an important role in cells because it can remove ubiquitin chains from its substrates. Until now it has been shown that USP7 can only remove Lys48-linked chains (Schaefer and Morgan, 2011). Therefore it protects proteins from proteasomal degradation. Several proteins were identified as potential substrates of USP7. These substrates are involved in the regulation of diverse cellular processes such as DNA repair and apoptotic signaling pathways (Li et al., 2002; Meulmeester et al., 2005a).

Among them, the regulation of the two main USP7 target proteins, p53 and Mdm2 will be described in more detail.

Mdm2 is a RING finger E3 ligase which regulates its own levels through autoubiquitination by Lys48-linked polyubiquitin chains leading to the proteasomal degradation of the enzyme (Honda and Yasuda, 2000). Moreover, Mdm2 is the main regulator of the tumor suppressor protein p53 which is upregulated after cellular stress and DNA damage and responsible for cell cycle arrest or apoptosis (Kubbutat et al., 1997). In unstressed cells, Mdm2 is present at high levels and leads to Lys48-linked polyubiquitination of p53 and subsequent degradation of the tumor suppressor protein by the proteasome (Li et al., 2003). In the presence of genotoxic stress, p53 is phosphorylated on multiple sites which prevents the binding to Mdm2 (Shieh et al., 1997). Subsequently, p53 is not ubiquitinated by Mdm2 anymore and thereby prevented from proteasomal degradation. This leads to the stabilization and activation of p53. This mechanism ensures the maintenance of low p53 levels in unstressed cells and fast upregulation of p53 and its target genes after genotoxic stress (Kubbutat et al., 1997).

The Mdm2-p53 axis is tightly regulated to ensure appropriate regulation. Several proteins, including USP7, are involved in the regulation of this process. (Sheng et al., 2006). USP7 can associate with p53 and Mdm2 and stabilize them through preventing proteasomal degradation by removing Lys48-linked polyubiquitin chains (Li et al., 2002). Therefore the question arises how USP7's substrate specificity is determined between p53 or Mdm2. In normal cycling cells, Mdm2 is strongly associated with USP7. It has been shown that Mdm2 is destabilized in USP7-null cells, resulting in the

consequent p53 accumulation. (Cummins et al., 2004). This substrate specificity seems to be a highly regulated mechanism after DNA damage. In response to DNA damage, ATM is activated and phosphorylates Mdm2 which lowers the affinity for USP7 (Meulmeester et al., 2005b). At the same time USP7 increases its affinity for p53 and stabilizes p53 by removing ubiquitin chains (Li et al., 2002). The consequence is a destabilization of Mdm2 and stabilization of p53, which can activate target genes and induce DNA damage repair or apoptosis (Fig. 7) (Li et al., 2002).

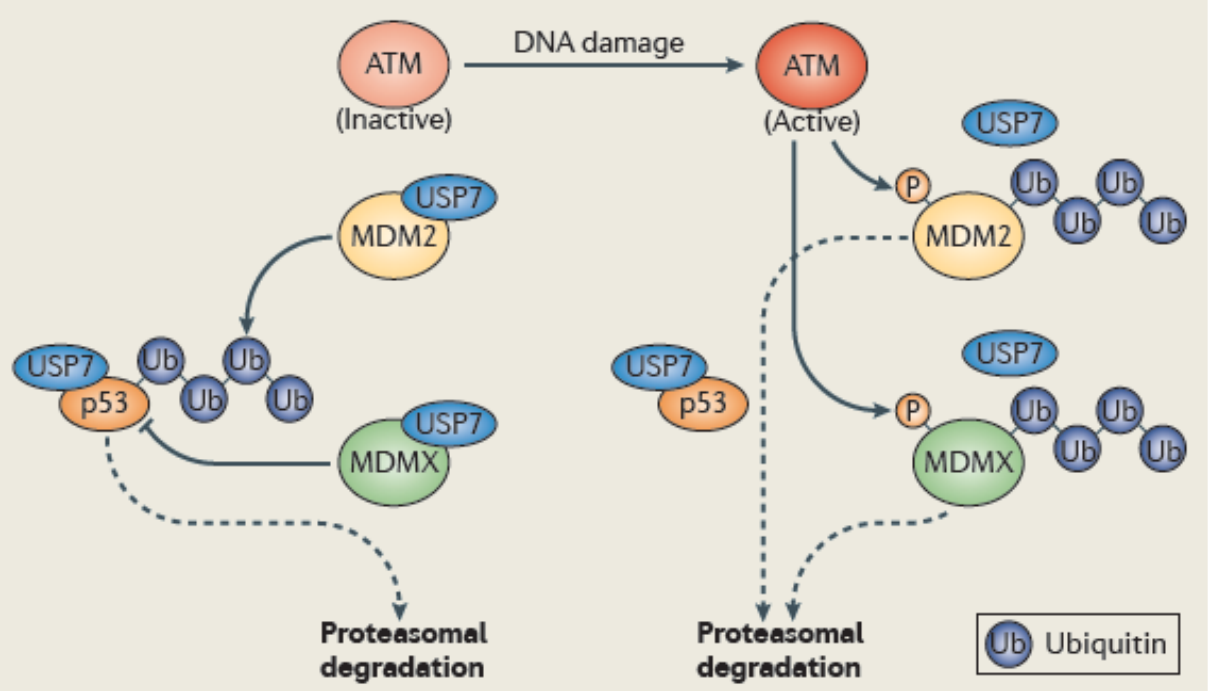


Fig. 7: The complex interrelationships between p53, Mdm2 and USP7. USP7 can associate with, deubiquitinate and stabilize p53 as well as Mdm2. After DNA damage ATM gets activated and phosphorylates Mdm2 so p53 inhibitors are no longer protected from degradation by USP7. This leads to stabilization of p53 and permitting to exert its tumor-suppressive functions. (Vucic et al., 2011)

In summary, USP7 is an important deubiquitiating enzyme by regulating apoptotic and DNA repair pathways through association with p53 and Mdm2 (Li et al., 2002).

1.3 Analogy between Phosphorylation and Ubiquitination

Phosphorylation and ubiquitination are important reversible post-translational modifications and regulating protein activity. Cells must respond immediately to environmental changes to maintain homeostasis and post-translational modifications are fast mechanisms to activate or inhibit signaling cascades. Controlling of cellular pathways can be induced through protein phosphorylation or ubiquitination and in many cases these mechanisms are interwoven (Nalepa et al., 2006).

With these modifications it is possible to change protein function in various ways, either through creation of new protein binding sites, by changing protein-protein interactions or through allosteric effects (Kubbutat et al., 1997; Yuan et al., 1998).

First of all, there are lots of similarities between the two modifications as both are involved in crucial cellular processes and both are ATP-dependent (Berg et al., 2002). Moreover, they are both catalyzed by a large number of transferases, until now more than 500 kinases and more than 600 E3 ubiquitin ligases were identified (Hunter, 2007). Subsequently, also the removal of these modifications is carried out by a high number of hydrolases, in detail by phosphatases and dubiquitinases. So far, there were around 140 phosphatases and more than 100 deubiquitinating enzymes identified in the human genome (Hunter, 2007). Another important similarity is that ubiquitination and phosphorlyation are reversible modifications which can positively or negatively modulate cellular processes. Both modifications are regulating the same physiological processes such as cell proliferation and apoptosis (Becker et al., 2008; Smith and Mayer, 2002). Appropriately, deregulation of these modifications through aberrant functions of the involved enzymes can have severe effects on cells and human health (Bhat et al., 1997).

Nevertheless, there are also differences. For instance, phosphorylation is exclusively a monoadduct event. The ubiquitination signal is more complex, ubiquitin can be added to proteins as a monomer or polymer (Hicke, 2001; Robzyk et al., 2000). Increasing diversity in the ubiquitination process is generated through the different types of ubiquitin chains (Yang et al., 2010).

The reciprocal regulation between phosphorylation and ubiquitination can be observed at many different levels. Kinases and phosphatases can be ubiquitinated as

well as ubiquitin enzymes and deubiquitinases can be phosphorylated (Fig. 8) (Hunter, 2007). These regulations are essential for the correct function of different cellular process like well described in the case of the cell cycle and its checkpoints (Hunter, 2007). The phosphorylation of E3 ligases can regulate the catalytic activity positively or negatively. Phosphorylation of E3 ligases containing a HECT domain can have an impact on protein binding whereas phosphorylation of E3s with a RING domain can affect allosteric activation or inhibition (Goldberg et al., 2002). For example, BRCA1 (Breast cancer type 1 susceptibility protein) an E3 ligase important in the DNA damage repair pathway is activated after phosphorylation in response to DNA damage by the serine/threonine kinase ATR (Ataxia telangiectasia and Rad3-related protein) (Tibbetts et al., 2000). While his is an example for the activation of an E3 ligase through phosphorylation, also the opposite regulation, the inhibition of the E3 ligase through phosphorylation, is possible. Indeed, phosphorylation of Mdm2 by Abl1 leads to the inhibition of Mdm2 and thereby to the stabilization of p53 (Goldberg et al., 2002; Kubbutat et al., 1997).

In addition, phosphorylation can also regulate the activity of deubiquitinating enzymes. Recent experiments showed that the phosphorylation of DUBs resultied in the regulation of their activity. The Ser/Thr kinase Akt (RAC-alpha serine/threonine-protein kinase) for example is directly associating with the the deubiquitinase USP4 (ubiquitin-specific protease 4) and phosphorylates the protein. This modification leads to a change of USP4 localization form the nucleus to the cytoplasm where it maintains protein stability (Zhang et al., 2012).

Furthermore, phosphorylation is regulating the substrates of ubiquitinating enzymes. For example, phosphorylation can prevent the substrates of E3 ligases from ubiquitination through phosphorylation-dependent transport of substrate or ligase into different cellular compartments (Hunter, 2007). Alternatively, phosphorylation can assign substrate proteins for degradation by the ubiquitin proteasome pathway - the induced phosphorylation allows the recognition of the protein by the ubiquitination system, mostly by the E3 ligases. The target for the regulation is in this case the substrate which becomes first phosphorylated and then ubiquitinated leading to its degradation. (Yang et al., 2010).

In addition to the regulation of E3 ligase activity through phosphorylation, also ubiquitination can modulate kinase activity. Kinases are targets of E3 ubiquitin ligases, which promote proteasomal degradation (Echarri and Pendergast, 2001). The tyrosine kinase Arg (Abelson-related gene protein), for example, is ubiquitinated and thereby degraded and inactivated depending on the degree of oxidative stress (Cao et al., 2005).

On the other hand, Lys63-linked ubiquitination can regulate the phosphorylation process through targeting kinases and phosphatases resulting in either activation or inhibition of the target protein (Yang et al., 2010).

The two post-translational modifications are essential for biological functions and cellular responses. The project identified USP7, a ubiquitin specific protease, as a substrate of the tyrosine kinase Abl1 and showed that USP7 is phosphorylated dependent on Abl1 kinase activity. Therefore, this result displays another example for the important crosstalk between kinases and deubiquitinating enzymes.

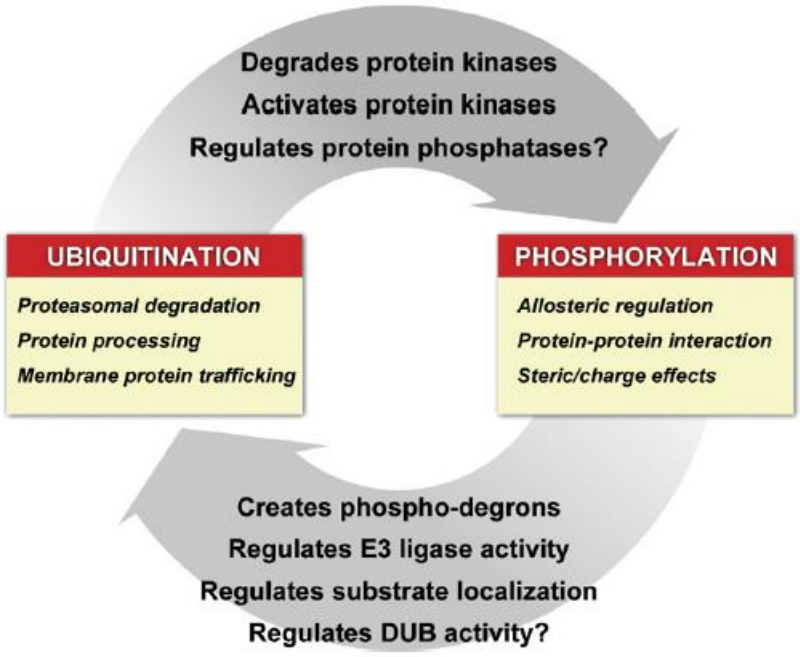


Fig. 8: Crosstalk between phosphorylation and ubiquitination (Hunter, 2007)

2. Results

2.1 USP7 as One of the Main Interactors of Nuclear Abl1

Proteins are often integrated in complexes and interacting among each other, which is important for their regulation and biological function. Indeed protein-protein interactions are essential for protein folding, changing activity and for altering the localization and the participation to molecular signal transduction pathways of each individual protein. Abl1 was shown to interact with several proteins depending on its sub-cellular localization. Moreover, its kinase activity is involved in the regulation of diverse biological pathways such as cell proliferation, cell motility and adhesion, apoptosis and DNA repair (Woodring et al., 2002; Yuan et al., 1997). Numerous proteins are described to interact with Abl1 and are capable of altering its activity; in addition various proteins are annotated as specific Abl1 substrates (Pendergast, 2002). To further elucidate the regulation of Abl1 and to identify new substrates, Abl1-affinity purifications were performed from nuclear and cytoplasmic extracts of mammalian cells. The pulldown data, already present in the lab, were generated using HEK293 flip-in cells, which allow doxycycline-dependent overexpression of Abl1 tagged with an Strep-HA tag at the C terminus (Fig. 9) (Glatter et al., 2009).

After the preparation of cytoplasmic and nuclear extracts, an HA-immunoprecipitation of the protein followed by liquid chromatography mass spectrometry analysis was conducted to identify specific interactors of Abl1 in the two different cellular compartments.

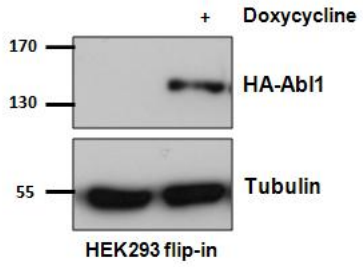


Fig.9: Overexpression of Abl1 in HEK293 flip-in cells after doxycyclin stimulation. Cells were seeded in a 6 well plate and treated with 1µg/ml doxycyclin for 24 hours: Then proteins were extracted in 100µl IP-buffer followed by western blot analysis.

