Fakultät für Biologie der Ludwig-Maximilians-Universität München

DISSERTATION

Investigation of the ubiquitin-specific protease UBP41 and of the lysosomal cysteine proteases cathepsin-L and cathepsin-B as potential mediators of proapoptotic signalling

Submitted by

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Short INDEX

PhD candidate declaration

I certify that the thesis entitled

"Investigation of the ubiquitin-specific protease UBP41 and of the lysosomal cysteine proteases cathepsin-L and cathepsin-B as potential mediators of proapoptotic signalling",

submitted for the degree of

Dr. rer. nat.,

is the result of my own, independent work, that all of the following material was written by myself, and that I did not use any other sources of information than those indicated by the given references.

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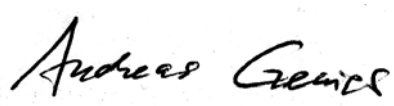
Hiermit versichere ich, dass die vorliegende Promotionsarbeit das Ergebnis meiner eigenständigen Arbeit ist und dass ich dieses Manuskript persönlich verfasst habe. Zudem habe ich keine anderen als die angegebenen Quellen und Hilfsmittel verwendet.



München, 10. November 2003 Andreas Gewies

I like to dedicate this work to my family,
to my parents Magdalena and Norbert Gewies,
to my sisters Christiane and Gabriela
as well as to my little brother Marcel.

I am looking back on those former times
that shaped me to what I am today,
that helped me to develop my various interests
and that gave me the opportunity to receive a decent education.

A special dedication to my beloved fiancée Bianca
who has accompanied me during the last two years of this work
and who means to me a wonderful world beyond science.

In memory of my grandfather Wilhelm Riedel,
who to me will always be an example of
modesty and decency.

Acknowledgement

The here presented doctoral thesis is based on the data and results obtained during my work at the Max-Planck-Institute for Biochemistry in Martinsried, Germany, from January 2000 until August 2003. Supervisor was PD Dr. Stefan Grimm as the principal investigator of the independent *Junior Research Group* with focus on "Programmed Cell Death". This work was supported by the Sonderforschungsbereich 469, by the Bavarian Government, Xantos Biomedicine AG, and Roche Diagnostics.

First of all, I would like to express my appreciation and thanks to my supervisor PD Dr. Stefan Grimm for giving me the opportunity to become a member of his group, for his support and his critical reviews of my work, by this guiding me to the completion of this thesis as well as to the successful publication of its results.

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Finally, I like to thank my former colleagues from the Department of Pathology at the University of Iowa in Iowa City, USA, especially Prof. Michael B. Cohen M.D. and Dr. Oskar W. Rokhlin for introducing me to the field of apoptosis research and for giving me the opportunity to independently practice the scientific research and writing process.

Apoptosis –

the end of an individual cell, at the same time a martyrdom in favor of the organism as a whole. A well-ordered process, genetically determined, also called "programmed cell death" what sounds somewhat artificial. However, apoptosis is as natural as the falling of leaves from a tree in autumn.

Long INDEX

Summary

Starting point for this thesis was the application of a genetic screening procedure for the identification of dominant apoptosis-inducing genes. Apoptosis is a form of programmed cell death by which cells of a multicellular organism die in a well-ordered and genetically determined sequence of morphologic and molecular events. Since apoptosis plays major roles during development and homeostasis, it is highly regulated by the action of a multitude of proteins that either promote or suppress the activation of the cell death program. Although a plethora of pro- or antiapoptotic proteins have already been described, many more, yet unidentified protein factors are expected to be involved in the induction, mediation and regulation of apoptosis. In this context, the main objective of this thesis was to identify and characterize proteins as novel regulatory factors of apoptosis pathways. To this end, a genetic screen was applied that aimed on the isolation and identification of cDNA clones which upon overexpression induce the typical morphological features of apoptosis in the chosen 293T cell system. Selected cDNA clones identified in this way, were subsequently characterized for their proapoptotic activity.

Two projects are described within the scope of this thesis (**Fig. S1**). The first project characterizes the ubiquitin-specific protease UBP41 as a protein which upon overexpression causes apoptosis induction in several mammalian cancer cell lines. The second project investigates a possible involvement of the lysosomal cysteine proteases cathepsin-L and cathepsin-B in apoptosis pathways induced by distinct death stimuli, in particular by the tumor necrosis factor α (TNF-α). Therefore, both projects examine a possible regulation of apoptosis induction by proteases that are part of one of the two major systems of protein degradation. UBP41 as a protease with deubiquitylating activity is expected to play a role in the ubiquitin/proteasome system which is the major proteolytic apparatus for the degradation of cytosolic proteins. Cathepsins, on the other hand, are lysosomal proteases that participate in the breakdown of membrane-associated proteins and of extracellular proteins that are taken up by endocytosis. Both, the ubiquitin/proteasome system as well as lysosomal proteases have been previously implicated in the regulation and mediation of apoptosis, and it therefore appeared particularly attractive to further study the effect of UBP41 and cathepsins on cell death signalling in more detail.

Project 1:

Identification and characterization of UBP41 as a dominant apoptosis-inducing factor

The mouse cDNA of the ubiquitin-specific protease UBP41 was one of the positive clones that induced cell death when overexpressed in 293T cells. Overexpression of the human UBP41 orthologue

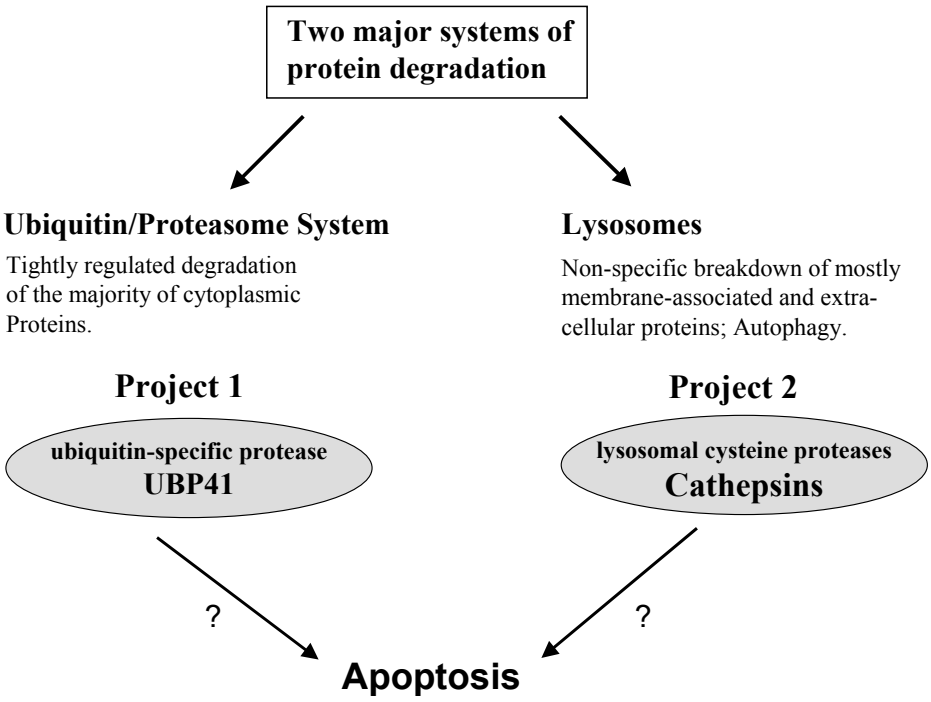


Fig. S1
The two projects of this thesis. *This thesis comprises two projects which both describe a possible interplay between components of the two major systems of protein degradation and apoptosis. The ubiquitin-specific protease UBP41 is part of the ubiquitin/proteasome system and induces apoptosis upon its overexpression, whereas cathepsins are part of the hydrolytic content of lysosomes and might contribute to proapoptotic signalling. Since UBP41 as well as the cathepsins-B and -L are cysteine proteases, both projects investigate the influence of non-caspase cysteine proteases on apoptosis pathways, a topic that recently gained increasing recognition.*

(hUBP41) also led to the induction of apoptosis with all its typical morphological and biochemical features. hUBP41-induced cell death was further characterized in human 293T and HeLa cells. An enzymatic inactive mutant form of hUBP41 did not induce significant cell death in 293T cells, indicating that the deubiquitinating activity of hUBP41 is necessary for its apoptosis-inducing effect. Indeed, overexpression of hUBP41 in 293T and HeLa cells resulted in a dramatic decrease of overall protein ubiquitination, suggesting that it is the interference with the ubiquitination system that causes cell death induction. The turnover of most cytoplasmic proteins is regulated by the ubiquitin/proteasome system. In a temporally and locally regulated process, specific proteins are covalently attached to the small ubiquitin protein and by this are targeted for recognition by the proteasome, a multi-subunit complex for the proteolysis of proteins. The untimely and inappropriate deubiquitination of proteins by hUBP41 might correspond to their rescue from proteasomal degradation, to an extension of their half life and therefore to an accumulation of proteins within the cell that normally are regulated by the ubiquitin/proteasome system. However, of several endogenous proteins tested, none was found to be stabilized by hUBP41 overexpression. Moreover, the proapoptotic effect of hUBP41 overexpression was clearly different from a general inhibition of

proteasomal protein degradation as provoked by the drug proteasome inhibitor MG132 which primarily leads to cell cycle arrest and only subsequently to moderate apoptosis induction. I found additional experimental evidence that hUBP41 in principle can rescue ubiquitylated proteins from degradation, leaving open the possibility that distinct short-lived substrates are stabilized by hUBP41. The accumulation of proteins with proapoptotic activity therefore might be responsible for apoptosis induction triggered by UBP41 overexpression.

According to the unified nomenclature for ubiquitin-specific proteases, UBP41 belongs to the family of USP2 splice isoforms whose gene is localized to chromosome 11q23.3. Deletions within this chromosomal region have been previously reported in cases of lymphoma, melanoma and cervical cancer. Results of northern hybridization experiments using a commercial tumor/normal profiling array indicate that the hUSP2 mRNA message might be specifically downregulated in tumor samples of the kidney when compared to normal tissue. Those observations tempt to speculate that UBP41 as a protein with proapoptotic regulatory functions might be considered a candidate tumor-suppressor.

The results of this project were published in Cancer Research [Gewies, 2003b].

Project 2:

Investigation of a possible role for cathepsin-L and cathepsin-B in proapoptotic signalling

Another cDNA clone selected from the screen was identified to encode mouse cathepsin-L. When murine or human cathepsin-L was overexpressed in 293T or HeLa cells, a minor fraction of cells displayed a small and round phenotype resembling apoptosis. It became apparent that the observed effect of cathepsin-L was too weak for being detected unequivocally by the available apoptosis standard assays such as DNA fragmentation or caspase activity assays. Therefore, studying the possible proapoptotic activity of cathepsin-L overexpression on its own was not feasible. Instead, it was further investigated whether enhanced human cathepsin expression levels might sensitize tumor cell lines, such as HeLa cells, to distinct apoptosis-inducing stimuli. Investigating this question was of special relevance because recent publications presented an accumulating body of evidence that cathepsins might be involved in the mediation of apoptosis. In many cases, cathepsin inhibitors were reported to render cells more resistant to the treatment with apoptosis inducers, e.g. TNF-α. Importantly, elevated cathepsin expression is frequently observed in various types of cancer and traditionally is regarded to be a tumor marker correlated with poor prognosis because cathepsins are believed to contribute to the degradation of the extracellular matrix, thereby promoting invasion and metastasis. Considering a possible proapoptotic activity of cathepsins, it has been hypothesized that cathepsin-B-like proteases, despite their implication as “bad prognosis” factors in the promotion of cancer progression, may prove useful in selectively targeting tumor cells for apoptosis-induction. Up to now there are no reports in the literature directly investigating the possible correlation between cathepsin expression levels and sensitivity to apoptosis.