Several proteins were identified (Fig. 10) which had been previously reported to interact with Abl1, such as CRKL (Crk-like protein) (Bhat et al., 1997), ABI1 (Abl interactor 1) (Shi et al., 1995), ABI2 (Abl interactor 2) (Dai and Pendergast, 1995) and 14-3-3 proteins (Yoshida et al., 2005), indicating that proteins found through this approach were specifically associated with Abl1. Looking at the list of the proteins identified by mass spectrometry, except for the chaperone proteins of the 14-3-3 family, USP7 was the protein strongest associated with Abl1, and this was exclusive for the nuclear form of the kinase. Although several proteins were identified as interactors or substrates of Abl1, it was never reported that Abl1 and USP7 are interacting. USP7 belongs to the family of ubiquitin specific proteases involved in DNA damage repair and apoptotic pathways (Meulmeester et al., 2005a). The crosstalk between phosphorylation and ubiquitination is essential in various signaling cascades, but until now Abl1 was mainly described as regulator of the activity of E3 ubiqitin ligases such as Mdm2 (Goldberg et al., 2002) and CBL (E3 ubiquitin-protein ligase CBL) (Andoniou et al., 1994).

On the other hand, USP7 activity was reported to be modulated by serine/threonine protein kinases such as Ck2 (Casein kinase 2) (Khoronenkova et al., 2012) but nothing is known about tyrosine phosphorylation events affecting USP7.

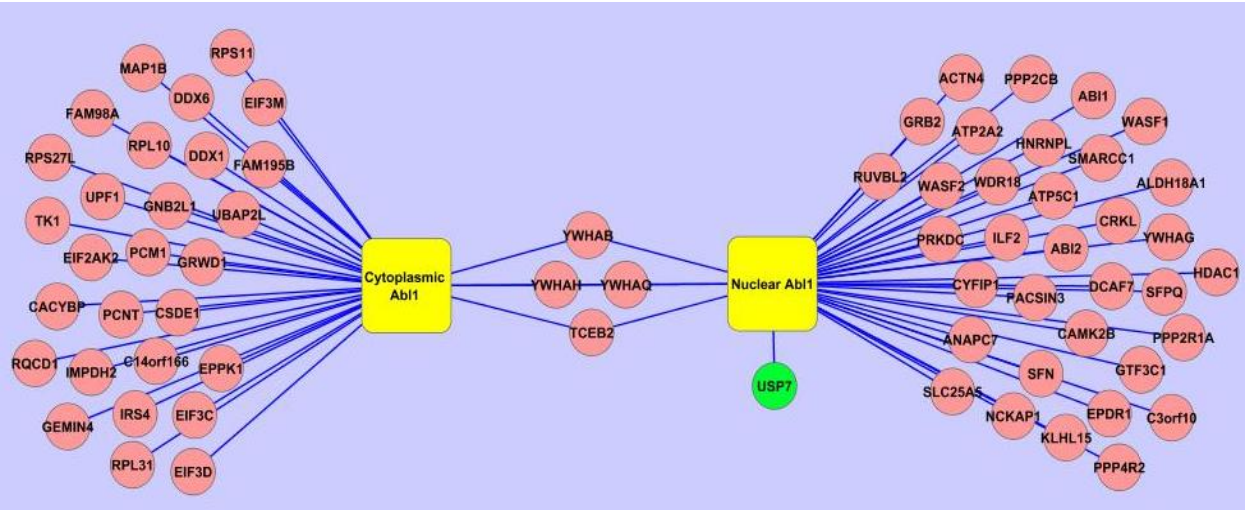


Fig. 10: Nuclear and cytoplasmic pulldown in HEK293 flip-in cells. Proteins identified to interact with nuclear or cytoplamic Abl1. USP7 is interacting only with nuclear Abl1.

As Abl1 is a tyrosine kinase, we hypothesized that USP7 is a substrate of Abl1 and the activity of the kinase might play a role in regulating USP7 activity or its signaling cascades. The phosphorylation event could lead to a positive or negative modulation of this DUB.

The amino acid sequence of USP7 was therefore analyzed for the presence of specific consensus motifs for Abl1 phosphorylation using the bioinformatic tool Scansite (Songyang et al., 1995). This program searches for motifs within amino acid sequences of proteins that are likely to be phosphorylated by specific kinases. As shown in figure 11, Scansite identified a phosphorylation site present in the USP7 protein sequence where the modification may take place, confirming that USP7 is a putative target of the tyrosine kinase Abl1.

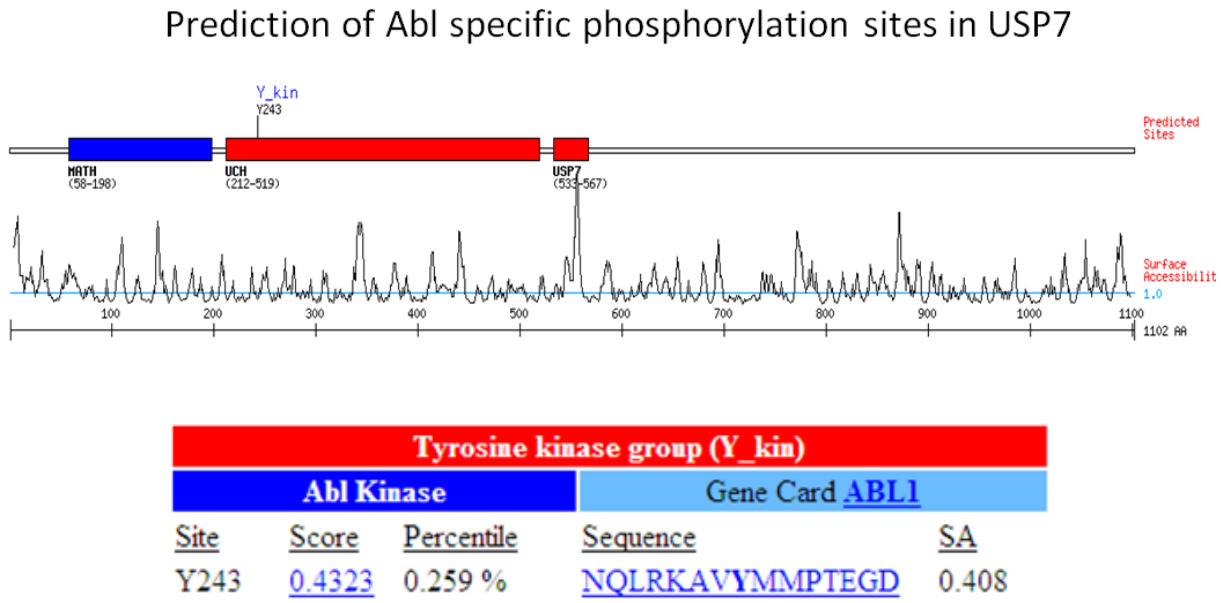


Fig. 11: Prediction of Abl specific phoshphorylation sites in USP7. One possible phosphorylation site has been identified in USP7 sequence using the program Scansite. Stringency scores correspond to the top 1% of sequence matches when compared to all subsequences matching the motif within the entire vertebrate collection of Swiss-Prot proteins, respectively.

To further investigate the effect of the interaction it was first necessary to confirm the result of the pulldown and validate USP7 as target of Abl1 kinase activity. Therefore USP7 cDNA was cloned into a pfMIG-Strep-HA plasmid and transiently overexpressed in HEK293 (Human Embryonic Kidney 293) cells in the presence of Abl1 cDNA. As a control, Mdm2, a known interactor of Abl1 in response to DNA damage, was also transiently expressed using a myc–tag together with Abl1 cDNA. Cells were harvested 48 hours after transfection and whole cell extracts were prepared. All proteins were correctly overexpressed (Fig. 12) therefore the interaction between USP7 and Abl1 was validated by immunoprecipitating Abl1. As shown in figure 4, the immunoprecipitation worked correctly purifying the majority of Abl1 expressed in the cells. A weak positive signal for HA-USP7 was observed in the lane where USP7 was co-expressed with Abl1, confirming the pulldown data (Fig. 12). Unfortunately the interaction of Abl1 and Mdm2 could not be detected under these conditions although Mdm2 was described as a substrate of Abl1 (Goldberg et al., 2002).

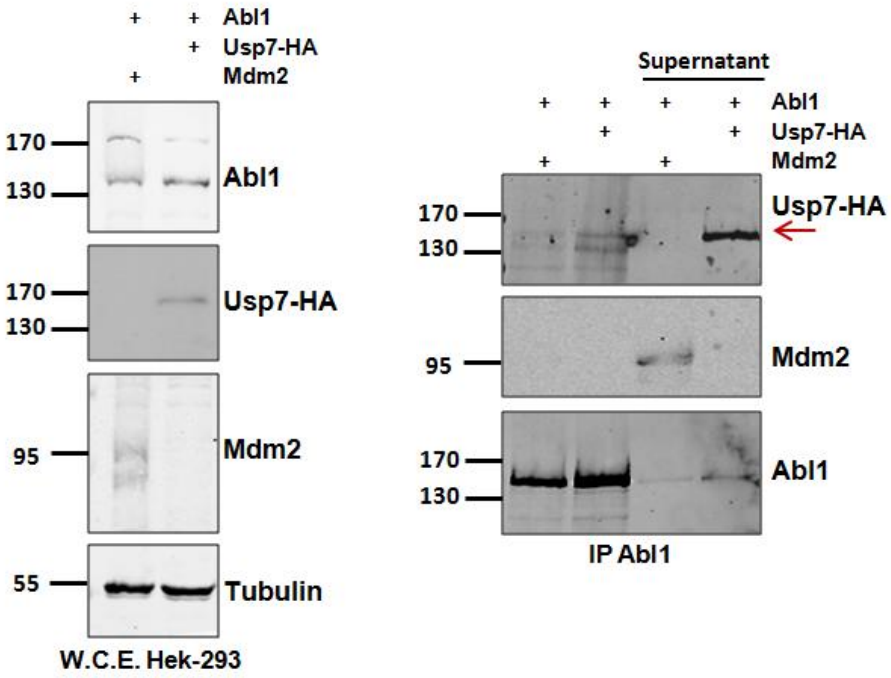


Fig. 12: Abl1 interacts with Usp7. HEK293 cells were transiently transfected with Abl1 and USP7-HA or Mdm2-myc. Then cells were harvested and whole-cell extracts (W.C.E.) were prepared in IP-buffer followed by immunoprecipitation (IP) with anti-Abl1 antibody and western blot analysis.

2.2 USP7 Is Phosphorylated in an Abl1-dependent manner

As USP7 displayed one possible phosphorylation site and was interacting with Abl1, the next step was to show that USP7 is a real substrate of Abl1. The experiment was performed using HEK293 cells transiently transfected with Abl1 and USP7-HA or with a corresponding amount of empty vector as negative control. The overexpression of the kinase induced its hyperactivation as displayed by the increased P-Y412 phosphorylation, a marker of the activation state of the kinase. In this condition, Abl1 phosphorylated its substrates on tyrosine residues, as displayed in figure 13, using phosphortyrosine-specific antibody.

To check the presence of tyrosine phosphorylation of USP7 mediated by Abl1, an immunoprecipitation of HA-USP7 was performed followed by western blot analysis using an antibody that recognizes proteins phosphorylated on tyrosine residues. As hypothesized, USP7 is phosphorylated on tyrosine residues in the presence of an active form of Abl1 (Fig. 13).

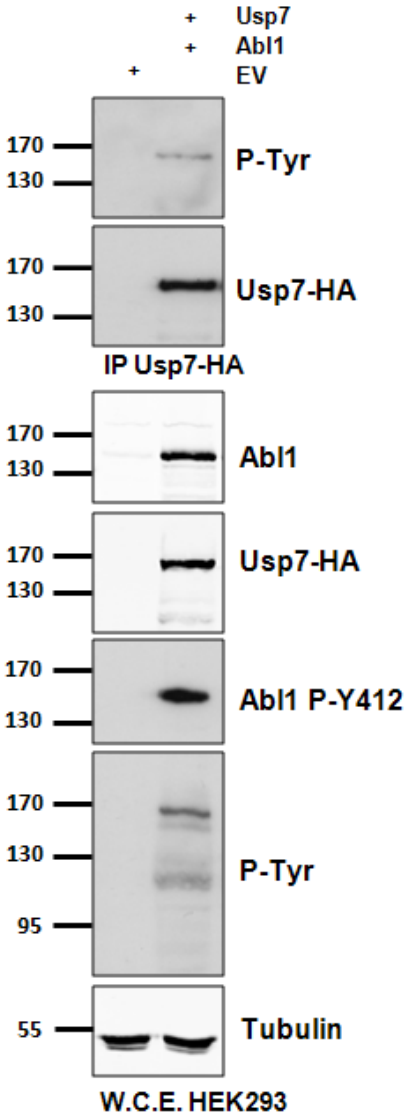


Fig. 13: Phosphorylation of USP7 by Abl1. HEK293 cells were transfected with an empty vector (EV) or USP7-HA and Abl1. Then whole-cell extracts (W.C.E.) were prepared in IP-buffer and analyzed by western blot. Subsequently, an immunoprecipitation (IP) for HA-USP7 and western blot analysis were performed.

2.3 Screening of Drugs that Trigger Abl1 Activation

Nuclear Abl1 was found to interact and phosphorylate USP7 in overexpression experiments using HEK293 cells. In mammalian cells, the physiological role of this interaction is still not clear, therefore different signal transduction pathways were investigated, in which the nuclear form of Abl1 can interact with USP7. It is known from the literature, that nuclear Abl1 can be activated in response to DNA double strand breaks (Kharbanda et al., 1995), triggering apoptosis in the case of severe DNA damage (Gong et al., 1999). Consistently, USP7 was reported to be essential for removing ubiquitin chains from p53 in response to DNA damage, thereby stabilizing p53 and contributing to the induction of p53-dependent apoptosis (Li et al., 2002). Because both proteins positively regulate apoptosis in response to DNA damage, we investigated whether Abl1 activation leads to the phosphorylation of USP7 and the consequent regulation of p53 levels and induction of apoptosis. Therefore, several genotoxic drugs were screened for their capability to trigger Abl1-dependent apoptosis. Based on the literature, four drugs - doxorubicin, etoposide, cisplatinum, and hydroxyurea - were reported to provoke DNA damage and trigger Abl1-dependent apoptosis in mammalian cells (Kharbanda et al., 1995; Kurz et al., 2004; Meresse et al., 2004; Wang et al., 2011). These drugs were tested for their ability to stimulate Abl1 kinase activity and trigger cell death in fibroblasts deficient for Abl1 and its homologous gene Arg (DKO Abl/Arg) (Gong et al., 1999). These cells were reconstituted with a human Abl1 cDNA or with an empty vector (EV) control plasmid. The western blot in figure 14 shows that Abl1 was expressed only in DKO Abl1/Arg fibroblasts reconstituted with the human Abl1 cDNA.

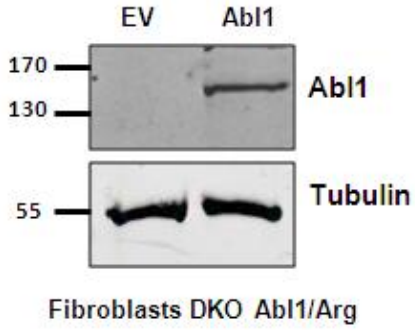


Fig. 14: Murine fibroblasts DKO (double knock-out) Abl1/Arg reconstituted either with an empty vector (EV) or human Abl1 cDNA. 4 * 10^5 cells/well were seeded in triplicates in a 6 well plate and extracted in 50µl Laemmli buffer followed by western blot analysis.

The different drugs were screened performing a dose-response curve in 96 well plates by measuring cell viability through Cell Titer Glo, a luminescent viability assay measuring the cellular ATP levels. The dose-response curves were performed after 24, 48 and 72 hours of drug exposure. After 24 and 48 hours of treatment, no significant differences in cell viability were observed in treated versus untreated cells for both, DKO cells and Abl1 reconstituted fibroblasts (data not shown). Instead, after 72 hours an induction of cell death was observed as displayed in figure 15C in response to hydroxyurea. The Abl1 reconstituted cells did not show any differences compared to the DKO cells even at higher concentrations (Fig. 15). On the other hand, treatments with etoposide and cisplatinum resulted in a mild difference in cell viability, with Abl1 DKO cells being more prone to die. These effects were only observed with higher doses of both drugs (Fig. 15A, 15B).

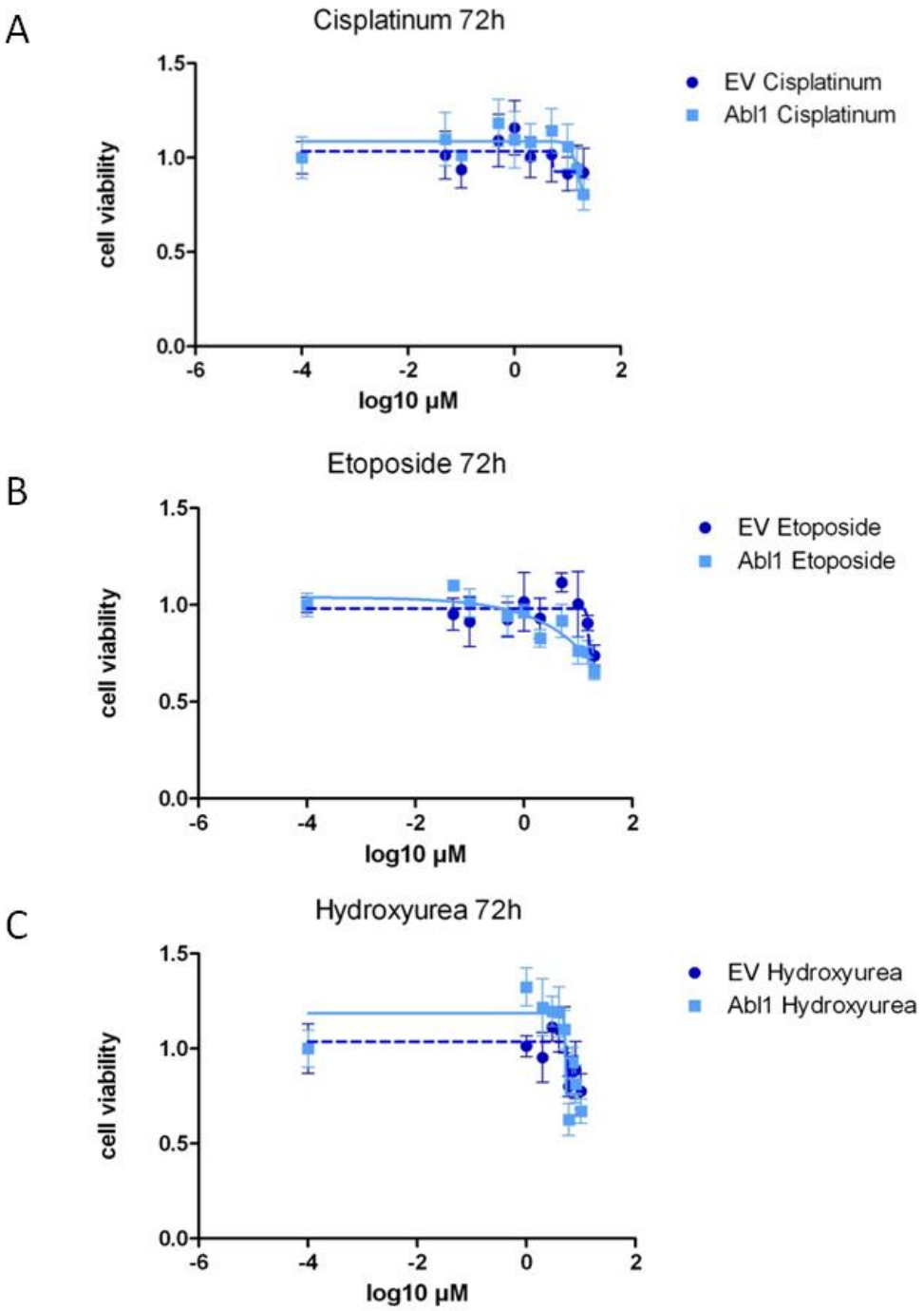
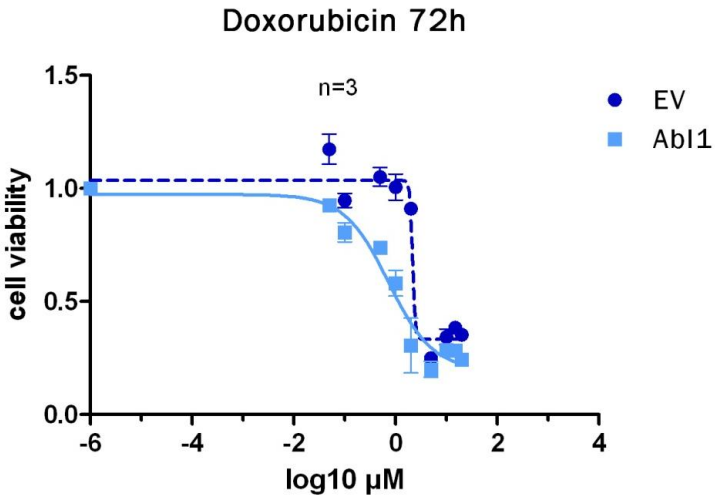


Fig. 15 (A-C): Drug screening of fibroblasts DKO Abl1/Arg reconstituted with an empty vector (EV) or human Abl1 cDNA. 0,8 * 10^4 cells/well cells were seeded in triplicates in a 96 well plate and treated with the indicated drugs for 72h. Then cell viability was determined through ATP level measurements using Cell Titer Glo reagent.

In contrast, doxorubicin treatment differently affected cell death of DKO fibroblasts reconstituted with Abl1 compared to DKO fibroblasts reconstituted with the empty vector. The difference in cell viability between the two cell types can be seen in figure 16. The IC50 (half maximal inhibitory concentration for cell viability) of doxorubicin for DKO EV fibroblasts was around 2,2µM whereas DKO fibroblasts reconstituted with Abl1 had an IC50 around 0,89µM - meaning that they were more sensitive to the treatment and died faster (Fig. 16). The dose-response curves for doxorubicin were repeated three times in triplicates to confirm the denoted result.

The drug doxorubicin is an anthracycline antibiotic and it is used in cancer chemotherapy for the treatment of solid tumors and soft tissue sarcomas. It generates DNA damage and free radical formation through topoisomerase II inhibition (Yoshida et al., 2005) thereby leading to SSB (single strand breaks) and DSB (double strand breaks) (Hortobagyi et al., 1997). Due to the result of the drug screening it was decided to continue working with doxorubicin to characterize the activation of Abl1 and the effect of this event on USP7.



	EV	Abl1	
IC50	2,186	0,890	µM

Fig. 16: Doxorubicin treatment of fibroblasts DKO Abl1/Arg reconstituted with an empty vector (EV) or human Abl1 cDNA. 0,8 * 10^4 cells/well cells were seeded in triplicates in a 96 well plate and treated with increasing concentrations of doxorubicin for 72h. Then cell viability was determined through measuring ATP levels using Cell Titer Glo reagent.

2.4 Activation of Nuclear Abl1 after Doxorubicin Treatment

Based on the cell viability results showing that Abl1 was required to enhance cell death in response to doxorubicin, it was necessary to investigate whether Abl1 kinase activity and the putative tyrosine phosphorylation of USP7 were required to determine this differential cellular phenotype. Overexpression experiments in HEK293 cells were performed to confirm that doxorubicin promotes the activation of Abl1. Therefore Abl1 and a point mutant with an inactive catalytic domain (Kin-) were transiently overexpressed in HEK293 cells followed by treatment with 2µM doxorubicin for 16 and 24 hours. In the absence of treatment, there were no detectable differences in Abl1 protein levels or in its activation state. After 16 hours of doxorubicin treatment, no Abl1 activation was detected, while a dramatic activation of Abl1 was observed after 24 hours. This event specifically required Abl1 kinase activity because the catalytically inactive mutant is not phosphorylated. The levels of Abl1 protein increased in response to doxorubicin in a time dependent way and this event was independent of Abl1 kinase activity (Fig. 17). The presence of DNA damage in cells was reflected by p53 levels. In addition, phosphorylation of p53 on Ser15 residue is a positive marker for its activation and stabilization, and this event is induced and regulated by ATM activity in response to DNA damage (Banin et al., 1998). Differently from Abl1 activation, p53 and in particular P-p53 Ser15 levels were strongly increased after 16 hours of treatment and decreasing after 24 hours (Fig. 17). The reduced phosphorylation of p53 on Ser15 after 24 hours of treatment was less pronounced in the presence of Abl1 compared to the Kin- mutant confirming a positive role of Abl1 kinase activity in the maintenance of the DNA damage response as previously described. Abl1 is indeed reported to phosphorylate ATM kinase, maintaining a positive feedback loop between these two kinases in the presence of genotoxic stress (Wang et al., 2011). No differences were detectable on USP7 protein levels after the treatment with doxorubicin. This indicates that the previously observed phosphorylation of USP7 by Abl1 does not influence protein stability after genotoxic stress.

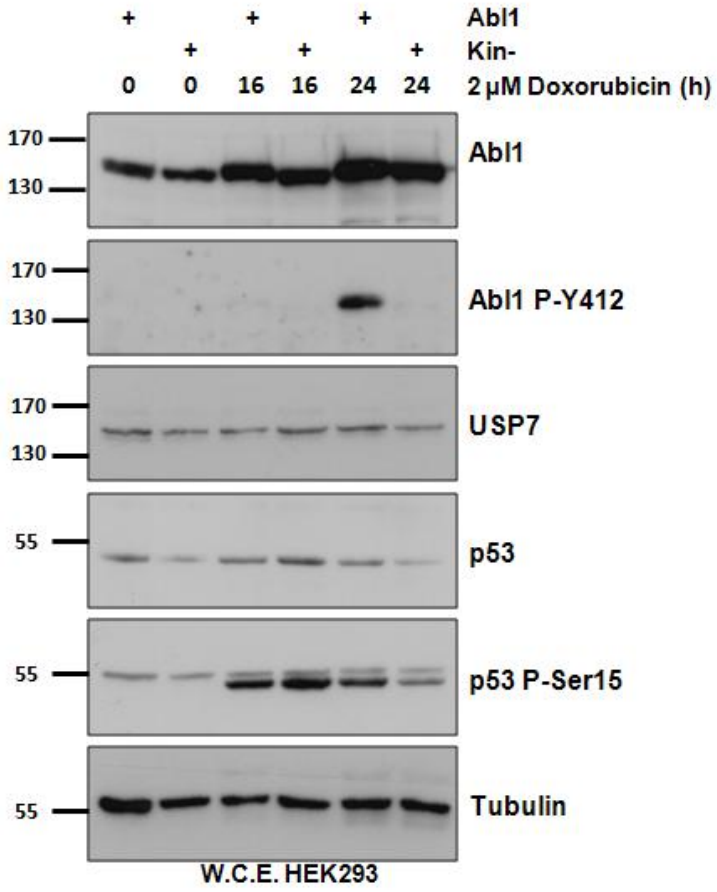


Fig. 17: Activation of Abl1 after doxorubicin treatment. HEK293 cells were transfected with Abl1 or Kin- and left untreated or treated with 2µM doxorubicin for 16 or 24 hours. Then whole cell extracts were prepared and analyzed by Western Blotting.

In response to genotoxic drugs, Abl1 accumulates in the nucleus where it is activated and determines the cellular response to DNA damage (Kharbanda et al., 1995). Therefore it was necessary to check if doxorubicin treatment triggers the activation of Abl1 kinase in the nucleus. HEK293 cells were transiently transfected with Abl1 or an empty vector and treated with 2µM doxorubicin for 24 hours. Then nuclear and cytoplasmic protein extraction was performed to separate the proteins of the different compartments. Tubulin levels present in the cytoplasmic fractions and Rcc1 (Regulator of chromosome condensation) levels, as nuclear marker, were monitored to ensure that the cellular fractionation was performed correctly.

While Abl1 was localized in both nuclear and cytoplasmic compartments, the active form of the kinase after doxorubicin treatment could be mainly found in the nuclear fraction. On the other hand, cytoplasmic Abl1 was also activated, but this event was modest. In summary, this experiment confirmed that doxorubicin treatment activates, as had been assumed, mainly the nuclear form of Abl1 (Fig. 18).

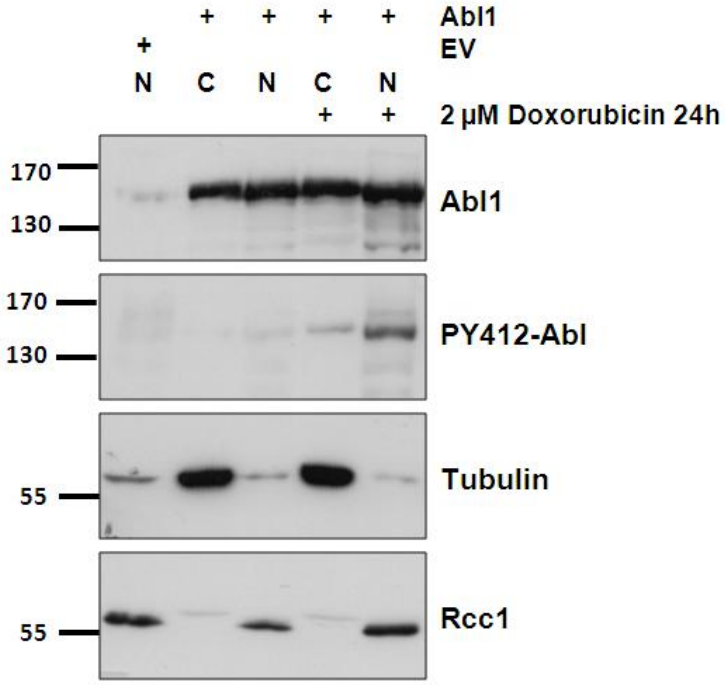


Fig. 18: Activation of nuclear Abl1 after doxorubicin treatment. HEK293 cells were transfected with Abl1 or an empty vector (EV) control and treated with doxorubicin for 24 hours. Then cell lysates form nuclear (N) and cytoplasmic (C) fractions were analyzed by western blotting.