In this context, I first of all studied the effect of various cathepsin inhibitors on TNF-α-induced cell

death. Treatment of HeLa cells with TNF-α resulted in DNA fragmentation that was significantly reduced in the presence of cathepsin-inhibitors, suggesting a possible role for cathepsins, such as cathepsin-B or –L, in this type of cell death. As a next step, it was examined whether sensitivity to TNF-α-mediated cell death might be influenced by increased cathepsin expression levels. Transient transfection of HeLa cells with cathepsin-B or -L resulted in strongly increased expression levels comparable to those found in many tumors but did not sensitize the cells to TNF-α-mediated apoptosis. As could be expected from previous reports, TNF-α caused a partial release of lysosomal content into the cytosol as was observed by anti-cathepsin-L immunocytochemical staining. In comparison, lysosomal integrity was efficiently disturbed during the treatment of HeLa cells with the lysosomotropic detergent N-dodecyl-imidazole-HCl (NDI-HCl), consequently resulting in cell death induction. Importantly, cell death induced by NDI-HCl also was not increased as a consequence of elevated cathepsin-B or –L expression levels.

Therefore, the data presented in this study suggest that cathepsin enzymatic activity contributes to certain aspects of TNF-α-mediated apoptosis, such as DNA fragmentation, but overexpression experiments do not support the hypothesis that the high cathepsin-B or -L expression levels frequently detected in tumor cells can be exploited to selectively target those tumors to an enhanced cell-death effect induced by e.g. TNF-α or lysosomotropic agents. The results of this projects were published in the British Journal of Cancer [Gewies, 2003a].

Conclusion:

The two projects presented in this thesis are a contribution to currently intensely studied fields of apoptosis research. The finding, that the ubiquitin-specific protease UBP41 might play a role as a proapoptotic regulatory factor adds to the growing body of evidence that there is intense crosstalk between apoptosis signalling pathways and the ubiquitin/proteasome system. In particular, the characterization of UBP41 as a dominant apoptosis-inducing protein suggests an important influence of deubiquitinating enzymes on the regulation of cell death, further supporting recently published observations. The second project tested the interesting hypothesis whether elevated cathepsin expression levels of tumor cells might be exploited to selectively target those tumor cells to cell death induction. My observation that high level overexpression of cathepsin-B and -L does not have any detectable impact on the sensitivity of HeLa cells to apoptosis induction does not support this hypothesis and stimulates further investigations of this kind in order to clarify the controversely debated role of cathepsins as proapoptotic signalling molecules. Since UBP41 as well as the cathepsins-B and -L are cysteine proteases, the here presented results additionally contribute to the growing knowledge about the influence of non-caspase cysteine proteases on the initiation, mediation, execution and regulation of apoptosis.

1 Introduction

1.1 Apoptosis

1.1.1 The development of the term *apoptosis*

Already since the mid-nineteenth century, many observations have indicated that cell death plays a considerable role during physiological processes of multicellular organisms, particularly during embryogenesis and metamorphosis [Gluecksmann, 1951; Lockshin, 2001]. The term *programmed cell death* was introduced in 1964, proposing that cell death during development is not of accidential nature but follows a sequence of controlled steps leading to locally and temporally defined self-destruction [Lockshin, 1964].

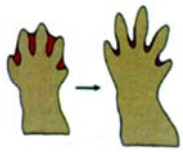
Eventually, the term *apoptosis* had been coined in order to describe the morphological processes leading to controlled cellular self-destruction and was first introduced in a publication by Kerr, Wyllie and Currie [Kerr, 1972]. *Apoptosis* is of greek origin, having the meaning "falling off or dropping off", in analogy to leaves falling off trees or petals dropping off flowers. This analogy emphasizes that the death of living matter is an integral and necessary part of the life cycle of organisms. The apoptotic mode of cell death is an active and defined process which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions. It should be stressed that apoptosis is a well-defined and possibly the most frequent form of programmed cell death, but that other, non-apoptotic types of cell death also might be of biological significance [Leist, 2001a].

1.1.2 The significance of apoptosis

The development and maintenance of multicellular biological systems depends on a sophisticated interplay between the cells forming the organism, it sometimes even seems to involve an altruistic behaviour of individual cells in favour of the organism as a whole. During development many cells are produced in excess which eventually undergo programmed cell death and thereby contribute to sculpturing many organs and tissues [see **Box 1.1** and Meier, 2000].

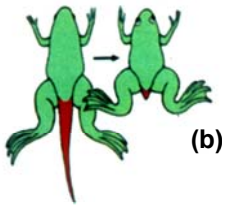
BOX 1.1 Examples of physiological cell death

In the human body about 100,000 cells are produced every second by mitosis and a similar number die by apoptosis (Vaux and Korsmeyer, 1999, Cell) !



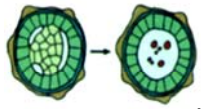
Development and Morphogenesis:

- 131 of the 1,090 somatic cells die during C.elegans development
- during limb formation separate digits evolve by death of interdigital mesenchymal tissue (a)
- ablation of cells no longer needed such as the amphibian tadpole tail during metamorphosis (b)
- demise of cells allows sculpturing of hollow structures (c)
- formation of reproductive organs (d)
  (Müllerian duct → uterus, deleted in males;Wolffian duct → male organs, deleted in females)
- massive cell death occurs during early development of the nervous system (> 50 percent of all neurons die)



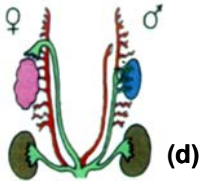
Homeostasis:

- a paradigm for the involvement of apoptosis in homeostasis is the immune system: several millions of B and T cells are generated every day and the majority (> 95 percent) of those die during maturation (death by neglect, negative selection) or by AICD of peripheral immune cells)



Deletion of damaged and dangerous cells:

- Cells with severely damaged DNA that cannot be repaired appropriately usually are removed by apoptosis
- Inappropriate mitogenic signalling that is in conflict with the environmental or cellular status of the cell usually results in cell cycle arrest or apoptosis
- Autoreactive cells of the immune system are deleted by apoptosis
- Elimination of infected cells



A particularly instructive example for the implication of programmed cell death in animal development is the formation of free and independent digits by massive cell death in the interdigital mesenchymal tissue [Zuzarte-Luis, 2002]. Other examples are the development of the brain, during which half of the neurons that are initially created will die in later stages when the adult brain is formed [Hutchins, 1998] and the development of the reproductive organs [Meier, 2000]. Also cells of an adult organism constantly undergo physiological cell death which must be balanced with proliferation in order to maintain homeostasis in terms of constant cell numbers. The majority of the developing lymphocytes die either during genetic rearrangement events in the formation of the antigen receptor, during negative selection or in the periphery, thereby tightly controlling the pool of highly efficient and functional but not self-reactive immune cells and at the same time keeping lymphocyte numbers relatively constant [Rathmell, 2002].

Taken together, apoptotic processes are of widespread biological significance, being involved in e.g. development, differentiation, proliferation/homoeostasis, regulation and function of the immune system and in the removal of defect and therefore harmful cells. Thus, dysfunction or dysregulation of the apoptotic program is implicated in a variety of pathological conditions. Defects in apoptosis can result in cancer, autoimmune diseases and spreading of viral infections, while neurodegenerative disorders, AIDS and ischaemic diseases are caused or enhanced by excessive apoptosis [Fadeel, 1999a].

Due to its importance in such various biological processes, programmed cell death is a widespread phenomenon, occuring in all kinds of metazoans [Tittel, 2000] such as in mammals, insects [Richardson, 2002], nematodes [Liu, 1999b], and cnidaria [Cikala, 1999]. Moreover, programmed cell death also might play a role in plant biology [Solomon, 1999], and apoptosis-like cell death mechanisms even have been observed and used as a model system in yeast [Frohlich, 2000; Skulachev, 2002]. Fascinating insights into the origin and evolution of programmed cell death might possibly be given by the fact, that programmed cell death is also an integral part of the life cycle of other unicellular eukaryotes (such as the kinetoplastid parasite *Trypanosoma brucei brucei*, the ciliate *Tetrahymena thermophila*, and the slime mold *Dictyostelium discoideum*) and that even prokaryotes (such as *Bacillus subtilis*, *Streptomyces* and *Myxobacteria*) sometimes undergo regulated cell death [Ameisen, 2002].

1.1.3 Morphological features of apoptosis

Apoptotic cells can be recognized by stereotypical morphological changes: the cell shrinks, shows deformation and looses contact to its neighbouring cells. Its chromatin condenses and marginates at the nuclear membrane, the plasma membrane is blebbing or budding, and finally the cell is fragmented into compact membrane-enclosed structures, called 'apoptotic bodies' which contain cytosol, the condensed chromatin, and organelles (**Fig. 1.1**). The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response. Those morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles [Saraste, 2000]. Apoptosis is in contrast to the necrotic mode of cell-death in which case the cells suffer a major insult, resulting in a loss of membrane integrity, swelling and disrupture of the cells. During necrosis, the cellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue [Leist, 2001a].

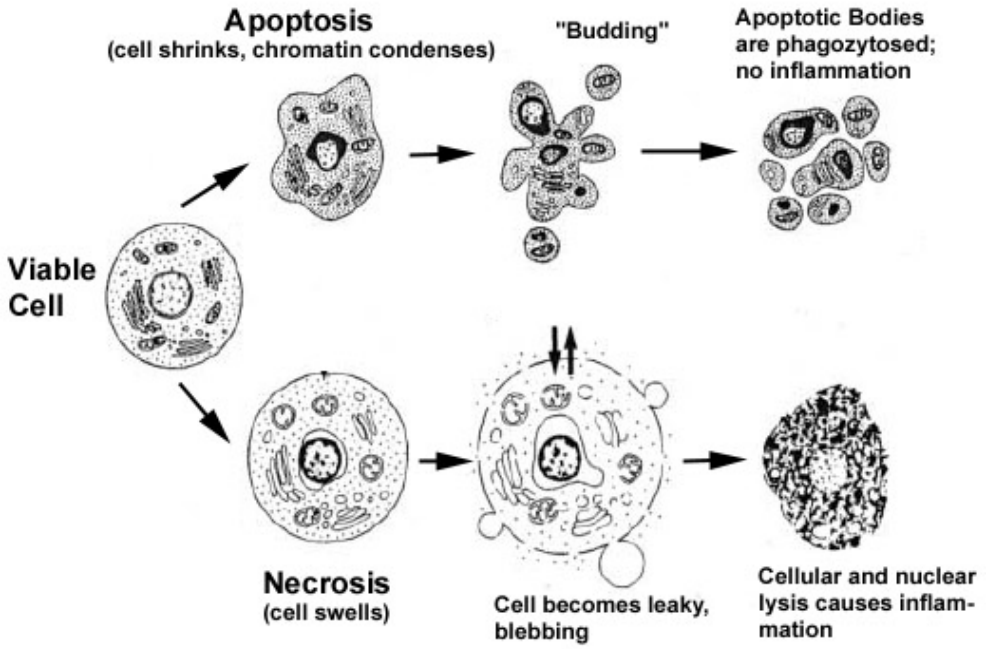


Fig. 1.1
Hallmarks of the apoptotic and necrotic cell death process. *Apoptosis includes cellular shrinking, chromatin condensation and margination at the nuclear periphery with the eventual formation of membrane-bound apoptotic bodies that contain organelles, cytosol and nuclear fragments and are phagocytosed without triggering inflammatory processes.The necrotic cell swells, becomes leaky and finally is disrupted and releases its contents into the surrounding tissue resulting in inflammation. Modified from [Van Cruchten, 2002].*

1.1.4 Molecular mechanisms of apoptosis signalling pathways

This paragraph is meant to provide a general overview of basic apoptotic signalling pathways and of the molecular machinery responsible for the induction and execution of apoptosis. The most important signalling molecules and cellular structures will be discussed in context of their function and of the mechanisms in which they are involved in the initiation, mediation, execution, and regulation of apoptosis. This chapter should give an impression of the sophisticated interplay between factors that promote or suppress apoptosis, resulting in a complicated regulatory network which determines the fate of an individual cell as part of its multicellular environment (**Fig. 1.5**).

1.1.4.1 Various death signals activate common signalling pathways

Apoptosis is a tightly regulated and at the same time highly efficient cell death program which requires the interplay of a multitude of factors. The components of the apoptotic signalling network are genetically encoded and are considered to be usually in place in a nucleated cell ready to be activated by a death-inducing stimulus [Ishizaki, 1995; Weil, 1996].

Apoptosis can be triggered by various stimuli from outside or inside the cell, e.g. by ligation of cell surface receptors, by DNA damage as a cause of defects in DNA repair mechanisms, treatment with cytotoxic drugs or irradiation, by a lack of survival signals, contradictory cell cycle signalling or by developmental death signals. Death signals of such diverse origin nevertheless appear to eventually activate a common cell death machinery leading to the characteristic features of apoptotic cell death.

Much of the understanding of cell death has come from genetic studies in the nematode *C. elegans* by which several genes have been identified that function in the apoptotic killing and elimination of 131 of the initially 1090 somatic cells that are generated druing hermaphrodite development [Hengartner, 1999]. The proximal cause of apoptosis in *C. elegans* is the activation of the cysteine protease ced-3, which is mediated by its oligomerization at the activator protein ced-4. Activity of the ced-3/ced-4 complex is regulated by the apoptosis inhibitor ced-9 and the apoptosis inducer egl-1 (**Fig. 1.2**). Subsequent studies in mammals and in the fly, *D. melanogaster*, have identidied counterparts for these *C. elegans* genes, demonstrating that the core components of the cell death machinery are conserved through evolution [Richardson, 2002]. Accordingly, ced-3 is the single *C. elegans* member of a family of cysteine proteases, the caspases, whereas ced-4 corresponds to the mammalian apoptotic protease activating factor 1, Apaf-1, which is the core of a caspase-activating signalling complex, the apoptosome. Egl-1 and ced-9 are members of the Bcl-2 family of pro- or antiapoptotic proteins, respectively, which play an important role in the mediation and regulation of apoptotic signalling pathways. All of those central components will be discussed within the following paragraphs.

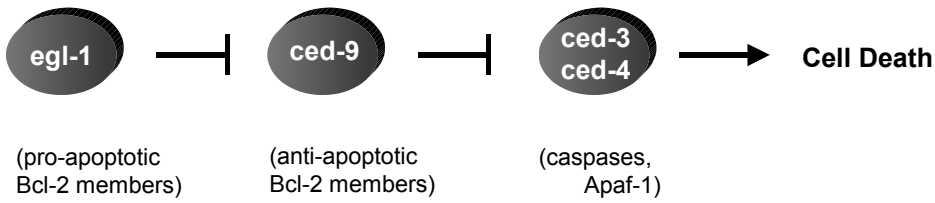


Fig. 1.2
C. elegans as a model system contains basic components of the cell death machinery. *Apoptosis regulation in C.elegans relies on a simple basic network of factors for which corresponding analogous components also can be found in higher organisms as given within brackets. Thus egl-1 is the worm representative for mammalian proapoptotic BH3-only proteins, ced-9 belongs to the antiapoptotic Bcl-2 family, ced-3 is the only worm caspase, and ced-4 is homologous to mammalian Apaf-1 (according to [Cecconi, 1999]).*

1.1.4.2 Caspases are central initiators and executioners of apoptosis

The caspases, cysteine proteases homologous to *C. elegans* ced-3, are of central importance in the apoptotic signalling network which are activated in most cases of apoptotic cell death [Bratton, 2000]. Actually, strictly defined, cell death only can be classified to follow a classical apoptotic mode if execution of cell death is dependent on caspase activity [Leist, 2001a].

The term caspases is derived from cysteine-dependent aspartate-specific proteases: their catalytical activity depends on a critical cysteine-residue within a highly conserved active-site pentapeptide QACRG, and the caspases specifically cleave their substrates after Asp residues. So far, 7 different caspases have been identified in *Drosophila*, and 14 different members of the caspase-family have been described in mammals, with caspase-11 and caspase–12 only identified in the mouse [Denault, 2002; Richardson, 2002]. According to a unified nomenclature, the caspases are referred to in the order of their publication: caspase-1 is ICE (Interleukin-1ß-Converting Enzyme), the first mammalian caspase described to be a homologue of Ced-3 [Creagh, 2001; Miura, 1993]. Caspase-1 as well as caspases-4, -5, -11, and –12 appear to be mainly involved in the proteolytic maturation of pro-inflammatory cytokines such as pro-IL-1ß and pro-IL-18 and their contribution to the execution of apoptosis remains questionable [Denault, 2002]. Indeed, mice deficient for caspase-1 or caspase-11 develop normally and cells from those knockout mice remain sensitive to various death stimuli [Li, 1995; Wang, 1998]. In contrast, gene knockout experiments targeting caspase-3 and caspase-9 resulted in perinatal mortality as a result of severe defects in brain development [Kuida, 1998; Kuida, 1996], whereas caspase-8 deficient embryos died after day 12 [Varfolomeev, 1998]. This and the observation that cell lines derived from those knockout experiments are resistant to distinct apoptosis stimuli underlines the importance of caspases as proapoptotic mediators. Indeed, caspase-3, caspase-9, caspase-8, and additionally caspases-2, -6, -7, and –10 have been recognized to play an important role in the apoptotic signalling machinery [Earnshaw, 1999].

In the cell, caspases are synthesized as inactive zymogens, the so called procaspases, which at their N-terminus carry a prodomain followed by a large and a small subunit which sometimes are separated by a linker peptide. Upon maturation, the procaspases are proteolytically processed between the large and small subunit, resulting in a small and a large subunit. The prodomain is also frequently but not necessarily removed during the activation process. A heterotetramer consisting of each two small and two large subunits then forms an active caspase. The proapoptotic caspases can be divided into the group of initiator caspases including procaspases-2, -8, -9 and –10, and into the group of executioner caspases including procaspases-3, -6, and –7. Whereas the executioner caspases possess only short prodomains, the initiator caspases possess long prodomains, containing death effector domains (DED) in the case of procaspases-8 and –10 or caspase recruitment domains (CARD) as in the case of procaspase-9 and procaspase-2.

Via their prodomains, the initiator caspases are recruited to and activated at death inducing signalling complexes either in response to the ligation of cell surface death receptors (extrinsic apoptosis pathways) or in response to signals originating from inside the cell (intrinsic apoptosis pathways).

In extrinsic apoptosis pathways (**Fig. 1.3**), e.g. procaspase-8 is recruited by its DEDs to the death inducing signalling complex (DISC), a membrane receptor complex formed following to the ligation of a member of the tumor necrosis factor receptor (TNFR) family [Sartorius, 2001]. When bound to the DISC, several procaspase-8 molecules are in close proximity to each other and therefore are assumed to activate each other by autoproteolysis [Denault, 2002].

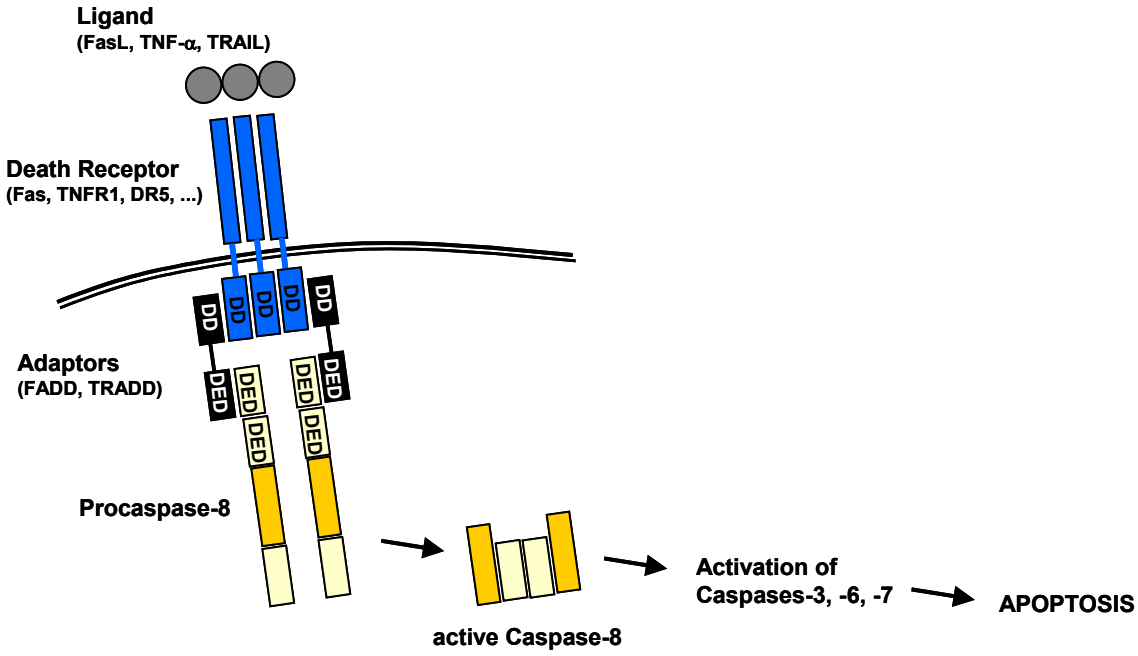


Fig. 1.3
Receptor-mediated caspase activation at the DISC. *Upon ligation by its cognate ligand, the trimeric death receptor recruits adaptor molecules via its cytoplasmic death domains (DD). Besides possessing DDs, the adaptors additionally contain death effector domains (DED) which recruit procaspase-8 to the receptor complex which now is called the death-inducing signalling complex (DISC). Procaspase-8 is activated by autoproteolytic cleavage and forms the active caspase-8 which is a heterotetramer of two small and two large subunits. The initiatior caspase-8 cleaves and thereby activates effector caspases for the execution of apoptosis.*

Intrinsic apoptosis pathways (**Fig. 1.4**) involve procaspase-9 which is activated downstream of mitochondrial proapoptotic events at the so called apoptosome, a cytosolic death signalling protein complex that is formed upon release of cytochrome c from the mitochondria [Salvesen, 2002b]. In this case it is the dimerization of procaspase-9 molecules at the Apaf-1 scaffold that is responsible for caspase-9 activation [Denault, 2002]. Once the initiator caspases have been activated, they can proteolytically activate the effector procaspases-3, -6, and -7 which subsequently cleave a specific set of protein substrates, including procaspases themselves, resulting in the mediation and amplification of the death signal and eventually in the execution of cell death with all the morphological and biochemical features usually observed [Earnshaw, 1999].