2.5 Phosphorylation of USP7 Dependent on Abl1 Kinase Activity

Having found the right stimulus to activate Abl1, the next step was to show that Abl1 phosphorylates USP7 in response to genotoxic stress. The phosphorylation of USP7 should be dependent on the tyrosine kinase activity of Abl1 which has to be confirmed by using a kinase inactive (Kin-) mutant of Abl1 as negative control. In addition, to confirm that USP7 is specifically phoshorylated in tyrosine residues, Abl1 kinase activity was blocked using the small molecule inhibitor nilotinib that is highly specific for this tyrosine kinase at the concentration of 100nM that had been used. Nilotinib binds the inactive conformation of the Abl1 catalytic domain and this event forces the activation loop into an inactive and unphosphorylated state (Weisberg et al., 2006). Subsequently the phosphorylation event in the tyrosine 412 residue necessary for the transition of the kinase in the active conformation cannot occur.

The experiment was conducted in HEK293 cells transiently overexpressing USP7 and Abl1 or the kinase inactive form of Abl1 (Kin-). Confirming the results of previous experiments, Abl1 was specifically activated after 24 hours of doxorubicin treatment, whereas the kinase inactive form as well as the wild-type form inhibited by nilotinib does not show any activity.

Furthermore, the levels of p53 P-Ser15 were upregulated after doxorubicin treatment and co-expression of Abl1 and USP7. This upregulation was decreased when Abl1 was inhibited using nilotinib or the kinase inactive form was used. On the other hand, the levels of p53 did not change in all conditions used. The phosphorylation state of USP7 was determined by an immunoprecipitation of HA-USP7 followed by western blot analysis using an antibody that recognizes proteins phosphorylated in tyrosine residues (p-Tyr). Looking specifically at the phosphorylation state of USP7, even though equal amounts of USP7 were immunoprecipitated, differences in the phosphorylation state of the protein were identified. In the absence of doxorubicin treatment it was possible to detect the phosphorylation of USP7 by Abl1 which was also shown in the previous experiments, indicating USP7 as a substrate of this tyrosine kinase. Furthermore the phosphorylation event on USP7 was increased after doxorubicin treatment and this event was correlated with the activation state of Abl1. In absence of Abl1 kinase activity, by either transiently overexpressing the catalytically

inactive form of Abl1 (Kin-) together with USP7 or through treatment of cells with nilotinib, the phosphorylation of USP7 was absent, in particular after doxorubicin treatment (Fig. 19). This clearly showed that USP7 is specifically phosphorylated in an Abl1-dependent way and this event is modulated by the activation of the kinase in response to genotoxic stress as doxorubicin.

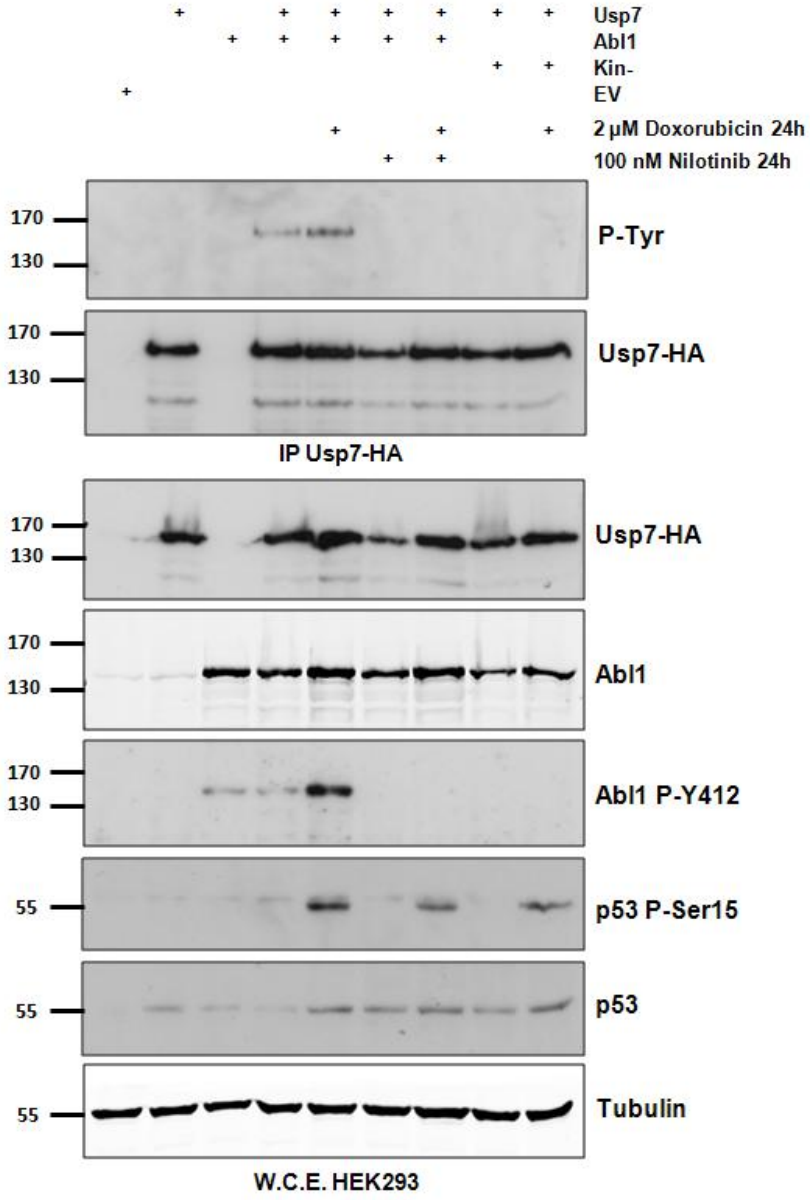


Fig. 19: Phosphorylation of USP7 dependent on Abl1 kinase activity. HEK293 cells were transfected with USP7 and Abl1 or Kin- and left untreated or treated with 2µM doxorubicin or 100nM nilotinib for 24 hours. Then whole-cell extracts (W.C.E.) were prepared and analyzed by western blot followed by an immunoprecipitation (IP) with anti-Abl1 antibody and western blot analysis.

As phosphorylation can modify substrate proteins in several ways, the next experiment was to determine if USP7 stability is regulated through the phosphorylation via Abl1 after doxorubicin treatment. The modification may stabilize the protein after genotoxic stress, or lead to increased degradation. Therefore endogenous protein levels of USP7 were monitored in nuclear and cytoplasmic fractions after doxorubicin treatment using western blots. For this experiment, HCT-116 (Human Colorecatal Carcinoma) cells were chosen, because they have an intact p53 signaling pathway and are therefore commonly used as model to study signal transduction pathways in response to DNA damage.

The cells were treated with a high (2 µM) and a lower concentration (0,5 µM) of doxorubicin to simulate severe and mild damage; then nuclear and cytoplasmic protein extraction was performed to analyze the proteins in the different compartments. The efficient separation was confirmed using tubulin and Rcc1 as respective markers for the cytoplasmic and nuclear compartments. The expression levels of endogenous proteins were analyzed by western blot to observe differences after genotoxic stress.

Abl1 and USP7 were localized in both - nucleus and cytoplasm of cells. The localization of the proteins was not altered by doxorubicin treatment. The same was true for Mdm2 and p53, both showing a more evident nuclear localization, which was also reported in the literature (Kubbutat et al., 1997). Levels of p53 and p53 phosphorylated on Ser15 were upregulated after doxorubicin treatment because p53 is activated in the presence of DNA damage in the cells. In contrast to the overexpression experiments in HEK293 cells shown before, increasing concentrations of doxorubicin were also positively correlating with p53 levels. In addition, the expression levels of the E3 ligase Mdm2, which is the main negative regulator of p53, were decreased during doxorubicin treatment. There were no differences in endogenous protein levels of Abl1 after increasing concentration of doxorubicin. Nevertheless, an increase in tyrosine-phosphorylated proteins was detected in the nuclear fraction, confirming the activation of Abl1 kinase (Fig. 20). Unfortunately, it is very difficult to detect Abl1 kinase activity using the phospho-specific Y412 antibody due to the lower endogenous Abl1 levels. USP7 levels were found stable during doxorubicin stimulation in cytoplasmic and nuclear fractions.

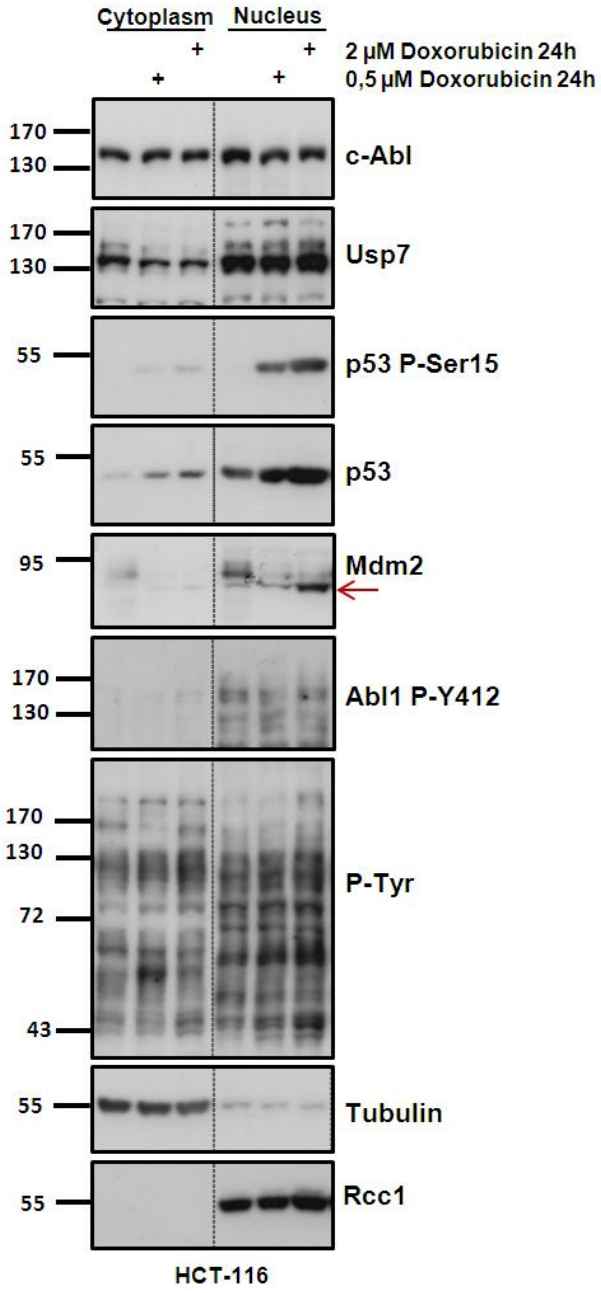


Fig. 20: Protein expression levels after Doxorubicin treatment. HCT-116 cells were treated with 0,5µM or 2µM doxorubicin for 24 hours. Then cell lysates from nuclear (N) and cytoplasmic (C) fractions were analyzed by western blotting.

2.6 Abl1 and USP7 Modulate p53 Activation and the Consequent Cell Death Signaling

To further monitor possible changes in endogenous protein levels through the consequent phosphorylation event of USP7 by Abl1 after doxorubicin treatment, another experiment was performed. This time cells were treated with two different concentrations of doxorubicin for shorter time points. HCT-116 cells were indeed treated with 0,5µM or 2µM doxorubicin for 0, 2, 4 or 8 hours and the activity of Abl1 was inhibited using nilotinib to investigate differences in protein expression levels when USP7 cannot be phosphorylated by Abl1.

Especially after a longer treatment with 2µM doxorubicin there were more proteins phosphorylated on tyrosine residues, indicating that Abl1 is active (Fig. 21). Furthermore the levels of p53 and P-p53 Ser15 were stabilized after doxorubicin treatment while Mdm2 levels were decreased. After nilotinib treatment, no increase in tyrosine-phosphorylated proteins was observed, confirming that the inhibition of Abl1 worked correctly. The protein levels of USP7 were higher after Abl1 inhibition using nilotinib (Fig.21). As previously shown, the phosphorylation and stabilization of p53 was prevented in the presence of nilotinib, confirming the importance of Abl1 activity in the regulation of p53 protein. As USP7 was shown to deubiquitinate p53 and thereby stabilizing it (Li et al., 2002), it is possible to speculate about an indirect effect of Abl1 on p53 through the activity of USP7.

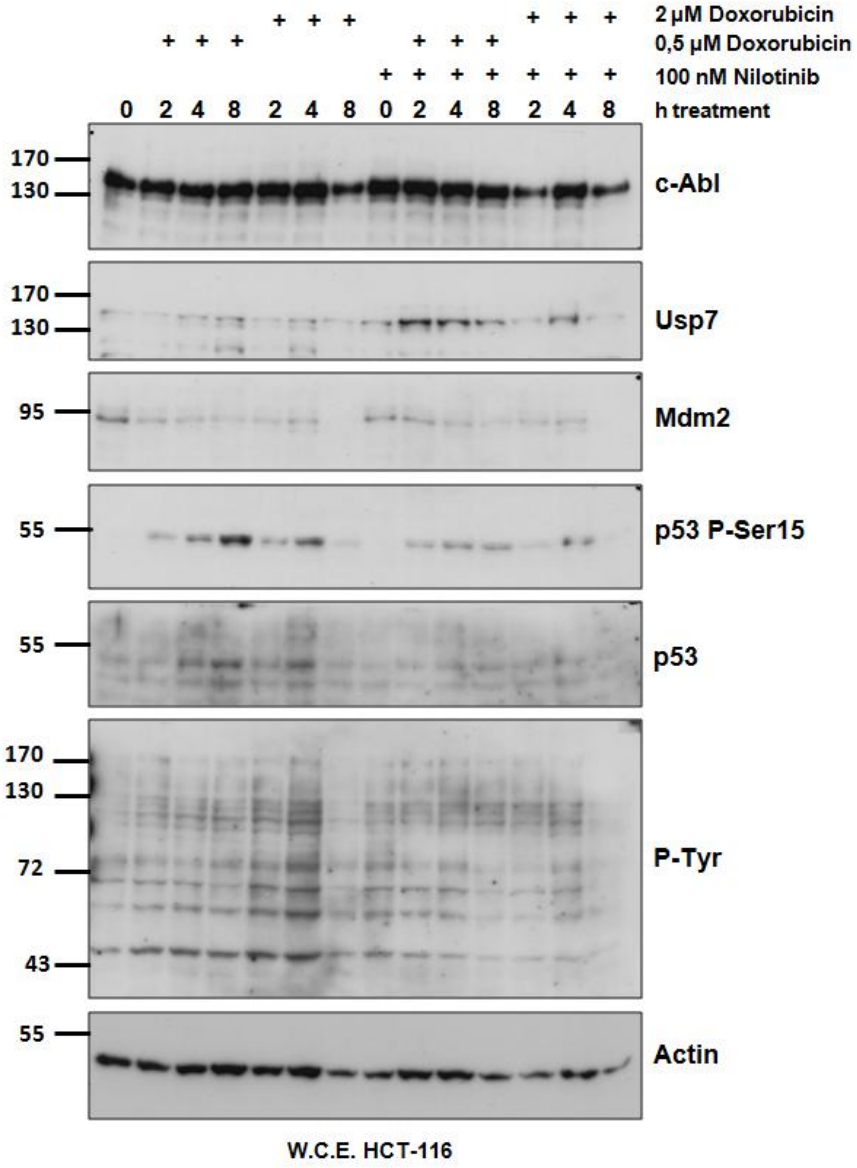


Fig. 21: Protein expression levels after different times of Doxorubicin treatment. HCT-116 cells were treated with 0,5µM or 2µM Doxorubicin for the indicated time points. Then cell lysates from nuclear (N) and cytoplasmic (C) fractions were analyzed by Western Blotting.