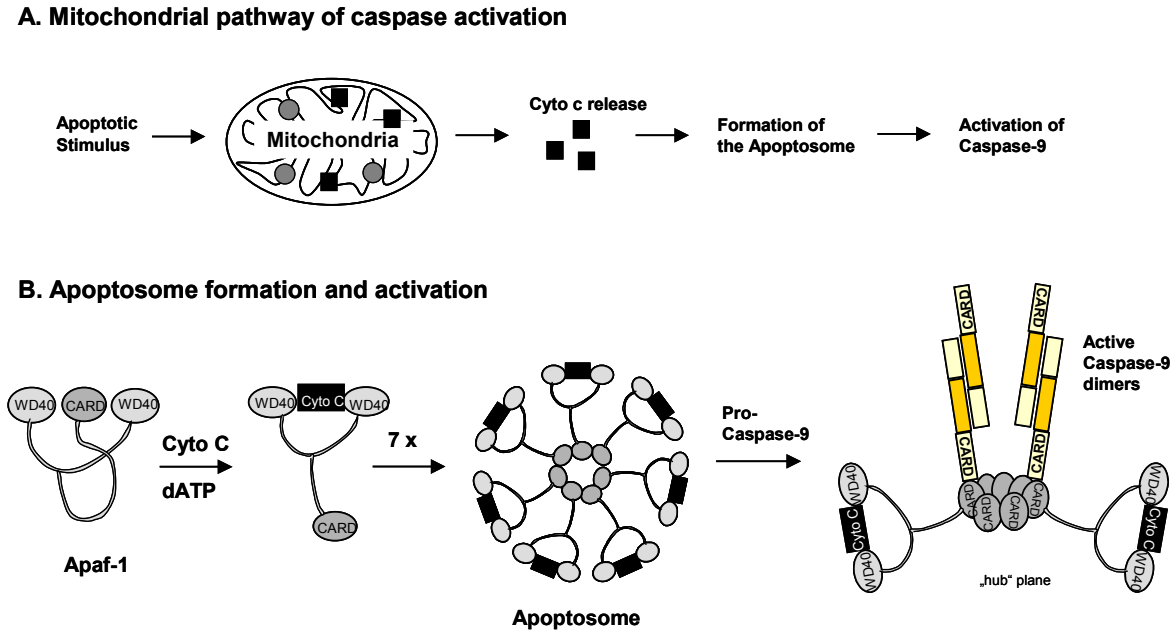


Fig. 1.4
Mitochondria-mediated caspase activation at the apoptosome. *A. Apoptotic stimuli trigger the release of apoptogenic factors from the mitochondrial intermembrane space to the cytosol, such as cytochrome c which induces the formation of the apoptosome and the activation of procaspase-9. B. By the action of cytochrome c (Cyto C) and dATP the Apaf-1 protein adopts a conformation that allows the formation of a heptameric, wheel-like structure, the apoptosome. Procaspase-9 molecules can bind to the inner "hub" region of the apoptosome and are activated by dimer formation. Active caspase-9 dimers further mediate activation of effector caspases [Acehan, 2002].*

1.1.4.3 Extrinsic apoptosis pathways of type I and type II

Extrinsic apoptosis signalling is mediated by the activation of so called "death receptors" which are cell surface receptors that transmit apoptotic signals after ligation with specific ligands. Death receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily, including TNFR-1, Fas/CD95, and the TRAIL receptors DR-4 and DR-5 [Ashkenazi, 2002]. All members of the TNFR family consist of cysteine rich extracellular subdomains which allow them to recognize their ligands with specificity, resulting in the trimerization and activation of the respective death receptor [Naismith, 1998]. Subsequent signalling is mediated by the cytoplasmic part of the death receptor which contains a conserved sequence termed the death domain (DD). Adapter molecules like FADD or TRADD themselves possess their own DDs by which they are recruited to the DDs of the activated death receptor, thereby forming the so-called death inducing signalling complex (DISC). In addition to its DD, the adaptor FADD also contains a death effector domain (DED) which through homotypic DED-DED interaction sequesters procaspase-8 to the DISC (**Fig. 1.3**). As described above (1.1.4.2, page 8), the local concentration of several procaspase-8 molecules at the DISC leads to their autocatalytic activation and release of active caspase-8. Active caspase-8 then processes downstream effector caspases which subsequently cleave specific substrates resulting in cell death. Cells harboring the capacity to induce such direct and mainly caspase-dependent apoptosis pathways were classified to belong to the so called type I cells [Scaffidi, 1998].

In type II cells, the signal coming from the activated receptor does not generate a caspase signalling cascade strong enough for execution of cell death on its own. In this case, the signal needs to be amplified via mitochondria-dependent apoptotic pathways. The link between the caspase signalling cascade and the mitochondria is provided by the Bcl-2 family member Bid. Bid is cleaved by caspase-8 and in its truncated form (tBID) translocates to the mitochondria where it acts in concert with the proapoptotic Bcl-2 family members Bax and Bak to induce the release of cytochrome c and other mitochondrial proapoptotic factors into the cytosol (see **Fig. 1.5** and [Luo, 1998]). Cytosolic cytochrome c is binding to monomeric Apaf-1 which then, in a dATP-dependent conformational change, oligomerizes to assemble the apoptosome, a complex of wheel-like structure with 7-fold symmetry, that triggers the activation of the initiator procaspase-9 (see **Fig. 1.4** and [Acehan, 2002]). Activated caspase-9 subsequently initiates a caspase cascade involving downstream effector caspases such as caspase-3, caspase-7, and caspase-6, ultimately resulting in cell death [Slee, 1999].

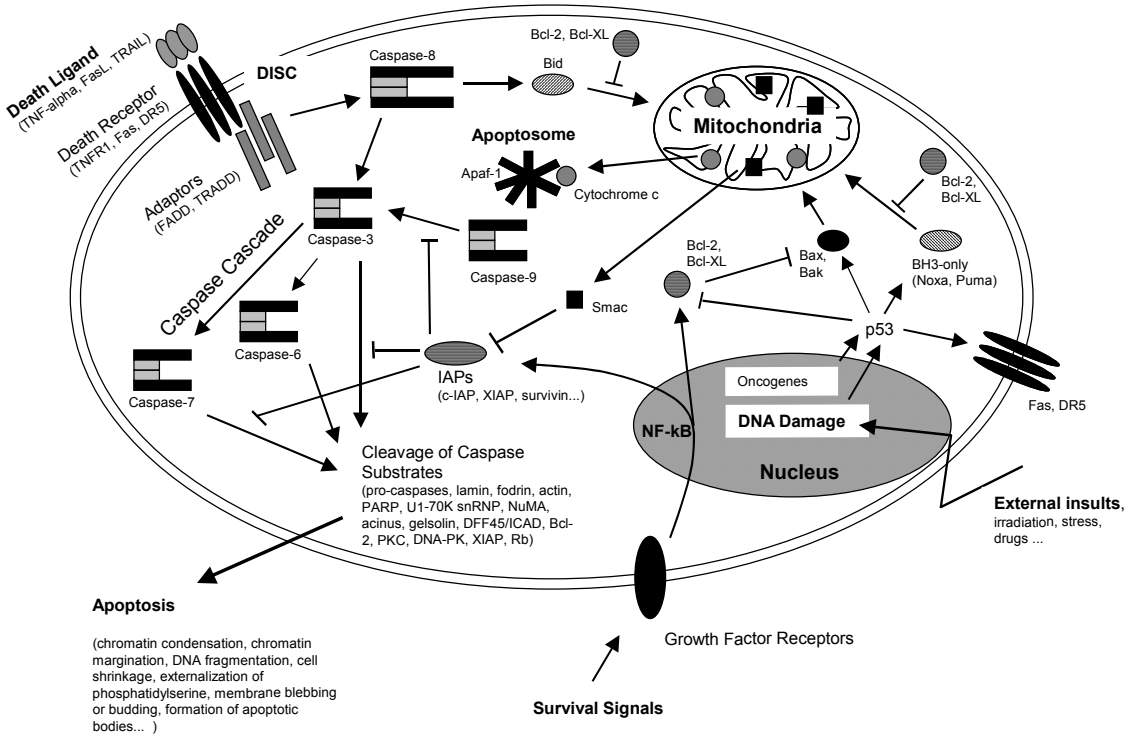


Fig. 1.5

Schematic representation of some major apoptotic signalling pathways. *Apoptosis can be induced in response to various signals from inside and outside the cell, e.g. by ligation of so called death receptors or by cellular stress triggered by oncogenes, irradiation or drugs. Signals emanating from death receptors initially activate the Death Inducing Signalling Complex (DISC) which mediates activation of the initiator caspase-8. Activated caspase-8 initiates a caspase cascade by processing the effector caspases-3, -6, and – 7 which in turn cleave a number of protein substrates. Cleavage of caspase substrates eventually leads to the characteristic morphological and biochemical features of apoptosis. In some cell systems, this direct caspase cascade is sufficient to elicit apoptosis on its own (type 1 signalling), whereas in other cases the signal coming from the DISC must be amplified by the proteolytic activation of the BH3-only protein Bid by caspase-8 with subsequent induction of apoptotic events at the mitochondria (type 2 signalling). Mitochondrial apoptotic signalling includes the release of cytochrome c from the mitochondrial intermembrane space to the cytosol where it contributes to the formation of the apoptosome which consists of cytochrome c, Apaf-1 and dATP. The apoptosome activates caspase-9 which is another initiator caspase and thus is able to mediate the caspase cascade by activating caspase-3. Another mitochondrial proapoptotic factor is Smac which acts by inhibiting the IAPs from blocking caspase activity. IAPs are a family of proteins with antiapoptotic activity by directly inhibiting caspases. IAP expression can be upregulated in response to survival signals such as those coming from growth factor receptors, e.g. by activation of the transcription factor NF-kB, therefore providing a means to suppress apoptosis signalling. Of central importance are the antiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-XL which counteract the action of BH3-only proteins such as Bid but also of proapoptotic Bax and Bak and thus can inhibit mitochondrial proapoptotic events. Apoptotic signals coming from the inside of the cell frequently have their origin within the nucleus, being a consequence of DNA damage induced by irradiation, drugs or other sort of stress. DNA damage in most cases eventually results in the activation of the p53 transcription factor which promotes expression of proapoptotic Bcl-2 members and suppresses antiapoptotic Bcl-2 and Bcl-XL. Other organelles besides mitochondria and the nucleus, such as the ER and lysosomes also have been implicated in apoptotic signalling pathways, and it should be kept in mind that presumably hundreds of proteins are part of an extremely fine-tuned regulatory network consisting of pro- and antiapoptotic factors.*

1.1.4.4 Mitochondria as central regulators of intrinsic apoptosis pathways

Besides amplifying and mediating extrinsic apoptotic pathways, mitochondria also play a central role in the integration and propagation of death signals originating from inside the cell such as DNA damage, oxidative stress, starvation, as well as those induced by chemotherapeutic drugs [Kaufmann, 2000; Wang, 2001]. Most apoptosis-inducing conditions involve the disruption of the mitochondrial inner transmembrane potential ($\Delta\psi$) as well as the so called permeability transition (PT), a sudden increase of the inner mitochondrial membrane permeability to solutes with a molecular mass below approximately 1.5 kDa. Concomitantly, osmotic mitochondrial swelling has been observed by influx of water into the matrix with eventual rupture of the outer mitochondrial membrane, resulting in the release of proapoptotic proteins from the mitochondrial intermembrane space into the cytoplasm [Bernardi, 1999; Loeffler, 2000]. Released proteins include cytochrome c, which activates the apoptosome and therefore the caspase cascade, but also other factors such as the apoptosis-inducing factor AIF [Susin, 1999], the endonuclease endoG [Li, 2001a], Smac/Diablo [Verhagen, 2000], and Htr/Omi [Verhagen, 2002]. Interestingly, PT is always followed by $\Delta\psi$, but $\Delta\psi$ is not always caused by PT, and cytochrome c release has been observed even in absence of $\Delta\psi$ [Bernardi, 1999; Kroemer, 2000]. In addition to the release of mitochondrial factors, the dissipation of $\Delta\psi$ and PT also cause a loss of the biochemical homeostasis of the cell: ATP synthesis is stopped, redox molecules such as NADH, NADPH, and glutathione are oxidized, and reactive oxygen species (ROS) are increasingly generated [Kroemer, 2000; Kroemer, 1997]. Increased levels of ROS directly cause the oxidation of lipids, proteins, and nucleic acids, thereby enhancing the disruption of $\Delta\psi$ as part of a positive feedback [Marchetti, 1997]. Several possible mechanisms for PT have been proposed, but there appears to exist consent that a so-called permeability transition pore (PTP) is formed consisting of the adenin nucleotide translocator (ANT) and the voltage-dependent anion channel (VDAC) as its core components. ANT is the most abundant protein of the inner mitochondrial membrane and as a transmembrane channel is responsible for the export of ATP in exchange with ADP (antiport). Overexpression of ANT-1 in human cancer cell lines dominantly induces apoptosis with all its characteristic features whereas its closely conserved homologue ANT-2 does not, indicating a specific mechanistic role of ANT-1 in mitochondrial apoptosis events [Bauer, 1999]. VDAC, also called porin, is the most abundant protein of the outer mitochondrial membrane and forms a non-selective pore through the outer membrane. Indicated by direct protein-protein interactions, VDAC-ANT complexes presumably connect inner and outer mitochondrial membrane to so-called ‘contact sites’, corresponding to a close association of the two membranes and thereby possibly constituting the PT pore [Beutner, 1998]. Since PT, loss of $\Delta\psi$, and release of mitochondrial proteins are of central importance in mediating and enhancing apoptotic pathways, those mitochondrial events must be kept under strict control of regulatory mechanisms which are in many ways dependent on members of the Bcl-2 family which will be discussed in the next paragraph.

1.1.5 Regulatory mechanisms in apoptosis signalling

Commonly, the activation of apoptosis is regarded to occur when a cell encounters a specific death-inducing signal such as the ligation of a death receptor by its cognate ligand or if cells are treated with a cytotoxic drug. This suggests that the apoptosis signalling pathways in viable cells are kept in an inactive state and are only turned on in response to a death stimulus. But it should be taken into account that the components of the apoptotic signalling network are genetically encoded and ready for action in most cell types. Therefore, an interesting and possibly more realistic alternative view would be as follows: all cells of a multicellular animal might be intrinsically programmed to self-destruct and indeed would die instantaneously unless cell death is continously repressed by survival signals such as provided by other cells of the organism, e.g. growth factors, hormones, nutrients. Those survival signals enhance the expression and/or activity of antiapoptotic regulatory molecules thereby keeping in check the activation of proapoptotic factors [Ameisen, 2002; Raff, 1993]. Indeed, a set of various antiapoptotic molecules and mechanisms has been identified, as well as proapoptotic factors that counteract those inhibitory molecules when apoptotic demise of a cell is timely and imperative.

1.1.5.1 The Bcl-2 family

Bcl-2, an oncogene which in follicular lymphoma is frequently linked to an immunoglobulin locus by the chromosome translocation t(14:18), was the first example of an oncogene that inhibits cell death rather than promoting proliferation. B cells transfected with *Bcl-2* were shown to be rendered resistant towards apoptosis induced by IL-3 withdrawal: for the first time it was shown that the pathway toward tumorigenesis depends not only on the ability to escape growth control but also depends on the ability to prevent apoptosis [Vaux, 1988].

When homologues of Bcl-2 had been identified, it became apparent that a Bcl-2 family of proteins can be defined by the presence of conserved sequence motifs known as Bcl-2 homology domains (BH1 to BH4). In mammals, up to 30 relatives have been decribed of which some belong to a group of pro-survival members and others to a group of proapoptotic members [Borner, 2003]. In addition to Bcl-2 itself, there are a number of other prosurvival proteins, e.g. Bcl-X_L, Bcl-w, A1, and Mcl-1, which all possess the domains BH1, BH2, BH3, and BH4. The proapoptotic group of Bcl-2 members can be devided into two subgroups: the Bax-subfamily consists of Bax, Bak, and Bok that all possess the domains BH1, BH2, and BH3, whereas the BH3-only proteins (Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, Puma, Blk, BNIP3, and Spike) have only the short BH3 motif, an interaction domain that is both necessary and sufficient for their killing action [Cory, 2002; Mund, 2003].

There has been quite some debate about how the Bcl-2 family controls apoptosis: one model proposes that Bcl-2 members might directly control caspase activation [Strasser, 2000], whereas another model claims that they mainly act by guarding mitochondrial integrity [Wang, 2001]. In support of the first model, the worm Bcl-2 orthologue ced-9 binds to the Apaf-1-like adaptor protein ced-4 and prevents it from activating the caspase ced-3 unless the BH3-only protein Egl-1 displaces ced-4 as shown in **Fig. 1.2** [Conradt, 1998]. In contrast, the mammalian ced-4 homologue Apaf-1 obviously does not interact with Bcl-2-like proteins [Moriishi, 1999] but is activated by cytosolic cytochrome c (see **Fig. 1.4**), and it is the release of cytochrome c from the mitochondria that can be controlled by Bcl-2 [Kluck, 1997; Yang, 1997]. Therefore it appears likely, that the central function of mammalian Bcl-2 family members is to guard mitochondrial integrity and to control the release of mitochondrial proteins into the cytoplasm [Cory, 2002].

How then is mitochondrial integrity affected by proapoptotic Bcl-2 family members? Central to this question are Bax and Bak, even though inactivation of the *Bax* gene alone affected apoptosis only slightly and disruption of *Bak* alone did not show any effect. However, the double knockout of Bax and Bak resulted in dramatic impairment of apoptosis during development in many tissues with superfluous cells accumulating in the hematopoietic system and in the brain. Additionally, cells derived from those Bax -/- Bak -/- mice are insensitive to treatment with e.g. etoposide or irradiation [Lindsten, 2000; Wei, 2001]. Bax is a cytosolic monomer in viable cells but during apoptosis changes its conformation, integrates into the outer mitochondrial membrane and oligomerizes [Nechushtan, 2001]. Although the mechanism is controversial, Bax and Bak oligomers are believed to provoke or contribute to the permeabilization of the outer mitochondrial membrane (PT), either by forming channels by themselves [Antonsson, 2000] or by interacting with components of the PT pore such as VDAC [Tsujimoto, 2000].

In contrast, antiapoptotic Bcl-2 members sequester proapoptotic Bcl-2 members by bindig to their BH3 domains and thereby ultimately prevent Bax or Bak activation/ oligomerization and consequently inhibit mitochondrial proapoptotic events: overexpression of Bcl-2 or Bcl-X_L potently inhibits apoptosis in response to many cytotoxic insults, among others by suppressing the generation of ROS, stabilizing $\Delta\psi$, preventing PT and consequently blocking the release of e.g. cytochrome c [Reed, 1998]. Besides eliciting its antiapoptotic effects on the mitochondrial level by indirectly controlling the activation of the apoptosome, Bcl-2 also appears to inhibit apoptotic pathways that are independent of Apaf-1/caspase-9 and which might depend on caspase-7 as a central effector [Marsden, 2002]. In this context one might even expect the existence of another but up to now unidentified Apaf-1 homologue that can be directly controlled by Bcl-2/Bcl-X_L [Puthalakath, 2002].

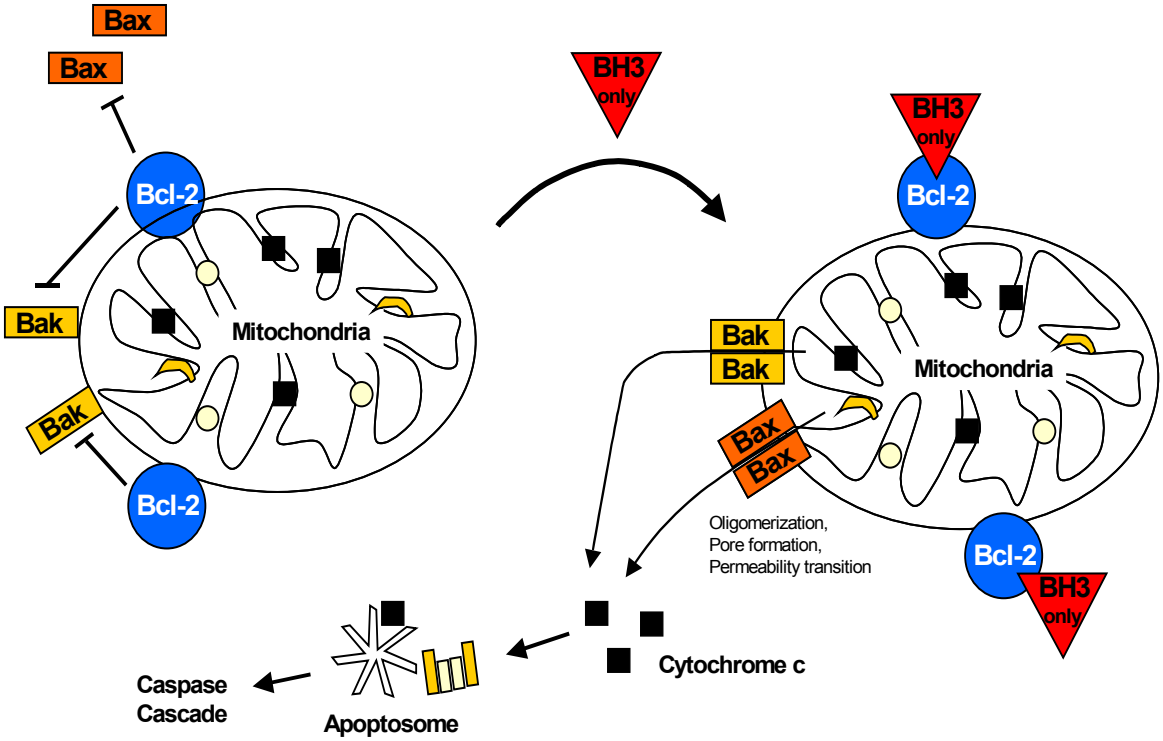


Fig. 1.6
Regulation of apoptosis by the Bcl-2 family. *In a viable cell, the proapoptotic Bcl-2 family members Bax, Bak, and BH3-only proteins are antagonized by antiapoptotic members such as Bcl-2. In response to an apoptotic stimulus, BH3-only members are activated by transcriptional upregulation (Bax, Noxa, Puma), subcellular relocalization (Bim, Bmf), dephosphorylation (Bad), or proteolysis (Bid). Activated BH3-only proteins prevent antiapoptotic Bcl-2 members from inhibiting proapoptotic members. In addition, they might directly induce a conformational change of Bax and Bak which subsequently oligomerize and insert into the mitochondrial membrane where they form pores either by themselves or by associating with the permeability transition pore complex. In consequence, proapoptotic factors are released from the inner mitochondrial membrane into the cytosol, such as cytochrome c which contributes to the formation of the apoptosome and the subsequent activation of the caspase cascade.*

Whereas Bax and Bak represent the central core of a proapoptotic Bcl-2 death machinery that is held in check by the pro-survival members Bcl-2 and Bcl-X_L, members of the BH3-only subfamily are required for the activation of proapoptotic Bax/Bak function [Bouillet, 2002]. On the other hand, the killing effect of BH3-only members depends on Bax/Bak, since cells double-deficient for Bax and Bak do not die upon overexpression of BH3-only proteins as it would be the case in wildtype cells, indicating that BH3-only members function upstream of Bax and Bak [Lindsten, 2000; Wei, 2001]. Importantly, just as the proapoptotic activity of multidomain proteins Bax and Bak is controlled by their interaction with the antiapoptotic guardians Bcl-2/Bcl-X_L, also most BH3-only members display a strong binding preference to antiapoptotic Bcl-2/Bcl-X_L and in this way are kept under control [Scorrano, 2003].