To further evaluate that the effect of Abl1 on p53 was really mediated by USP7 activity, the cellular response to doxorubicin was tested in cells silenced for USP7. Therefore U2OS (Human Bone Osteosarcoma Cells) cells expressing a shRNA for USP7 or for GFP were generated and the Cell Titer Glo Assay was used to measure cell viability. For silencing of USP7, two cell lines expressing different shRNAs for USP7 were tested and both of them showed an efficient knock-down of the protein (Fig. 22).

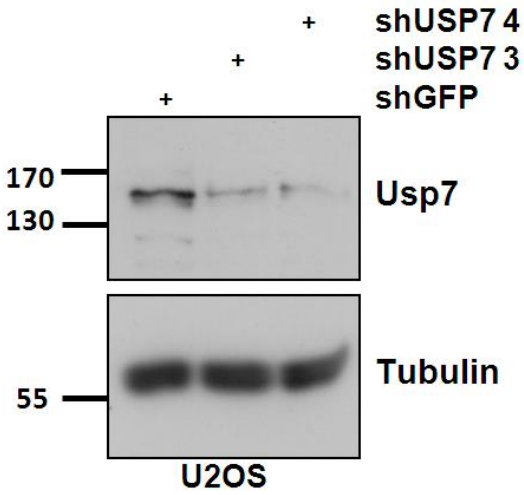


Fig. 22: Conformation of the knock-down of USP7 in U2OS cells shGFP (control) and shUSP7 (3 and 4, two different shRNA were used). 4 * 10^5 cells/well were seeded in triplicates in a 6 well plate and extracted in 50µl Laemmli-buffer followed by western blot analysis.

The three different cell lines were seeded in 96 well plates and treated with increasing concentrations of Doxorubicin for 2, 8 and 24 hours. After these timepoints the drug was removed and cells were kept in normal growth medium for 72 subsequent hours. After 2 and 8 hours of treatment we observed a reduction in cell viability but only at the higher concentration, which was independent of USP7 protein levels. On the other hand, after 24 hours of treatment it was possible to observe a significant difference in cell viability between the control cells and the USP7 silenced cells. Two independent shRNAs targeting USP7 render cells more resistant to cell death, as detected after 0,5µM and 0,75µM doxorubicin treatment (Fig. 23). This indicated that a longer time of relative mild DNA damage was required to activate Abl1 and stimulate USP7 activity, which regulates p53 levels. In the absence of USP7, the levels of p53 were less

stabilized, thereby reducing the apoptotic rate in response to genotoxic stress. In case of a too severe DNA damage, as for 2μM doxorubicin treatment, Abl1 modulation of USP7 activity was not required and cells were committed to death via different signal transduction pathways.

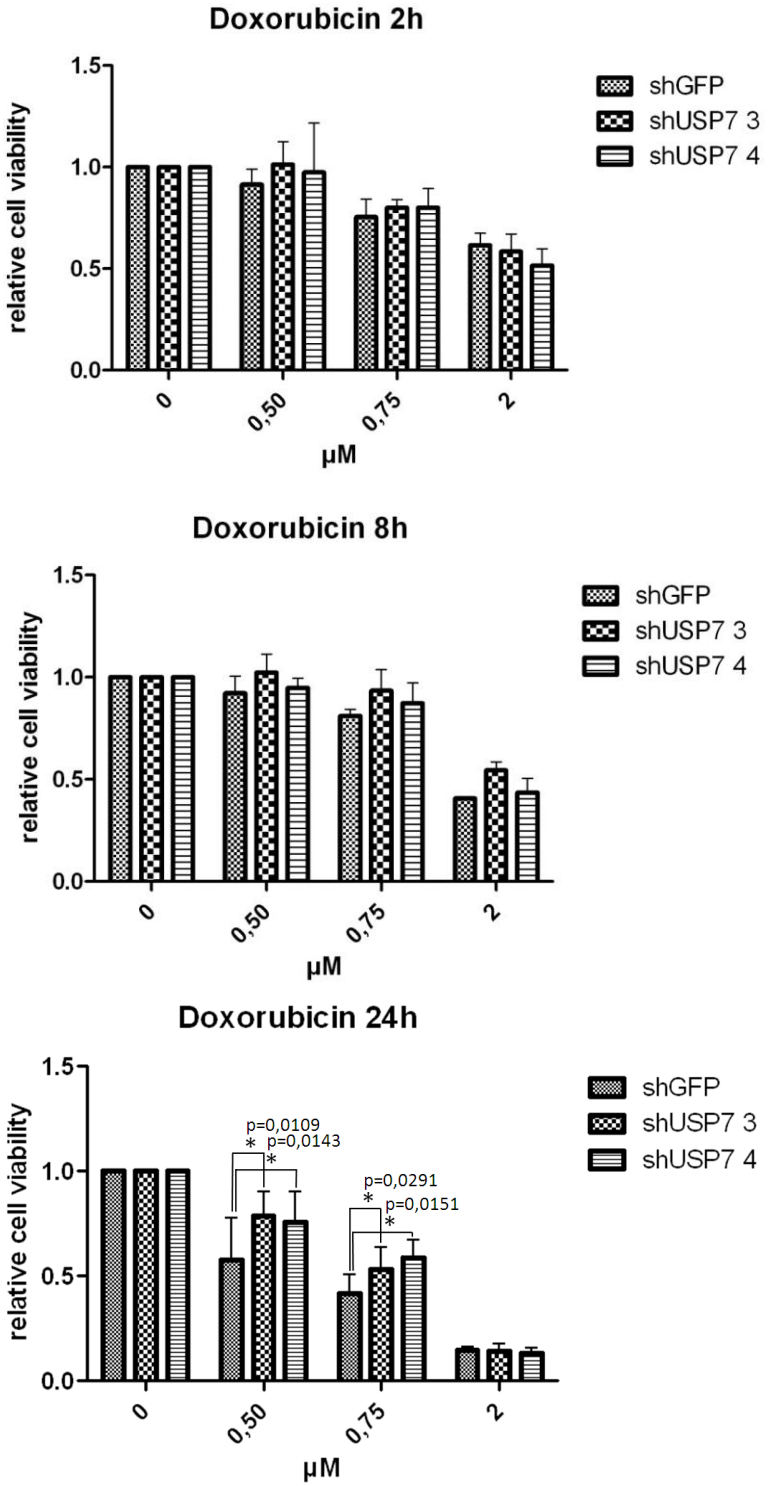


Fig. 23: Doxorubicin treatment of U2OS shGFP, shUSP7 3, shUSP7 4 for 2, 8 or 24 hours. 0,5 * 10^4 cells/well of each cell line were seeded in a 96 well plate and treated with doxorubicin for the indicated time points. Then cell viability was determined through measuring ATP levels using Cell Titer Glo reagent. Data were analyzed from sixplexs of two independent experiments using student's t test, *p<0,05.

In summary, this project identified USP7 as a new substrate of the tyrosine kinase Abl1 and leads to the formation of the following model in response to DNA damage (Fig.24). Nuclear Abl1 was activated after genotoxic stress and phosphorylated USP7. This event was dependent on the kinase activity of Abl1, as the absence of Abl1's catalytic activity prevented the phosphorylation of USP7. This post-translational modification did not regulate the stabilization or degradation of USP7 after genotoxic stress. Therefore it is possible to speculate that the phosphorylation event is regulating the activity of the deubiquitinase although data supporting this hypothesis are still missing. It is known from the literature that USP7 is deubiquitinating p53 leading to upregulation and stabilization of p53 levels which leads to apoptosis (Li et al., 2002). Indeed, when USP7 was phosphorylated by Abl1, we observed an increase in p53 levels and at its phosphorylation state (Fig. 24). Furthermore the absence of USP7 increased cell viability after genotoxic stress, probably due to a decreased induction of p53-mediated apoptosis.

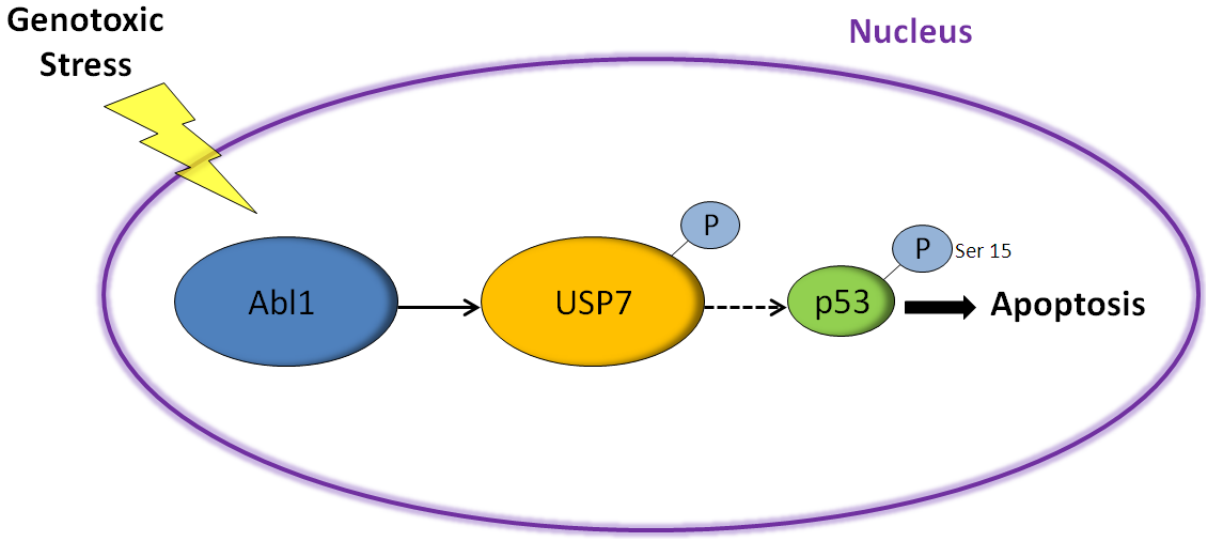


Fig. 24: Proposed model for USP7 phosphorylation by Abl1 after genotoxic stress. Nuclear Abl1 gets activated after genotoxic stress and phosphorylates USP7. This event leads to the increase of activated p53 that promotes apoptosis.

3. Discussion

Various cellular signaling networks, each composed by hundreds of proteins, are essential for correct functioning of a cell. Disruption of one or more network components can have severe consequences. Abl1 plays a role in several cellular pathways depending on its subcellular localization and on the proteins it is interacting with. To further evaluate the networks Abl1 is participating to, an affinity purification coupled mass spectrometry approach using Abl1 as bait was used. This method represents a straightforward way to reveal new interactors of the kinase, as numerous proteins were described to interact with Abl1. Several proteins, already known to be associated with Abl1, were identified by this system, confirming the specificity of the approach. In addition, the deubiquitinating enzyme USP7 was identified as a new interactor of Abl1 exclusively in the nucleus of mammalian cells.

After validation of the interaction by immunoprecipitation, overexpression experiments conducted in Hek293 cells showed that USP7 is phosphorylated in an Abl1-dependent manner. Overexpression of Abl1 led to the phosphorylation of USP7 in the nucleus. A candidate residue for phosphorylation is Y243 of USP7, as predicted by the bioinformatic tool Scansite. The mutation of this specific USP7 tyrosine residue should therefore impair the Abl1 mediated phosphorylation, a hypothesis which has to be confirmed by experimental approaches.

Nuclear Abl1 is shown to be an important player in response to DNA damage. Treatment of mammalian cells with doxorubicin provokes the formation of DNA double strand breaks and induces the activation of DNA damage signal responses. The first cellular reaction is driven by the serine/threonine kinase ATM, the sensor and key player of the DNA damage signaling. This protein orchestrates the cellular response together with its main cellular effector, the transcription factor p53. In the presence of persistent DNA damage, other important players like Abl1 are activated and modulate the cellular response. Particularly, in the presence of severe genotoxic stress, such as high concentrations of doxorubicin, Abl1 is activated and phosphorylates different targets, such as p73 (Yuan et al., 1999), triggering cell death. Among these proteins, also USP7 was phosphorylated by nuclear Abl1 in response to doxorubicin. As the

phosphorylation event on USP7 was neither regulating the stability nor degradation of the protein, it can be speculated that the post-translational modification might change the activity of USP7. Indeed, the main substrate of USP7, the transcription factor p53, was present at higher levels after genotoxic stress in cells expressing an active nuclear form of Abl1 and consequently higher levels of tyrosine phosphorylated USP7. This event also correlated with the induction of cell death observed in Abl1 proficient cells, while in the absence of Abl1 kinase activity, cells were more resistant to cell death triggered by doxorubicin treatment. In addition, cells shRNA-mediated silencing of USP7 resulted in a decreased rate of cell death after genotoxic stress, in line to the postulated model.

By identifying USP7 as a potential new substrate of the tyrosine kinase Abl1, a new link between these two proteins involved in the DNA damage response was provided. The Abl1-USP7-p53 pathway was therefore identified as an additional way leading to cell death in response to severe DNA damage.

USP7 was shown to be important for cell cycle regulation and the regulation of oncogenes and tumor suppressors (Reverdy et al., 2012). Therefore the question arises how the different cellular functions of USP7 are determined. The diverse functions of USP7 wild-type protein versus the mutant forms can be dependent on different subcellular localizations of the protein or on different post-translational modifications, such as phosphorylation events. It is known that in response to DNA damage, the deubiquitinating acitivity of USP7 is regulated through phosphorylation events. In particular, the kinase ATM phosphorylates USP7 on serine residues, which triggers the activation of the protein as well as its nuclear localization (Meulmeester et al., 2005b). Therefore we hypothesize that the tyrosine phosphorylation of USP7, as observed in our experiments, is also regulating the activity of the protein. In the presence of severe or persistent DNA damage, this modification could stabilize the nuclear form of USP7 and reinforce its activation state, thereby irreversibly committing cells to death. Consequently, it could be interesting to determine the levels and activity of a USP7 Y243 mutant in response to DNA damage. This could help to clarify the mechanistic role of the phosphorylation on USP7 mediated by Abl1 in response to genotoxic stress.

As p53 is deubiquitinated by USP7 and stabilized after DNA damage (Li et al., 2002) it would also be interesting to investigate how the mutant USP7 form influences this event. Hence, the levels of p53 and the consequent cell death in response to DNA damage should be checked in the presence of a non-phosphorylatable USP7 Y243 mutant. This would elucidate the signaling pathway connecting Abl1 with p53 in response to DNA damage that is still under debate.

Additionally, it was shown that USP7 itself plays a role in disease by regulating pathways important in tumorigenesis. Studies reported that USP7 activity can be associated with aggressiveness of the tumor in bladder or colon cancer (Hussain et al., 2009; Song et al., 2008). Therefore it would be interesting to look for mutations in the USP7 gene of patients suffering from diseases with aberrant USP7 function. They might display a mutation in the tyrosine 243 that is therefore affecting the activity of the protein. Based on this model, the absence of Abl1-mediated phosphorylation of USP7 could correlate with a lower rate of apoptosis and consequently the high aggressiveness of the tumor.

Moreover, these findings might also help to further understand the role of Abl1 and USP7 in diseases. The pro-apoptotic functions of Abl1 are reported to play an important role in neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Schlatterer et al., 2011a). The expression of active Abl1 in neurons leads to severe neurodegeneration. Consequently, an increased tyrosine phosphorylation of proteins was observed which contributes to the progress of the neuronal loss (Schlatterer et al., 2011b). In addition, treatments with tyrosine kinase inhibitors such as imatinib or nilotinib increased the neuronal survival, confirming the importance of the fact that Abl1 kinase activity could trigger cell death (Schlatterer et al., 2011b). Thus, it would be interesting to test if the phosphorylation of USP7 contributes to Abl1 dependent neuronal loss observed in these neurodegenerative diseases.

As reported in this project, USP7 is phosphorylated by the active form of nuclear Abl1 and thereby contributing to execute apoptotic signals in particular by stabilizing p53 levels. Consequently, USP7 could play a central role in the process of neuronal loss caused by Abl1 activation. At the end of the last year, new molecules inhibiting USP7 activity were synthesized (Reverdy et al., 2012). Therefore, these drugs could be tested in patients affected by neurodegenerative diseases. The combinatorial use of

two drugs affecting the same pathway might lead to a higher specificity and potency of currently available treatments.

In summary, our work identified an Abl1-mediated phosphorylation of USP7 in response to genotoxic stress. This event was shown to be important for triggering p53-dependent cell death, although more experiments are required to definitely address this cellular response. Furthermore, these findings provide a new possible therapy target in diseases characterized by altered levels of apoptosis due to deregulated Abl1 or USP7 activities. In future, this might help to improve therapy outcome of affected patients by developing new synergistic treatments.

4. Material and Methods

4.1 DNA Cloning and Amplification

cDNA constructs were cloned into mammalian vectors using the Gateway technology (Invitrogen). The obtained DNA was then transformed by heat-shock in *E. coli* (DH5α) (Invitrogen). The bacteria were left on ice for 20 minutes together with the DNA, then the heat-shock was performed for 45 seconds at 42°C followed by 2 minutes back on ice. Then the bacteria were put in SOC (Super Optimal broth with Catabolite repression) nutrient enriched media for 1h at 37°C to recover. Subsequently, the bacteria were plated on LB (Lysogeny broth) agar plates containing selective antibiotics and were grown over night at 37°C. Then the bacterial colonies were inoculated over night shaking at 37°C in LB media containing the selective antibiotics. The DNA was extracted by Mini or Maxi DNA Preparations (Qiagen) according to the manufacturer's instructions. The DNA concentrations were measured using Nanodrop (Peqlab) and sequenced at Microsynth (Switzerland) to confirm the cloning of the correct cDNA sequence.

4.2 Cell Culture

HEK293 (Human Embryonic Kidney 293 cells, DSMZ), HCT-116 (Human Colorecatal Carcinoma cells) and U2OS (Human Bone Osteosarcoma cells) cells expressing different shRNAs were cultured in DMEM (Dulbecco's Modified Eagle's Medium) containing high-glucose (PAA), Penicillin, Streptomycin (PAA) and 10% heat-inactivated fetal calf serum (Gibco) at 37°C with 5% CO_2. Cells were washed with PBS (PAA) and detached using 1x Trypsin-EDTA (PAA). Fibroblasts DKO (double-knock out) Abl/Arg were cultivated in DMEM containing 15%FCS.

Cells were frozen in freezing medium containing FCS + 10% DMSO (Merck) in Cryotubes (Nunc).

For cells with an inducible shRNA the expression of the shRNA was induced using 1µg/ml Doxycyclin for 24h (SIGMA-Aldrich).

4.3 Transient Transfection

Transfection Using the Transfection Reagent Polyfect

Cells were grown until 70% confluence and transfected using Polyfect (Qiagen). The reagent ensures the uptake of DNA by assembling it into compact structures which are binding to the cell surface and uptaken by endocytosis. For transfection of a 10cm plate the DNA (1-6µg) was added to 300µl DMEM (Dulbecco's Modified Eagle's Medium) containing 80µl Polyfect. Then the solution was vortexed for 10 seconds and incubated for 10 minutes at room temperature. This solution was then added dropwise on cells in the presence of fresh DMEM medium without antibiotics. Cells were incubated for 48 hours before harvesting.

Transfection with Calcium Phosphate

Solutions:

Mix A: 0,5 M $CaCl_2$ (SIGMA-Aldrich), DNA, deionized water (Gibco)

Mix B: 50 mM HEPES dissolved together with 280 mM NaCl (Merck) pH 7,1

70 mM Na_2HPO_4 (SIGMA-Aldrich) pH 7,1

One day before transfection, HEK293 cells were seeded to 50-60% confluence in 10cm culture plates. All solutions were brought to room temperature before start. For Mix A the DNA for transfection was added to 500µl water and 500µl 0,5M $CaCl_2$. Then 4,9ml of HEPES-NaCl solution was complemented with 100µl Na_2HPO_4 (=Mix B). Using a 2ml pipette, air was blown in the falcon where mix A was present and dropwise 1ml of Mix B was added. Then the solution was distributed drop by drop on the 10cm culture dish. The next day, the medium was changed and cells were harvested 48 hours after the medium change. For transfection of cells in a 6 well plate the procedure was the same but half of the volumes for the solutions were used.

4.4 Retroviral Infection of Fibroblasts DKO Abl/Arg

Hek-platE cells stably expressing gag, pol and env genes were grown in 10cm culture plates for 24 hours before transfection with 8µg of different pMSCV cDNA constructs using 60µl Polyfect (Qiagen) in a final volume of 6ml. On the same day the target cells fibroblasts DKO Abl/Arg were seeded at a concentration of 0,5 * 10^5 cells/ml in a 6 well plate.

The day after, 6ml of viruses produced from HEK-platE were centrifuged at 1300rpm for 5 minutes and 5ml supernatant was added to fibroblasts DKO Abl/Arg. Furthermore 2µl of the adjuvant Polybrene (SIGMA-Aldrich) was added and a final concentration of 15%FCS in the medium was reached by adding FCS. Fresh 10%FCS-DMEM was given to Hek-platE and after 24 hours of incubation the whole procedure was repeated. After these two rounds of infection fibroblasts DKO Abl/Arg were supplemented with 2ml fresh 15%FCS-DMEM. The used pMSCV constructs carried a Puromycin resistance gene therefore selection was done using Puromycin (5µg/ml) for 48h.

4.5 Drug Screening

For drug screening fibroblasts DKO c-Abl/Arg were seeded in triplicates at a concentration of 0,8 * 10^4 cells/well in a 96 well plate. After 24 hours of incubation the treatment with different drugs was performed.

The following drugs (all SIGMA-Aldrich) and respective concentrations were used:

- Doxorubicin: 0,05µM; 0,1µM; 0,5µM; 1µM, 2µM, 5µM, 10µM, 15µM, 20µM
- Etoposide: 0,05µM; 0,1µM; 0,5µM; 1µM, 2µM, 5µM, 10µM, 15µM, 20µM
- Cisplatinum: 0,05µM; 0,1µM; 0,5µM; 1µM, 2µM, 5µM, 10µM, 15µM, 20µM
- Hydroxyurea: 1mM, 2mM, 3mM, 4mM, 5mM, 8mM, 10mM, 15mM, 20mM

The drugs were dissolved in DMSO, therefore an equal amount of it was used in non-treated cells to compensate eventual toxic effect due to the solvent. The treatments were performed for 72h and cell viability was determined using CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the instructions of the manufacturer. The reagent allows determination of viable cells present in each well by generating a luminescent signal proportional to ATP-levels. Generation of ATP indicates the presence of active metabolic cells. Luminescence was measured using SpectraMax M5 (Molecular Devices).

Treatment of Cells

Treatment was performed using HEK293, U2OS or HCT-116 cells in 10 cm dishes or in a 6 well plate. Cells were treated using doxorubicin (SIGMA-Aldrich) at concentrations of 0,5µM or 2µM for different timepoints. Cells without doxorubicin treatment received DMSO (Merck) for the same time to compensate eventual toxic effect due to the solvent. Abl1 kinase activity was inhibited adding 100nM nilotinib one hour before doxorubicin treatment.

4.6 Cell Lysis and Protein Extraction

IP-buffer:

50mM Tris (SIGMA-Aldrich)-HCl (Merck) (ph 7,5), 150mM NaCl (Merck), 5mM EDTA (Fluka), 5mM EGTA (Fluka), 1% NP-40 (Calbiochem)

Following inhibitors were added freshly: 1mM Na_3VO_4 (SIGMA-Aldrich), Inhibitor Cocktail (Roche), 50mM NaF (SIGMA-Aldrich), 0,1mM PMSF (Phenylmethanesulfonyl fluoride, SIGMA-Aldrich), 0,005mg/ml TPCK (Tosyl phenylalanyl chloromethyl ketone, Sanova Pharma)

Laemmli Sample Buffer(4×):

0,2M Tris-HCl pH 6,8; 40% glycerol (Merck), 8% SDS (Fisher Scientific), 0,004% bromphenol blue (Sigma) in deionized water (MilliQ, Millipore), before usage 10% of β-Mercaptoethanol (SIGMA-Aldrich) was added

After the harvest, cells were washed once with 1x PBS and lysed in Laemmli buffer or IP-buffer.

Cell lysis using Laemmli buffer: After addition of Laemmli buffer, cells were immediately scraped off the plate using a rubber scraper. Then the lysate was boiled for 10 minutes and centrifuged for 10 minutes at 14000rpm. The supernatant fraction, containing the soluble proteins, was collected and analyzed by SDS-PAGE.

Protein extraction using IP-buffer: For protein extraction with IP-buffer all steps were performed on ice. After the addition of IP-buffer, cells were scraped off the plate with a rubber scraper and collected in a chilled Eppendorf tube. The lysate was then incubated on ice for 10 minutes followed by centrifugation at 14000rpm at 4°C for 10 minutes. The supernatant fraction was taken and its protein concentration was determined using Bradford reagent (Bio-Rad) and Ultrospec2100pro (Amersham-Biosciences).

Nuclear and Cytoplasmic Protein Extraction

Buffer N:

300mM sucrose (Merck), 10mM HEPES pH 7,9, 10mM KCl (SIGMA-Aldrich), 0,1mM EDTA (Fluka), 0,1mM EGTA (Fluka), 1mM DTT (DL-Dithiothreitol, SIGMA-Aldrich), 0,75mM spermidine (SIGMA-Aldrich), 0,15mM spermine (SIGMA-Aldrich), 0,1% (w/v) NP-40 substitute (Calbiochem), 10% NaF (SIGMA-Aldrich), 0,1% Na_3VO_4 (SIGMA-Aldrich), 0,1% PMSF (SIGMA-Aldrich), 0,1% TPCK (Sanova Pharma), 0,1% Inhibitor cocktail (Roche), H_2O (Gibco)

Buffer C:

20mM HEPES pH 7,9, 420mM NaCl (Merck), 25% glycerol (Merck), 1mM EDTA (Fluka), 1mM EGTA (Fluka), 1mM DTT (SIGMA-Aldrich), 1M NaF (SIGMA-Aldrich), 0,1% Na_3VO_4 (SIGMA-Aldrich), 0,1% PMSF (SIGMA-Aldrich), 0,1% TPCK (Sanova Pharma), 0,1% Inhibitor cocktail (Roche), H_2O (Gibco)

For the separation of the cytoplasmic fraction, cells were first harvested, washed once with PBS and then collected in a falcon tube. Cells were then centrifuged for 5 minutes at 600g and the cell pellet was resuspended in more than 3 volumes of buffer N and vortexed briefly. The solution was then incubated for 5 minutes on ice and then centrifuged for 5 minutes at 500g at 4°C. The supernatant fraction containing the cytoplasmic proteins was collect in a chilled Eppendorf tube. The pellet was washed once in buffer N and resuspended in 3 volumes of buffer C to extract nuclear proteins. The solution was shaken for 15 minutes in a thermo mixer at 4°C. Afterwards, the sample was centrifuged for 10 minutes at 14000rpm at 4°C and the supernatant fraction containing the nuclear proteins was collected in a chilled Eppendorf tube. Protein concentration was determined using Bradford reagent (Bio-Rad) and Ultrospec2100pro (Amersham-Biosciences).

4.7 SDS-PAGE and Western Blot

Solutions

- Gel Buffer 1 (4x) for running gel: 1,5M Tris (SIGMA-Aldrich)-HCl (Merck) pH 8,8; 10% SDS (Fisher Scientific) in deionized water (MilliQ, Millipore)
- Gel Buffer 2 (4x) for stacking gel: 0,5M Tris-HCl pH 6,8; 10% SDS
- SDS Running Buffer (5×): 250mM Tris; 1,9M Glycin (Merck), 35mM SDS
- 10% APS: 10% APS (Ammoniumperoxodisulfate, Merck) in deionized water
- Laemmli Sample Buffer(4×): 0,2M Tris-HCl pH 6,8; 40% glycerol (Merck), 8% SDS, bromphenol blue (SIGMA-Aldrich)
- Western Blot Buffer (1x): 2mM Tris-HCl pH 8,3; 96mM glycine, 20% methanol (SIGMA-Aldrich), before use 10% β-mercaptoethanol (SIGMA-Aldrich) added
- PBST: 10% 10x PBS (PAA), 0,1% TWEEN-20 (SIGMA-Aldrich)
- 5% milk/PBST: 5% Blotting-Grade Blocker nonfat dry milk (Bio-Rad) diluted in PBST

SDS-Polyacrylamide Gel

SDS-Polyacrylamide gels were prepared using Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad). Running gels with different percentages of polyacrylamide were prepared according to the following table.
Dilution table for preparation of one running gel (5 ml):

% Acrylamide	7%	10%	15%
Gel buffer 1	1,25ml	1,25ml	1,25ml
30% Acrylamide/Bis (Bio-Rad)	1,17ml	1,67ml	2,50ml
Water	2,56ml	2,09ml	1,25ml

Then 50µl 10% APS solution and 5µlTEMED per 5ml gel mixture was added to initiate the acrylamide polymerization. Stacking gel was prepared using 2,5ml gel buffer 2 and 25µl 10%APS and 3,8µl TEMED. The gel was inserted into a running-chamber (Bio-Rad) filled with 1xSDS-Running buffer. Samples were prepared by adding 4xLaemmli buffer before loading. PageRuler Prestained Protein Ladder (Fermentas)

was used as a marker. Electrophoresis was performed at 120V for one hour and 20 minutes (Bio-Rad).