Individual BH3-only proteins are believed to transduce specific death signals since they can be activated for apoptosis signalling by sensing cell stress, such as DNA damage (*Noxa* and *Puma* are p53-inducible genes), growth factor deprivation (*Hrk* and *Bim* mRNA expression is increased), or anoikis (Bmf is activated by subcellular relocalization) [Borner, 2003]. As another example, cytoplasmic Bid is processed by caspase-8 to its truncated form tBid, which after myristoylation translocates to the mitochondria where it triggers cytochrome c release by affecting Bax/Bak oligomerization and/or by mobilizing cytochrome c stores in cristae [Cory, 2002; Scorrano, 2002].

In general, BH3-only proteins are thought to interfere with the fine-tuned balance of homo- or heterooligomerization between proapoptotic multidomain members Bax/Bak and antiapoptotic members Bcl-2/Bcl-XL (**Fig. 1.6**). It has been proposed that Bid and Bim possess BH3 domains (Bid-like BH3 domain) which can directly mediate Bax/Bak oligomerization, whereas Bad and Bik possess Bad-like BH3 domains which do not directly act on Bax/Bak but preferentially interact with antiapoptotic Bcl-2/Bcl-XL. As a consequence, activated Bad/Bik might be able to displace Bid/Bim from the binding pocket of antiapoptotic Bcl-2/Bcl-XL, and - in this way released - Bid/Bim might provoke Bax/Bak oligomerization and cytochrome c release even at subliminal levels [Letai, 2002].

In summary, a current model of how Bcl-2 family members regulate apoptosis can be descibed as follows (**Fig. 1.6**): specific apoptotic stress signals trigger the activation of particular BH3-only proteins which then interact with antiapoptotic members on the outer mitochondrial (but also nuclear/ER) membrane, resulting in the release of Bax-like proapoptotic factors. Bax-like factors undergo a conformational change (possibly assisted by some BH3-only proteins), insert into the outer mitochondrial membrane where they provoke PT and the release of apoptogenic factors [Borner, 2003]

1.1.5.2 Regulation of apoptosis by IAPs

Expression levels of antiapoptotic proteins such as Bcl-2, Bcl-X_L, and A1 were reported to be upregulated by the transcription factor NF-κB which besides being a central regulator of the innate and adaptive immune response is commonly described as an antiapoptotic transcription factor [Heckman, 2002; Karin, 2002], although under certain circumstances NF-κB also might positively contribute to apoptosis induction [Grimm, 1996b]. Besides inducing the expression of pro-survival Bcl-2 members, NF-κB additionally transactivates a number of other antiapoptotic genes, such as the IAPs (inhibitors of apoptosis proteins).

IAPs are a family of antiapoptotic proteins whose prototype originally was described in baculovirus with many homologues found to be conserved across several species. So far, eight human IAP homologues have been identified, among others NAIP, c-IAP1, c-IAP2, XIAP and survivin. All IAPs contain baculovirus IAP repeat (BIR) domains, 70 amino acid motifs, which are essential for the antiapoptotic properties of IAPs [Takahashi, 1998] because it is the interaction between the BIR domains and caspases that is believed to confer most of the antiapoptotic activity of IAPs. Indeed, XIAP, c-IAP1 and c-IAP2 are thought to directly inhibit caspases-3, -7, and –9 [Salvesen, 2002a]. In case of XIAP, it is the BIR3 domain that directly binds to the small subunit of caspase-9, whereas it is the BIR2 domain that interacts with the active-site substrate binding pocket of caspases-3 and –7 [Huang, 2001; Srinivasula, 2001].

In addition to the BIR domains, c-IAP1, c-IAP2, and XIAP contain a highly conserved RING domain at their C-terminal end which possesses E3 ubiquitin ligase activity (see paragraph 1.2.1). Via this RING domain, IAPs are able to catalyze their own ubiquitination, thereby targeting themselves for degradation by the proteasome [Yang, 2000], but they also might target other proteins such as caspase-3 and –7 for ubiquitination and degradation [Huang, 2000; Suzuki, 2001]. Direct inhibition of caspase activity by c-IAPs is certainly a very important means of regulation when considered that signalling cascades mediated by proteolytic enzymes such as caspases is irreversible once activated and therefore must be precisely regulated in order to prevent locally and temporally inappropriate demise of cells. Importantly, Smac/Diablo, when released from the mitochondrial intermembrane space during mitochondrial apoptotic events, is able to counteract the inhibitory effect of IAPs on caspases since Smac/Diablo can bind to e.g. XIAP in a manner that displaces caspases from XIAP and enables their activation. Thus, Smac/Diablo is a negative regulator of IAPs and in this way unfolds its apoptosis-enhancing property [Du, 2000]. Essential for the ability of Smac/Diablo to bind to XIAP and to release caspases is a conserved tetrapeptide motif which is also present in HtrA2/Omi, another mitochondrial proapoptotic factor [van Loo, 2002], as well as in the *Drosophila* proapoptotic proteins Reaper, Hid, Grim, and Sickle which can bind to DIAP1, a *Drosophila* IAP and thereby support activation of the fly caspase Dronc [Hawkins, 2000].

1.1.6 Disease as a consequence of dysregulated apoptosis

In the adult human body several hundred thousand cells are produced every second by mitosis, and a similar number die by apoptosis for the maintenance of homeostasis and for specific tasks such as the regulation of immune cell selection and activity [Fadeel, 1999b]. Dysregulation of apoptotic signalling can play a primary or secondary role in various diseases with insufficient apoptosis leading to e.g. cancer (cell acumulation, resistance to therapy, defective tumor surveillance by the immune system), autoimmunity (failure to eliminate autoreactive lymphocytes), persistent infections (failure to eradicate infected cells), whereas excessive apoptosis contributes to e.g. neurodegeneration (Alzheimers' disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis), autoimmunity (uncontrolled apoptosis induction in specific organs), AIDS (depletion of T lymphocytes), and ischaemia (stroke, myocardial infarction) [Reed, 2002]. Malfunction of the death machinery results from the mutation of genes that code for factors directly or indirectly involved in the initiation, mediation, or execution of apoptosis, and several mutations in apoptosis genes have been identified as a causing or contributing factor in human diseases [Mullauer, 2001].

Of special interest is the involvement of defective apoptosis pathways in tumor formation, progression, and metastasis as well as the occurrence of multidrug resistance during cancer therapy [Johnstone, 2002]. During the last years it became more and more evident that tumorigenesis is not merely the result of excessive proliferation due to the activation of oncogenes but to the same extent depends on the – frequently concurrent - impairment of apoptosis checkpoints [Hanahan, 2000; Wang, 1999]. Intriguingly, many of the alterations that induce malignant transformation, such as oncogene-driven deregulated proliferation and invasion, actually sensitize a cell to apoptosis, and therefore only those oncogenic transformed cells will survive and become malignant which additionally acquire defects in apoptosis pathways and therefore are protected against cell death induction [Vousden, 2002]. A transformed cell can achieve protection against apoptosis by inappropriate activation or expression of antiapoptotic proteins (which usually act as oncogenes), or by the inactivation of proapoptotic factors (which usually are tumor-suppressors).

As an example and as already mentioned (paragraph 1.1.5.1, page 13), *Bcl-2* was the first apoptosis-related gene that was recognized to play a role in tumorigenesis, and indeed, Bcl-2 is overexpressed in a variety of cancers, contributing to cancer cell survival through direct inhibition of apoptosis [Hockenbery, 1990; Reed, 1999]. Conversely, mutated or downregulated Bax and Bak are observed in certain cancers [Kondo, 2000; Rampino, 1997] and disruption of those genes promotes tumorigenesis in mice [Yin, 1997]. Further, proapoptotic Bad and procaspase-9 are negatively regulated by the oncogenic Akt/PKB kinase, which on the other hand is frequently constitutively active or amplified in many types of human cancer [Nicholson, 2002], and its antagonist, the phosphatase PTEN, is one of

the most commonly mutated tumor-suppressors [Yamada, 2001]. Even more underlining its potential as an oncogene, Akt/PKB is also stimulating the NF-κB survival pathway by phosphorylation of IκB kinase α (IKK α) and it is suppressing p53 proapoptotic signalling by phosphorylation of the oncogene Mdm2 which thereby is activated for inhibition of p53 [Mayo, 2002]. Both, NF-κB and Mdm2, are themselves inappropriately activated or overexpressed in the process of transformation [Chene, 2003; Orlowski, 2002].

A paradigm for the central importance of apoptosis checkpoints in the defense against malignant transformation presents the tumor suppressor p53, which is presumably the most intensely studied apoptosis factor contributing to cancer because it is inactivated in presumably more than 50% of all human cancers [Hainaut, 2000]. p53 is a tumor suppressor protein which is activated as a transcription factor in response to e.g. oncogene activation, hypoxia and especially DNA damage, resulting in growth arrest and/or apoptosis by stimulating the expression of various p53 target genes such as p21, Bax, Puma, Noxa, Apaf-1, Fas, and DR5 [Vousden, 2002] or by repressing the expression of antiapoptotic proteins, e.g. Bcl-2, Bcl-XL or survivin [Hoffman, 2002; Wu, 2001]. Recent evidence suggests transcription-independent p53 apoptosis pathways in which p53 translocates to the mitochondria, interacts with Bcl-X_L, induces PT and the release of cytochrome c [Mihara, 2003].

In non-stressed, undamaged cells p53 therefore must be kept under stringent control: it is present only at low cellular concentrations, it is retained in the cytosol and prevented to enter the nucleus, and its transactivation domain is inactivated [Chene, 2003]. Central to p53 regulation is the oncogene Mdm2 which binds to and thereby inhibits p53 (see **Fig. 1.7**). Mdm2 is a ubiquitin-ligase which mediates ubiquitination of p53, thereby targeting it for degradation by the proteasome. In this way, p53 levels are kept low in normal cells [Kubbutat, 1997]. The importance of Mdm2 in the control of p53 is demonstrated by *mdm2* gene knockout mice which die early during development but are rescued from death by additional deletion of the *p53* gene [Montes de Oca Luna, 1995].

In response to cellular stress (such as DNA damage), p53 is phosphorylated at specific serine/threonine residues which prevents the Mdm2-p53 interaction, and thus p53 is stabilized and activated [Schon, 2002]. Moreover, p53 is central to oncogene-induced cell death because it is induced by oncogenes such as c-myc, adenovirus E1A, and ras as well as by loss of the retinoblastoma tumor suppressor pRb [Henriksson, 2001]. All those oncogenes activate the transcription factor E2F-1 which not only can promote cell cycle progression and proliferation but at the same time directly triggers expression of the tumor suppressor ARF which leads to stabilization and activation of p53 [Ginsberg, 2002]. This explains in part why oncogene activation not always leads to uncontrolled cell proliferation but under certain circumstances to the stabilization of p53 and activation of cell death, provided that p53 signalling pathways are intact [Eischen, 1999].