Semi Dry Transfer and Immunoblot

After SDS-PAGE, proteins were transferred from the polyacrylamide gel to a nitrocellulose transfer membrane (A. Hartenstein) using a semi dry transfer system. Blotting was performed using 1mA per square centimeter of the membrane for 1 hour (15% gel), 1,15 hour (10% gel) or 1,5 hour (7% gel) using a Hoefer Semi-Phor semi-dry transfer unit. After the transfer a Ponceau-Staining (Serva) was done to ensure that the transfer worked correctly.

Membranes were blocked using 5% milk/PBST for 30 minutes. Afterwards membranes were incubated with specific primary antibodies for 2 hours at room temperature or over night at 4°C. Incubation with secondary antibody was done for one hour at room temperature.

Membranes were developed using Li-Cor (Odyssey) for fluorescently labeled secondary antibodies. Alternatively the membranes were incubated with HRP (horseradish peroxidase)-conjugated secondary antibodies and developed with ECL Western Blotting Detection Reagents (GE Healthcare) according to the protocol of the manufacturer.

Antibodies

The following primary antibodies were used: c-Abl 21-63, P-Tyrosine (both home-made), Mdm2, p53, Rcc1 (Santa Cruz), HA-11 (Covance), p53 P-Ser15, Abl1 P-Y412, USP7 (Cell Signalling), Tubulin (Abcam), Myc (IRdye 800, Rockland)

Following secondary antibodies were used: Rabbit IgG, Mouse IgG (Alexa Fluor 680, Molecular probes), Rabbit IgG, Mouse IgG, Goat IgG, HA-7 (HRP conjugated, Jackson ImmonResearch)

4.8 Immuno-Precipitation

Depending on the targeted bait-protein either HA-beads (Sigma) or protein G sepharose beads (GE Healthcare) were used for the IP (Immuno-Precipitation). Beads were washed three times with 1 ml IP-buffer and resuspended 1:1 in IP-buffer.
Two milligrams of protein extracts were then pre-cleared using 30µl slurry beads to a final volume of 500µl per sample in IP-buffer. Samples were incubated for one hour rotating on a wheel at 4° C.
After centrifugation at 2000rpm for 3 minutes at 4°C the cleared supernatant was transferred into a new tube. 3µl of antibody was added to each sample and incubated on a rotating wheel for 2 hours at 4° C. Afterwards the solutions were transferred in new Eppendorf tubes containing 30µl protein G sepharose beads and incubated on a rotating wheel at 4°C for an additional hour.
Afterwards samples were centrifuged at 2000rpm for 2 minutes at 4°C and the beads with the bound materials were washed three times with 400µl IP-buffer. After carefully removing all liquid, the beads were resuspended in 20µl Laemmli buffer.
For the HA-IP, beads directly conjugated to the HA-7 antibody clone (SIGMA) were used. In this case, the cleared supernatants were directly incubated on a rotating wheel for 2h at 4°C. Then the beads were washed three times in IP-buffer and resuspended in Laemmli buffer, as described above.

5. References

Abelson, H.T., and Rabstein, L.S. (1970). Lymphosarcoma: virus-induced thymic-independent disease in mice. Cancer research *30*, 2213-2222.

Andoniou, C.E., Thien, C.B., and Langdon, W.Y. (1994). Tumour induction by activated abl involves tyrosine phosphorylation of the product of the cbl oncogene. The EMBO journal *13*, 4515-4523.
Arora, A., and Scholar, E.M. (2005). Role of tyrosine kinase inhibitors in cancer therapy. J Pharmacol Exp Ther *315*, 971-979.

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C.W., Chessa, L., Smorodinsky, N.I., Prives, C., Reiss, Y., Shiloh, Y.*, et al.* (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science *281*, 1674-1677.

Baskaran, R., Wood, L.D., Whitaker, L.L., Canman, C.E., Morgan, S.E., Xu, Y., Barlow, C., Baltimore, D., Wynshaw-Boris, A., Kastan, M.B.*, et al.* (1997). Ataxia telangiectasia mutant protein activates c-Abl tyrosine kinase in response to ionizing radiation. Nature *387*, 516-519.

Becker, K., Marchenko, N.D., Palacios, G., and Moll, U.M. (2008). A role of HAUSP in tumor suppression in a human colon carcinoma xenograft model. Cell Cycle *7*, 1205-1213.

Berg, J.M., Tymoczko, J.L., and Stryer, L. (2002). Biochemistry, 5th edn (New York: W.H. Freeman).

Bhat, A., Kolibaba, K., Oda, T., Ohno-Jones, S., Heaney, C., and Druker, B.J. (1997). Interactions of CBL with BCR-ABL and CRKL in BCR-ABL-transformed myeloid cells. The Journal of biological chemistry *272*, 16170-16175.

Bhoj, V.G., and Chen, Z.J. (2009). Ubiquitylation in innate and adaptive immunity. Nature *458*, 430-437.

Brasher, B.B., and Van Etten, R.A. (2000). c-Abl has high intrinsic tyrosine kinase activity that is stimulated by mutation of the Src homology 3 domain and by autophosphorylation at two distinct regulatory tyrosines. J Biol Chem *275*, 35631-35637.

Cao, C., Li, Y., Leng, Y., Li, P., Ma, Q., and Kufe, D. (2005). Ubiquitination and degradation of the Arg tyrosine kinase is regulated by oxidative stress. Oncogene *24*, 2433-2440.

Chauhan, D., Tian, Z., Nicholson, B., Kumar, K.G., Zhou, B., Carrasco, R., McDermott, J.L., Leach, C.A., Fulcinniti, M., Kodrasov, M.P.*, et al.* (2012). A small molecule inhibitor of ubiquitin-specific protease-7 induces apoptosis in multiple myeloma cells and overcomes bortezomib resistance. Cancer Cell *22*, 345-358.

Cohn, M.A., Kee, Y., Haas, W., Gygi, S.P., and D'Andrea, A.D. (2009). UAF1 Is a Subunit of Multiple Deubiquitinating Enzyme Complexes. Journal of Biological Chemistry *284*, 5343-5351.

Converso, A., Hartingh, T., Garbaccio, R.M., Tasber, E., Rickert, K., Fraley, M.E., Yan, Y., Kreatsoulas, C., Stirdivant, S., Drakas, B.*, et al.* (2009). Development of thioquinazolinones, allosteric Chk1 kinase inhibitors. Bioorganic & medicinal chemistry letters *19*, 1240-1244.

Cummins, J.M., Rago, C., Kohli, M., Kinzler, K.W., Lengauer, C., and Vogelstein, B. (2004). Tumour suppression: disruption of HAUSP gene stabilizes p53. Nature *428*, 1 p following 486.

Dai, Z., and Pendergast, A.M. (1995). Abi-2, a novel SH3-containing protein interacts with the c-Abl tyrosine kinase and modulates c-Abl transforming activity. Gene Dev *9*, 2569-2582.

Daley, G.Q., Van Etten, R.A., and Baltimore, D. (1990). Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. Science *247*, 824-830.

Deininger, M., Buchdunger, E., and Druker, B.J. (2005). The development of imatinib as a therapeutic agent for chronic myeloid leukemia. Blood *105*, 2640-2653.

Echarri, A., and Pendergast, A.M. (2001). Activated c-Abl is degraded by the ubiquitin-dependent proteasome pathway. Curr Biol *11*, 1759-1765.

Everett, R.D., Meredith, M., Orr, A., Cross, A., Kathoria, M., and Parkinson, J. (1997). A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. Embo Journal *16*, 1519-1530.

Faesen, A.C., Dirac, A.M., Shanmugham, A., Ovaa, H., Perrakis, A., and Sixma, T.K. (2011). Mechanism of USP7/HAUSP activation by its C-terminal ubiquitin-like domain and allosteric regulation by GMP-synthetase. Mol Cell *44*, 147-159.

Finley, D., Ciechanover, A., and Varshavsky, A. (2004). Ubiquitin as a central cellular regulator. Cell *116*, S29-32, 22 p following S32.

Glatter, T., Wepf, A., Aebersold, R., and Gstaiger, M. (2009). An integrated workflow for charting the human interaction proteome: insights into the PP2A system. Molecular systems biology *5*, 237.

Goga, A., Liu, X., Hambuch, T.M., Senechal, K., Major, E., Berk, A.J., Witte, O.N., and Sawyers, C.L. (1995). p53 dependent growth suppression by the c-Abl nuclear tyrosine kinase. Oncogene *11*, 791-799.

Goldberg, Z., Vogt Sionov, R., Berger, M., Zwang, Y., Perets, R., Van Etten, R.A., Oren, M., Taya, Y., and Haupt, Y. (2002). Tyrosine phosphorylation of Mdm2 by c-Abl: implications for p53 regulation. EMBO J *21*, 3715-3727.

Gong, J.G., Costanzo, A., Yang, H.Q., Melino, G., Kaelin, W.G., Jr., Levrero, M., and Wang, J.Y. (1999). The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. Nature *399*, 806-809.

Gorre, M.E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P.N., and Sawyers, C.L. (2001). Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science *293*, 876-880.

Grebien, F., Hantschel, O., Wojcik, J., Kaupe, I., Kovacic, B., Wyrzucki, A.M., Gish, G.D., Cerny-Reiterer, S., Koide, A., Beug, H.*, et al.* (2011). Targeting the SH2-kinase interface in Bcr-Abl inhibits leukemogenesis. Cell *147*, 306-319.

Hantschel, O., Nagar, B., Guettler, S., Kretzschmar, J., Dorey, K., Kuriyan, J., and Superti-Furga, G. (2003). A myristoyl/phosphotyrosine switch regulates c-Abl. Cell *112*, 845-857.

Hantschel, O., and Superti-Furga, G. (2004). Regulation of the c-Abl and Bcr-Abl tyrosine kinases. Nat Rev Mol Cell Biol *5*, 33-44.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu Rev Biochem *67*, 425-479.
Hicke, L. (2001). Protein regulation by monoubiquitin. Nature reviews Molecular cell biology *2*, 195-201.

Honda, R., and Yasuda, H. (2000). Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase. Oncogene *19*, 1473-1476.

Hortobagyi, G.N., Holmes, F.A., Theriault, R.L., Rahman, Z., and Buzdar, A.U. (1997). Combination chemotherapy with paclitaxel and doxorubicin for metastatic breast cancer. Semin Oncol *24*, S11-13-S11-19.

Hu, M., Li, P., Li, M., Li, W., Yao, T., Wu, J.W., Gu, W., Cohen, R.E., and Shi, Y. (2002). Crystal structure of a UBP-family deubiquitinating enzyme in isolation and in complex with ubiquitin aldehyde. Cell *111*, 1041-1054.

Hu, M., Li, P., Song, L., Jeffrey, P.D., Chenova, T.A., Wilkinson, K.D., Cohen, R.E., and Shi, Y. (2005). Structure and mechanisms of the proteasome-associated deubiquitinating enzyme USP14. The EMBO journal *24*, 3747-3756.

Hubbard, S.R., and Miller, W.T. (2007). Receptor tyrosine kinases: mechanisms of activation and signaling. Curr Opin Cell Biol *19*, 117-123.

Hunter, T. (2007). The age of crosstalk: phosphorylation, ubiquitination, and beyond. Mol Cell *28*, 730-738.

Hurley, J.H., Lee, S., and Prag, G. (2006). Ubiquitin-binding domains. Biochem J *399*, 361-372.

Husnjak, K., and Dikic, I. (2012). Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. Annual review of biochemistry *81*, 291-322.

Hussain, S., Zhang, Y., and Galardy, P.J. (2009). DUBs and cancer The role of deubiquitinating enzymes as oncogenes, non-oncogenes and tumor suppressors. Cell Cycle *8*, 1688-1697.

Ito, Y., Pandey, P., Mishra, N., Kumar, S., Narula, N., Kharbanda, S., Saxena, S., and Kufe, D. (2001). Targeting of the c-Abl tyrosine kinase to mitochondria in endoplasmic reticulum stress-induced apoptosis. Mol Cell Biol *21*, 6233-6242.

Jabbour, E., Cortes, J., Giles, F., O'Brien, S., and Kantarijan, H. (2007). Drug evaluation: Nilotinib - a novel Bcr-Abl tyrosine kinase inhibitor for the treatment of chronic myelocytic leukemia and beyond. IDrugs *10*, 468-479.

Janssens, S., and Tschopp, J. (2006). Signals from within: the DNA-damage-induced NF-kappaB response. Cell Death Differ *13*, 773-784.

Kharbanda, S., Ren, R., Pandey, P., Shafman, T.D., Feller, S.M., Weichselbaum, R.R., and Kufe, D.W. (1995). Activation of the c-Abl tyrosine kinase in the stress response to DNA-damaging agents. Nature *376*, 785-788.

Khoronenkova, S.V., Dianova, II, Ternette, N., Kessler, B.M., Parsons, J.L., and Dianov, G.L. (2012). ATM-dependent downregulation of USP7/HAUSP by PPM1G activates p53 response to DNA damage. Mol Cell *45*, 801-813.

Kirisako, T., Kamei, K., Murata, S., Kato, M., Fukumoto, H., Kanie, M., Sano, S., Tokunaga, F., Tanaka, K., and Iwai, K. (2006). A ubiquitin ligase complex assembles linear polyubiquitin chains. EMBO J *25*, 4877-4887.

Komander, D., and Barford, D. (2008). Structure of the A20 OTU domain and mechanistic insights into deubiquitination. Biochemical Journal *409*, 77-85.

Komander, D., Clague, M.J., and Urbe, S. (2009). Breaking the chains: structure and function of the deubiquitinases. Nat Rev Mol Cell Biol *10*, 550-563.

Komander, D., Lord, C.J., Scheel, H., Swift, S., Hofmann, K., Ashworth, A., and Barford, D. (2008). The structure of the CYLD USP domain explains its specificity for Lys63-linked polyubiquitin and reveals a B box module. Molecular cell *29*, 451-464.

Konopka, J.B., Watanabe, S.M., and Witte, O.N. (1984). An alteration of the human c-abl protein in K562 leukemia cells unmasks associated tyrosine kinase activity. Cell *37*, 1035-1042.

Krause, D.S., and Van Etten, R.A. (2005). Tyrosine kinases as targets for cancer therapy. N Engl J Med *353*, 172-187.

Kubbutat, M.H., Jones, S.N., and Vousden, K.H. (1997). Regulation of p53 stability by Mdm2. Nature *387*, 299-303.

Kurz, E.U., Douglas, P., and Lees-Miller, S.P. (2004). Doxorubicin activates ATM-dependent phosphorylation of multiple downstream targets in part through the generation of reactive oxygen species. The Journal of biological chemistry *279*, 53272-53281.

Lam, Y.A., Xu, W., DeMartino, G.N., and Cohen, R.E. (1997). Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. Nature *385*, 737-740.

Lehninger, A.L., Nelson, D.L., and Cox, M.M. (2008). Lehninger principles of biochemistry, 5th edn (New York: W.H. Freeman).

Li, M., Brooks, C.L., Wu-Baer, F., Chen, D., Baer, R., and Gu, W. (2003). Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. Science *302*, 1972-1975.

Li, M., Chen, D., Shiloh, A., Luo, J., Nikolaev, A.Y., Qin, J., and Gu, W. (2002). Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature *416*, 648-653.

Lynch, T.J., Bell, D.W., Sordella, R., Gurubhagavatula, S., Okimoto, R.A., Brannigan, B.W., Harris, P.L., Haserlat, S.M., Supko, J.G., Haluska, F.G.*, et al.* (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. The New England journal of medicine *350*, 2129-2139.

Maiani, E., Diederich, M., and Gonfloni, S. (2011). DNA damage response: the emerging role of c-Abl as a regulatory switch? Biochem Pharmacol *82*, 1269-1276.

Mayer, B.J., and Baltimore, D. (1994). Mutagenic analysis of the roles of SH2 and SH3 domains in regulation of the Abl tyrosine kinase. Mol Cell Biol *14*, 2883-2894.

Meresse, P., Dechaux, E., Monneret, C., and Bertounesque, E. (2004). Etoposide: discovery and medicinal chemistry. Curr Med Chem *11*, 2443-2466.

Meulmeester, E., Maurice, M.M., Boutell, C., Teunisse, A.F., Ovaa, H., Abraham, T.E., Dirks, R.W., and Jochemsen, A.G. (2005a). Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. Mol Cell *18*, 565-576.

Meulmeester, E., Pereg, Y., Shiloh, Y., and Jochemsen, A.G. (2005b). ATM-mediated phosphorylations inhibit Mdmx/Mdm2 stabilization by HAUSP in favor of p53 activation. Cell Cycle *4*, 1166-1170.

Miller, W.T. (2003). Determinants of substrate recognition in nonreceptor tyrosine kinases. Acc Chem Res *36*, 393-400.

Nagar, B., Hantschel, O., Young, M.A., Scheffzek, K., Veach, D., Bornmann, W., Clarkson, B., Superti-Furga, G., and Kuriyan, J. (2003). Structural basis for the autoinhibition of c-Abl tyrosine kinase. Cell *112*, 859-871.

Nalepa, G., Rolfe, M., and Harper, J.W. (2006). Drug discovery in the ubiquitin-proteasome system. Nat Rev Drug Discov *5*, 596-613.

Neet, K., and Hunter, T. (1996). Vertebrate non-receptor protein-tyrosine kinase families. Genes to cells : devoted to molecular & cellular mechanisms *1*, 147-169.

Newton, K., Matsumoto, M.L., Wertz, I.E., Kirkpatrick, D.S., Lill, J.R., Tan, J., Dugger, D., Gordon, N., Sidhu, S.S., Fellouse, F.A.*, et al.* (2008). Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies. Cell *134*, 668-678.

Pendergast, A.M. (2002). The Abl family kinases: mechanisms of regulation and signaling. Adv Cancer Res *85*, 51-100.

Pickart, C.M. (2001). Mechanisms underlying ubiquitination. Annu Rev Biochem *70*, 503-533.

Resh, M.D. (1994). Myristylation and palmitylation of Src family members: the fats of the matter. Cell *76*, 411-413.

Reverdy, C., Conrath, S., Lopez, R., Planquette, C., Atmanene, C., Collura, V., Harpon, J., Battaglia, V., Vivat, V., Sippl, W*., et al.* (2012). Discovery of specific inhibitors of human USP7/HAUSP deubiquitinating enzyme. Chem Biol *19*, 467-477.

Robzyk, K., Recht, J., and Osley, M.A. (2000). Rad6-dependent ubiquitination of histone H2B in yeast. Science *287*, 501-504.

Schaefer, J.B., and Morgan, D.O. (2011). Protein-linked ubiquitin chain structure restricts activity of deubiquitinating enzymes. J Biol Chem *286*, 45186-45196.

Schindler, T., Bornmann, W., Pellicena, P., Miller, W.T., Clarkson, B., and Kuriyan, J. (2000). Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. Science *289*, 1938-1942.

Schlatterer, S.D., Acker, C.M., and Davies, P. (2011a). c-Abl in neurodegenerative disease. J Mol Neurosci *45*, 445-452.

Schlatterer, S.D., Tremblay, M.A., Acker, C.M., and Davies, P. (2011b). Neuronal c-Abl overexpression leads to neuronal loss and neuroinflammation in the mouse forebrain. Journal of Alzheimer's disease : JAD *25*, 119-133.

Shafman, T., Khanna, K.K., Kedar, P., Spring, K., Kozlov, S., Yen, T., Hobson, K., Gatei, M., Zhang, N., Watters, D*., et al.* (1997). Interaction between ATM protein and c-Abl in response to DNA damage. Nature *387*, 520-523.

Shah, N.P., and Sawyers, C.L. (2003). Mechanisms of resistance to STI571 in Philadelphia chromosome-associated leukemias. Oncogene *22*, 7389-7395.

Shaul, Y. (2000). c-Abl: activation and nuclear targets. Cell Death Differ *7*, 10-16.

Sheng, Y., Saridakis, V., Sarkari, F., Duan, S., Wu, T., Arrowsmith, C.H., and Frappier, L. (2006). Molecular recognition of p53 and MDM2 by USP7/HAUSP. Nat Struct Mol Biol *13*, 285-291.

Shi, Y., Alin, K., and Goff, S.P. (1995). Abl-interactor-1, a novel SH3 protein binding to the carboxy-terminal portion of the Abl protein, suppresses v-abl transforming activity. Gene Dev *9*, 2583-2597.
Shieh, S.Y., Ikeda, M., Taya, Y., and Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell *91*, 325-334.

Shtivelman, E., Lifshitz, B., Gale, R.P., Roe, B.A., and Canaani, E. (1986). Alternative splicing of RNAs transcribed from the human abl gene and from the Bcr-Abl fused gene. Cell *47*, 277-284.

Sirvent, A., Benistant, C., and Roche, S. (2008). Cytoplasmic signalling by the c-Abl tyrosine kinase in normal and cancer cells. Biol Cell *100*, 617-631.

Smith, J.M., and Mayer, B.J. (2002). Abl: mechanisms of regulation and activation. Front Biosci *7*, d31-42.

Song, M.S., Salmena, L., Carracedo, A., Egia, A., Lo-Coco, F., Teruya-Feldstein, J., and Pandolfi, P.P. (2008). The deubiquitinylation and localization of PTEN are regulated by a HAUSP-PML network. Nature *455*, 813-817.

Songyang, Z., Carraway, K.L., 3rd, Eck, M.J., Harrison, S.C., Feldman, R.A., Mohammadi, M., Schlessinger, J., Hubbard, S.R., Smith, D.P., Eng, C*., et al.* (1995). Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. Nature *373*, 536-539.

Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J*., et al.* (1993). SH2 domains recognize specific phosphopeptide sequences. Cell *72*, 767-778.

Sun, X.G., Wu, F., Datta, R., Kharbanda, S., and Kufe, D. (2000). Interaction between protein kinase C delta and the c-Abl tyrosine kinase in the cellular response to oxidative stress. Journal of Biological Chemistry *275*, 7470-7473.

Taagepera, S., McDonald, D., Loeb, J.E., Whitaker, L.L., McElroy, A.K., Wang, J.Y., and Hope, T.J. (1998). Nuclear-cytoplasmic shuttling of C-ABL tyrosine kinase. Proc Natl Acad Sci U S A *95*, 7457-7462.

Tibbetts, R.S., Cortez, D., Brumbaugh, K.M., Scully, R., Livingston, D., Elledge, S.J., and Abraham, R.T. (2000). Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. Gene Dev *14*, 2989-3002.

Tonks, N.K., and Neel, B.G. (1996). From form to function: signaling by protein tyrosine phosphatases. Cell *87*, 365-368.

Ubersax, J.A., and Ferrell, J.E., Jr. (2007). Mechanisms of specificity in protein phosphorylation. Nat Rev Mol Cell Biol *8*, 530-541.

Van Etten, R.A., Jackson, P., and Baltimore, D. (1989). The mouse type IV c-abl gene product is a nuclear protein, and activation of transforming ability is associated with cytoplasmic localization. Cell *58*, 669-678.

Varadan, R., Assfalg, M., Haririnia, A., Raasi, S., Pickart, C., and Fushman, D. (2004). Solution conformation of Lys63-linked di-ubiquitin chain provides clues to functional diversity of polyubiquitin signaling. J Biol Chem *279*, 7055-7063.

Vlahovic, G., and Crawford, J. (2003). Activation of tyrosine kinases in cancer. Oncologist *8*, 531-538.

Vucic, D., Dixit, V.M., and Wertz, I.E. (2011). Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. Nat Rev Mol Cell Biol *12*, 439-452.

Wang, X., Zeng, L., Wang, J., Chau, J.F., Lai, K.P., Jia, D., Poonepalli, A., Hande, M.P., Liu, H., He, G*., et al.* (2011). A positive role for c-Abl in Atm and Atr activation in DNA damage response. Cell Death Differ *18*, 5-15.

Watanabe, D., Ezoe, S., Fujimoto, M., Kimura, A., Saito, Y., Nagai, H., Tachibana, I., Matsumura, I., Tanaka, T., Kanegane, H*., et al.* (2004). Suppressor of cytokine signalling-1 gene silencing in acute myeloid leukaemia and human haematopoietic cell lines. British journal of haematology *126*, 726-735.

Weisberg, E., Manley, P., Mestan, J., Cowan-Jacob, S., Ray, A., and Griffin, J.D. (2006). AMN107 (nilotinib): a novel and selective inhibitor of BCR-ABL. Br J Cancer *94*, 1765-1769.

Welch, P.J., and Wang, J.Y. (1993). A C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-Abl tyrosine kinase in the cell cycle. Cell *75*, 779-790.

Wen, S.T., and Van Etten, R.A. (1997). The PAG gene product, a stress-induced protein with antioxidant properties, is an Abl SH3-binding protein and a physiological inhibitor of c-Abl tyrosine kinase activity. Genes Dev *11*, 2456-2467.

Woodring, P.J., Litwack, E.D., O'Leary, D.D.M., Lucero, G.R., Wang, J.Y.J., and Hunter, T. (2002). Modulation of the F-actin cytoskeleton by c-Abl tyrosine kinase in cell spreading and neurite extension. Journal of Cell Biology *156*, 879-892.

Yadav, S.S., and Miller, W.T. (2008). The evolutionarily conserved arrangement of domains in SRC family kinases is important for substrate recognition. Biochemistry *47*, 10871-10880.

Yang, W.L., Zhang, X., and Lin, H.K. (2010). Emerging role of Lys-63 ubiquitination in protein kinase and phosphatase activation and cancer development. Oncogene *29*, 4493-4503.

Yoshida, K., Yamaguchi, T., Natsume, T., Kufe, D., and Miki, Y. (2005). JNK phosphorylation of 14-3-3 proteins regulates nuclear targeting of c-Abl in the apoptotic response to DNA damage. Nat Cell Biol *7*, 278-285.

Yuan, Z.M., Huang, Y., Ishiko, T., Kharbanda, S., Weichselbaum, R., and Kufe, D. (1997). Regulation of DNA damage-induced apoptosis by the c-Abl tyrosine kinase. Proc Natl Acad Sci U S A *94*, 1437-1440.
Yuan, Z.M., Huang, Y., Ishiko, T., Nakada, S., Utsugisawa, T., Kharbanda, S., Wang, R., Sung, P., Shinohara, A., Weichselbaum, R*., et al.* (1998). Regulation of Rad51 function by c-Abl in response to DNA damage. J Biol Chem *273*, 3799-3802.

Yuan, Z.M., Shioya, H., Ishiko, T., Sun, X., Gu, J., Huang, Y.Y., Lu, H., Kharbanda, S., Weichselbaum, R., and Kufe, D. (1999). p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage. Nature *399*, 814-817.

Zhang, J., Adrian, F.J., Jahnke, W., Cowan-Jacob, S.W., Li, A.G., Iacob, R.E., Sim, T., Powers, J., Dierks, C., Sun, F*., et al.* (2010). Targeting Bcr-Abl by combining allosteric with ATP-binding-site inhibitors. Nature *463*, 501-506.

Zhang, L., Zhou, F., Drabsch, Y., Gao, R., Snaar-Jagalska, B.E., Mickanin, C., Huang, H., Sheppard, K.A., Porter, J.A., Lu, C.X*., et al.* (2012). USP4 is regulated by AKT phosphorylation and directly deubiquitylates TGF-beta type I receptor. Nature cell biology *14*, 717-726.

Zwickl, P., Baumeister, W., and Steven, A. (2000). Dis-assembly lines: the proteasome and related ATPase-assisted proteases. Curr Opin Struct Biol *10*, 242-250.

Curriculum Vitae

Personal information

First name/ Surname	**Tanja Plessl**
Nationality	Austrian
Gender	female

Education and training

Dates	2010 -2013
Title of qualification awarded	**Master of Science in Molecular Biology**
Name and type of organisation providing education and training	University of Vienna, Austria
Dates	2007 - 2010
Title of qualification awarded	**Bachelor of Science in Molecular Biotechnology**
Name and type of organisation providing education and training	University of Applied Sciences, FH Campus Wien, Vienna, Austria

Work experience

Dates	January 2012 – January 2013
Occupation or position held	**Master thesis** Characterization of the Molecular Signal Transduction Pathway of the Tyrosine Kinase Abl1 in Response to Genotoxic Stress
Name and address of employer	CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences Pathological Networks in Leukemia and Immunity: Dr. Giulio Superti-Furga Lazarettgasse 14, AKH BT 25.3 A-1090 Vienna
Dates	October 2011 – November 2011
Occupation or position held	**Internship for 2 months**
Name and address of employer	CCRI Children's Cancer Research Institute Genetics of Leukemia: Dr. Sabine Strehl Zimmermannplatz 10 A-1090 Vienna

Dates	July 2011- Seotember 2011
Occupation or position held	**Internship for 3 months**
	Performance of transfer runs for recombinant proteins, Chromatography Systems
Name and address of employer	Boehringer Ingelheim RCV GmbH & Co KG Process Science Downstream: Dipl. Ing. Johannes Salzbrunn Dr. Boehringer-Gasse 5-11, A-1121 Vienna
Dates	January 2010 – June 2010, August 2010
Occupation or position held	**Bachelor thesis, internship**
	Bacelor thesis about DNA-methylation based tumor markers Elucidating biomarkers from clinical tumor samples
Name and address of employer	Austrian Institute of Technology Biomarker and Assay Development: Dr. Andreas Weinhäusel A-2444 Seibersdorf