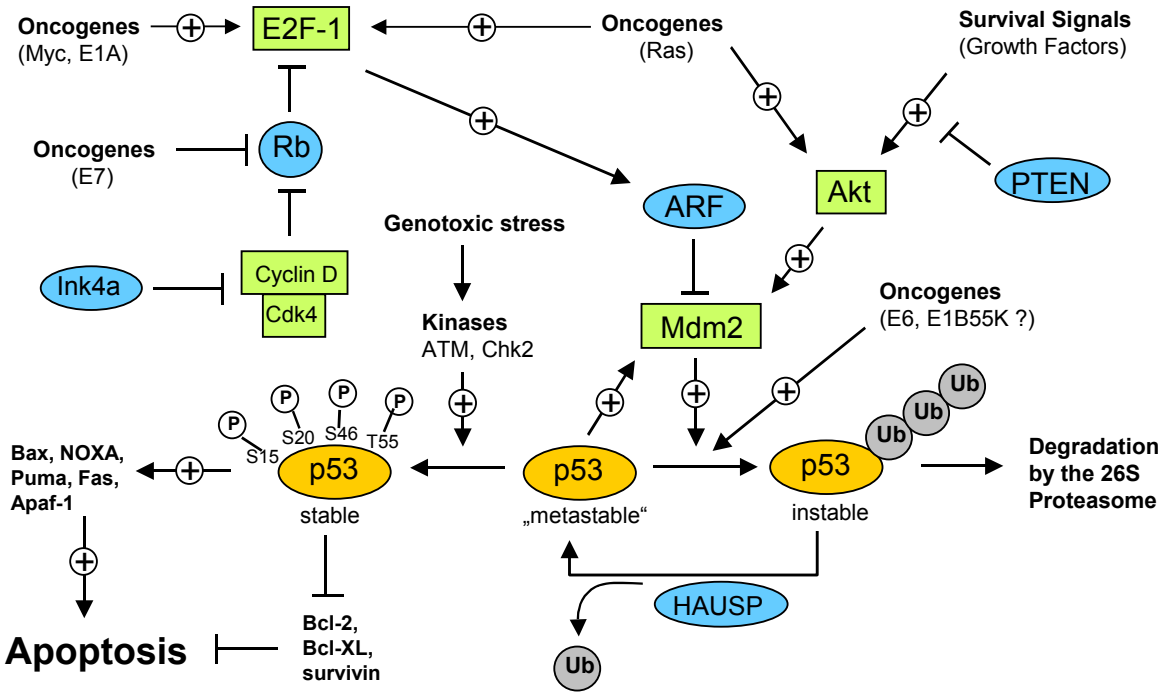


Fig. 1.7
The p53 network – survival and cell death regulation. *In a normal growing viable cell, the p53 protein is in a metastable state, i.e. p53 is susceptible to targeted ubiquitination and subsequent proteasomal degradation. Mdm2 directly interacts with p53 and thereby catalyzes ubiquitination of p53. Ubiquitination of p53 can be reversed by the action of the deubiquitinating enzyme HAUSP which thereby can rescue p53 from degradation (see also paragraph 1.2.3.2). p53 is stabilized in response to genotoxic stress such as DNA damage which leads to the phosphorylation of p53 at several specific serine and threonine residues. Stabilized and activated p53 can translocate into the nucleus where it activates the transcription of proapoptotic genes and suppresses the transcription of antiapoptotic genes what under certain conditions can result in the induction of apoptosis. p53-mediated apoptosis signalling is dependent on the interplay of many regulatory factors, including protooncogenes as well as tumor-suppressors. Mdm2 activity is positively regulated by the action of the Akt kinase: when Mdm2 is phophorylated by Akt, Mdm2 is able to translocate from the cytosol to the nucleus where it unfolds its inhibitory effect on p53. Akt kinase, on the other hand, is activated in response to survival signals coming from growth factor receptors. This is therefore an instructive example for the negative regulation of proapoptotic, p53-mediated signals by survival signalling. Whereas Akt kinase positively regulates Mdm2 activity, Mdm2-mediated suppression of p53 is blocked by the action of the ARF tumor suppressor. By binding to Mdm2, ARF prevents the interaction between Mdm2 and p53 and therefore stabilizes and activates p53. ARF expression is dependent on the transcription factor E2F-1 which is regulated by the retinoblastoma (Rb) tumor-suppressor and by the action of oncogenes. As an example, mitogenic signals lead to the activation of oncogenes such as c-myc and ras which among others activate E2F-1, resulting in increased ARF activity, stabilization of p53 and induction of apoptosis. Therefore, increased mitogenic signalling or inappropriate oncogenic activity not necessarily causes excessive proliferation but in cells with intact p53 signalling pathways can act as apoptosis inducers.*

Therefore in many instances, an oncogenic insult only results in increased proliferation and eventually malignant transformation if activators of p53 (such as ARF, Chk2, or ATM), p53 itself (e.g. by Mdm2, the adenovirus E1B, papillomavirus E6, or SV40 large T antigen), or p53 downstream signalling components (p53 target genes) are inactivated (**Fig. 1.7**).

On the other hand, p53-mediated apoptosis pathways can be suppressed by survival signals, such as growth factors binding to their cognate growth factor receptors what eventually results in activation of the Akt kinase [Datta, 1999]. Akt kinase is known to mediate a number of antiapoptotic mechanisms, such as the direct phosphorylation and inactivation of Bad and caspase-9, the activation of NF-κB antiapoptotic signalling via phosphorylation of IκB, but also phoshorylation and activation of Mdm2 as an inhibitor of p53 [Mayo, 2002]. Besides phosphorylation, ubiquitination and protein-protein interactions, p53 is also regulated by acetylation what affects its transcriptional activity, as well as by sumoylation [Melchior, 2003; Appella, 2001].

Gaining insight into the mechanisms and alterations by which components of the apoptotic machinery contribute to pathogenic processes, should allow the development of more effective, higher specific and therefore better-tolerable therapeutic approaches. Those may include the targeted activation of proapoptotic tumor suppressors or alternatively the blockade of antiapoptotic oncogenes in the case of cancer, whereas for the treatment of premature cell death during e.g. neurodegeneration the inhibition of proapoptotic key components such as the caspases might be promising [Reed, 2002].

1.1.7 Non-caspase proteases also play a role in cell death processes

Contrary to earlier expectations, the inhibition of caspases does not always prevent cell death, and some researchers even claim that e.g. the pan-caspase inhibitor zVAD-fmk never can completely save cells from dying, whatever death stimulus is used [Borner, 2003; Leist, 2001a]. Usually, the activation of caspases and the consequent cleavage of their specific substrates is responsible for many of the typical morphological and molecular features of apoptosis. Examples are the cleavage of genomic DNA into oligonucleosomal fragments by the CAD/ICAD system [Nagata, 2000], chromatin condensation mediated by activation of acinus [Sahara, 1999], nuclear disassembly by cleavage of lamins [Lazebnik, 1995], cytoplasmic morphological changes by cleavage of actin, alpha-fodrin and gelsolin [Earnshaw, 1999], and plasma membrane blebbing by the activation of ROCK kinases [Sebbagh, 2001]. Cell death occurring in the absence of caspase activity, for example in presence of zVAD-fmk or in cells deficient for caspase-3 such as Mcf-7, still displays incomplete chromatin condensation and a varying degree of apoptosis-like morphological features or results in a necrosis-like form of programmed cell death, i.e. in the absence of chromatin condensation but still in a not totally uncontrolled process [Leist, 2001a]. Thus, it must be assumed that besides caspases also other proteases might play a role in apoptosis-like cell death processes. Indeed, many non-caspase proteases,

e.g. calpains, can cleave at least some of the classical caspase substrates, indicating that they might mimic or substitute for some of the cellular effects of caspases [Orrenius, 2003; Johnson, 2000; Leist, 2001b; Wang, 2000].

During recent years, the cathepsins, lysosomal cysteine proteases, were reported to possibly contribute to the apoptotic proteolytic network, and also the ubiquitin/proteasome system has been implicated in the mediation and regulation of apoptosis pathways. The next two chapters will provide an insight into what is known about the involvement of the ubiquitin/proteasome system in the control of apoptosis and about the role of cathepsins in caspase-independent cell death pathways.

1.2 Apoptosis and the ubiquitin/proteasome system

Apoptosis signalling pathways are tightly controlled by diverse regulatory mechanisms, including protein-protein interactions, transcriptional regulation, phosphorylation, but also ubiquitination and degradation of apoptotic signalling factors by the proteasome. Indeed, accumulating evidence supports the importance of the ubiquitin/proteasome system as a regulator of cell proliferation and apoptosis, influencing the delicate balance between a cell's life and death depending on the cellular and environmental context [Jesenberger, 2002; Naujokat, 2002; Yang, 2003]. Besides regulating apoptosis, ubiquitin-dependent pathways also have been shown to play major roles in a number of other biological processes, including cell cycle regulation, transcription, DNA repair, protein trafficking, endocytosis, and regulated intramembrane proteolysis [Hicke, 2001; Muratani, 2003; Varshavsky, 2000]. Regulation of cellular processes involves the accurate modulation of steady state levels of key regulatory proteins, which can be achieved at the level of protein biosynthesis but also at the level of protein degradation.

1.2.1 The ubiquitin/proteasome system

The ubiquitin/proteasome system is the major pathway of selective protein degradation in eukaryotic cells. In order to target a protein for degradation, it is covalently attached to several units of the 76 amino acid protein ubiquitin, resulting in a polyubiquitin chain tag that labels the protein to be recognized and eventually degraded by the 26S proteasome [Kornitzer, 2000]. 26S proteasomes are large multisubunit proteolytic complexes consisting of a central catalytic machine, the 20S proteasome, and two regulatory 19S subcomplexes [Baumeister, 1998]. Proteasomes are localized to the cytoplasm and to the nuclei [Wojcik, 2003]. The 20S proteasome exhibits several proteolytic activities, including chymotrypsine-like and trypsine-like activities, the active sites of which are burried in the interior of the barrel-like structure and thus are shielded from the cytoplasm and therefore are prevented from uncontrolled protein degradation. The 19S subcomplexes consist of several proteins, some of which possess ATPase-activity and are involved in the ATP-dependent steps of proteasomal protein degradation. The 19S complex presumably functions to recognize ubiquitylated proteins, unfold them, and allow them to enter the proteolytic interior chamber of the 20S particle by possibly changing their conformation [Kornitzer, 2000].

Covalent conjugation of ubiquitin to a target protein is achieved by the sequential action of an elaborate enzymatic machinery (**Fig. 1.8**). Initially, ubiquitin is activated by E1 (ubiquitin-activating enzyme) which in a ATP-dependent reaction forms a thioester bond between a cysteine residue of its active site and the carboxyl group of ubiquitin's C-terminal glycine residue. In a trans-esterification

reaction, the activated ubiquitin is then transferred to a cysteine of a E2 enzyme (ubiquitin-conjugating enzyme). E3 proteins (ubiquitin-protein ligase) are required for specific substrate recognition and for mediating the transfer of ubiquitin from E2 to the ε-amino group of an internal lysine residue of the corresponding target protein. After covalent attachment of one ubiquitin moiety to a substrate protein, in most cases a polyubiquitin chain is generated by the conjugation of additional ubiquitin molecules to the ε-amino group of the internal Lys^{48} of a previously added ubiquitin moiety [Ciechanover, 2000; Kornitzer, 2000]. Polyubiquitylated proteins are recognized by the proteasome and are degraded. In case that a protein is modified by a single ubiquitin (mono-ubiquitination), this modification does not result in its degradation but is essential for a regulatory function of the protein in various cellular processes such as endocytosis or histone regulation [Hicke, 2001].

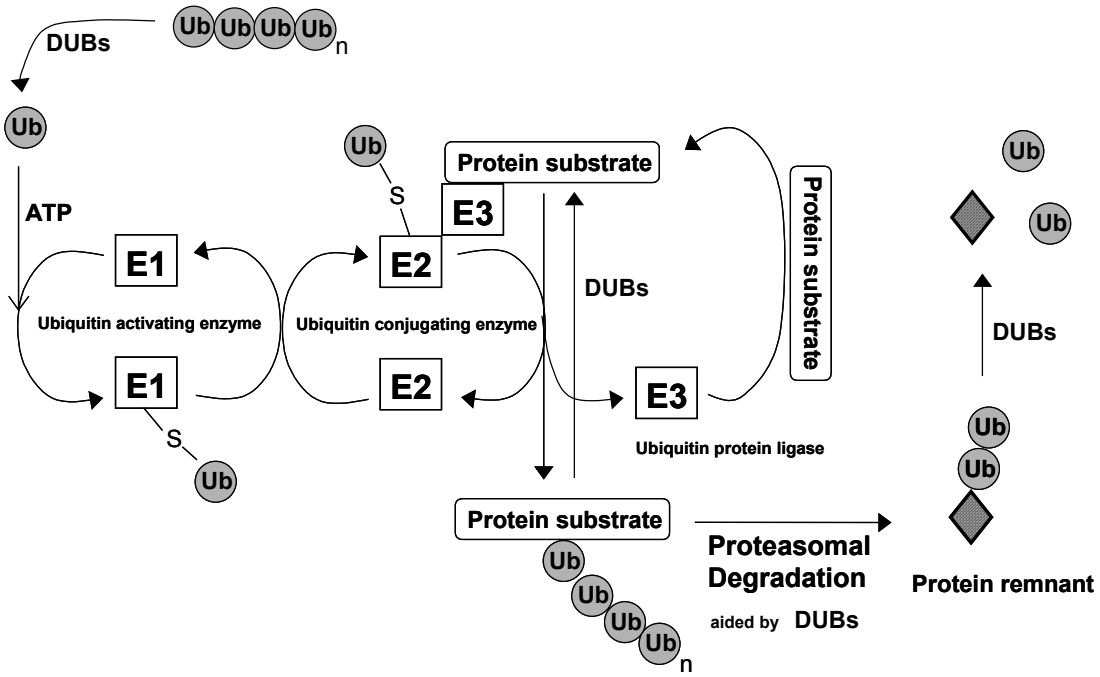


Fig. 1.8
The ubiquitin/proteasome system. *The ubiquitin/proteasome system is the major molecular machinery for the controlled degradation of specific protein substrates within the cell. A protein substrate destined for degradation by the proteasome must be labelled by the covalent attachment of usually several ubiquitin molecules which form a polyubiquitin chain. Ubiquitination of a protein substrate involves the coordinated action of the ubiquitin-activating enzyme (E1), several ubiquitin-conjugating enzymes (E2), and numerous ubiquitin ligases (E3) in an ATP consuming process. Multiubiquitylated proteins are generated through the sequential addition of further ubiquitin molecules to an already ubiquitylated protein substrate, a process that is catalysed by the same E1, E2, and E3 enzymatic complex, although sometimes requiring an additional E4 enzyme (not shown here). The attachment of one or more ubiquitin moieties to the substrate can be reversed by the action of deubiquitinating enzymes (DUBs) which in addition also serve other functions within the ubiquitin/proteasome system such as the generation of monoubiquitin from polyubiquitin precursors or from still ubiquitylated protein remnants which originate from proteasomal degradation.*

Interestingly, whereas there is only one single known E1 enzyme, several E2 enzymes (>25) and even more E3 enzymes have been identified in mammals [Pickart, 2001], also arguing that it is the combined action of E2/E3 complexes which confers substrate specificity. Up to now, two main types of E3 ubiquitin ligases have been identified, one class containing the HECT domain, the other containing RING finger domains [Yang, 2003]. The prototype for a HECT (homologous to E6-AP carboxyl terminus) domain E3 enzyme is E6-AP (E6-associated protein) which in concert with the human papillomavirus (HPV) protein E6 functions to catalyze the ubiquitination and degradation of p53 [Scheffner, 1993]. More than 30 different mammalian HECT domain E3s are expected to exist which are believed to bind, on one hand, to their specific substrate proteins by protein-protein interaction motifs such as WW domains and, on the other hand, to their cognate E2 enzyme, eventually transfering ubiquitin to the substrate via an active site cysteine of the E3 [Jackson, 2000]. Interestingly, inactivation of E6-AP leads to the neurological disease Angelman syndrome [Kishino, 1997], whereas mutation of Nedd4, another HECT domain E3, is causative for Liddle's syndrome [Abriel, 1999].

Recently, it has been recognized that all other E3 proteins (non-HECT domain E3s) share a distinct structural element, the RING finger domain [Lorick, 1999; Pickart, 2001] which consists of eight conserved cysteine or histidine residues, forming a cross-brace structure to chelate two zinc ions [Jackson, 2000]. Several E3 proteins have been identified (including Mdm2 and IAPs) which contain RING finger domains that proved to be essential for autoubiquitination and the ubiquitination of substrates [Fang, 2000; Joazeiro, 2000; Yang, 2000]. Little is known about the catalytic mechanisms by which RING fingers catalyze ubiquitination, they apparently do not directly participate in the transfer of ubiquitin from E2 to the substrate but presumably are responsible for bringing E2 and substrate together. Besides functioning as single polypeptide E3s, such as Mdm2 or IAPs, small RING finger proteins are essential components of the multi-subunit E3 complexes SCF (Skp1/Cullin/F-Box protein), APC (anaphase-promoting complex), and VHL-CBC (von Hippel-Lindau/Cul2/elongin B/elongin C). In the case of yeast SCF, the cullin protein cdc53 forms a scaffold that associates with the Ring finger protein Rbx1, which further recruits and activates the E2 protein Cdc34p. Skp1 binds to this cdc53/Rbx1/Cdc34p complex and mediates recruitment of various F-box proteins which confer substrate specificity to this type of E3s [Jackson, 2002; Joazeiro, 2000]. Interestingly, Rbx1 is also part of the VHL-CBC complex, consisting of the VHL protein (pVHL), elongin B, elongin C, and Cul2. In analogy to SCF, Cul2 corresponds to the cullin scaffold, elongin C to Skp1, and the pVHL may serve the function of a F-box protein. Indeed, VHL-CBC exhibits ubiquitin ligase activity, targeting the Hypoxia Inducible Factor-α (HIF-α) subunit (a transcription factor subunit) for polyubiquitination and subsequent degradation. pVHL-defective cells are unable to degrade HIF-α, resulting in the overproduction of HIF-α target genes such as VEGF and EPO, which indeed are frequently increased in hemangioblastomas and renal cell carcinomas, malignancies frequently associated with the von Hippel-Lindau hereditary tumor syndrome [Kaelin, 2002; Kondo, 2001].

Unexpectedly, at the same time, the tumor-suppressor pVHL appears to protect renal carcinoma cells from apoptosis induced by various stimuli possibly by upregulating Bcl-2 levels [Devarajan, 2001].

1.2.2 Regulation of the ubiquitin/proteasome system

The ubiquitin/proteasome pathway is required for a multitude of cellular functions, and therefore it is not surprising that its general components – the proteasome and the ubiquitin-activating enzymes – are constitutively active. Regulation of the proteasomal degradation of specific substrates is mainly achieved at the level of ubiquitination, in many instances involving the post-translational modification of potential substrates or by the dependence on interactions with accessory proteins [Kornitzer, 2000].

Short-lived proteins usually carry a degradation signal, the so called N-degron, consisting of a destabilizing N-terminal amino acid residue and a specific internal Lys residue, the site for the attachment of a polyubiquitin chain [Varshavsky, 1992]. Proteins with destabilizing residues (especially basic and hydrophobic amino acids) at their N-terminus are preferentially recognized by certain E2/E3 complexes such as Ubc2p/Ubr1p and consequently are ubiquitylated and degraded [Varshavsky, 2000].

An increasing number of proteins is known to require phosphorylation prior to their ubiquitination, including the mammalian G_1 cyclins D and E, the cyclin-dependent kinase (CDK) inhibitors $p21^{Waf1,Cip1}$, $p27^{Kip1}$, and the inhibitor of NF-κB, IκB-α [Bornstein, 2003; Ganoth, 2001; Tanaka, 2001]. In all those cases, phosphorylation stimulates ubiquitination by E3 ubiquitin ligases of the SCF type. Phosphorylation can also inhibit ubiquitination as has been described for e.g. Bcl-2: dephosphorylation of Bcl-2 following apoptotic stimuli renders it susceptible to degradation by the ubiquitin/proteasome pathway [Dimmeler, 1999]. Another important expample is the regulation of p53 stability by targeted phosphorylation at specific serine/threonine residues. Besides phosphorylation, the modification of proteins by SUMO (small ubiquitin-like modifier) might interfere with ubiquitination as has been reported for Mdm2 and IκB-α [Muller, 2001].

1.2.3 Deubiquitinating enzymes (DUBs)

Deubiquitinating enzymes (DUBs) are cysteine proteases that specifically cleave ubiquitin conjugates at the ubiquitin carboxy terminus. There are two major families of DUBs, the UCH (ubiquitin carboxy terminal hydrolase) family and the USP (ubiquitin-specific protease) family. The UCHs are small enzymes most probably involved in co-translational processing of polyubiquitin chains or in the release of ubiquitin from adducts with small peptides or amino acids. In contrast, USPs are usually

much larger proteins which besides the core catalytic domain (consisting of a cysteine domain, an aspartate domain, a histidine domain, and three additional homology domains) possess N- or C-terminal extensions which might allow the selective or even specific interaction with distinct substrate proteins or determines cellular localization [D'Andrea, 1998]. DUBs (especially USPs) are able to cleave off intact ubiquitin molecules conjugated to other proteins, potentially providing reversibility to the ubiquitination system. Therefore one might expect a major impact of DUBs on the regulation of signalling pathways that are modulated by the ubiquitin/proteasome system, especially when regarded in analogy to protein kinase/phosphatase signalling systems. On the other hand, DUBs act at multiple levels in the ubiquitin pathway, particularly also serving some quite basic functions (**Fig. 1.9**).

1.2.3.1 General functions of DUBs

Ubiquitin is encoded by a multigene family of which many ubiquitin genes express multimeric head-to-tail ubiquitin repeat proteins (polyubiquitins) or ubiquitin fused to ribosomal proteins L40 and S27a [Archibald, 2003].

Since only monomeric ubiquitin can be used by the ubiquitin/proteasome system, the linear polyubiquitins and the ubiquitin fusion proteins are assumed to be processed into monomeric ubiquitin units by deubiquitinating enzymes, although the responsible enzyme(s) have not been identified thus far [Wilkinson, 1997]. Moreover, proteasomal degradation often produces a protein remnant that is still attached to its branched chain polyubiquitin tag, and DUBs such as the yeast Doa4 are able to remove the remnant from the polyubiquitin chain which subsequently is disassembled to monoubiquitin molecules by the action of isopeptidase T /USP5 [Papa, 1993; Wilkinson, 1995]. Therefore, certain DUBs serve general housekeeping functions by maintaining a sufficient pool of free monomeric ubiquitin and/or by preventing the accumulation of polyubiquitin chains, which might interfere with proteasomal degradation [Wilkinson, 1997]. Also a more general role might play the PA700 isopeptidase which is associated with the 19S proteasomal subcomplex, potentially inhibiting ubiquitin-mediated proteolysis by releasing ubiquitin chains from substrates before they reach the proteasome. PA700 thus provides a correction or proofreading function by preventing degradation of mistakenly ubiquitylated proteins [Kornitzer, 2000; Lam, 1997].

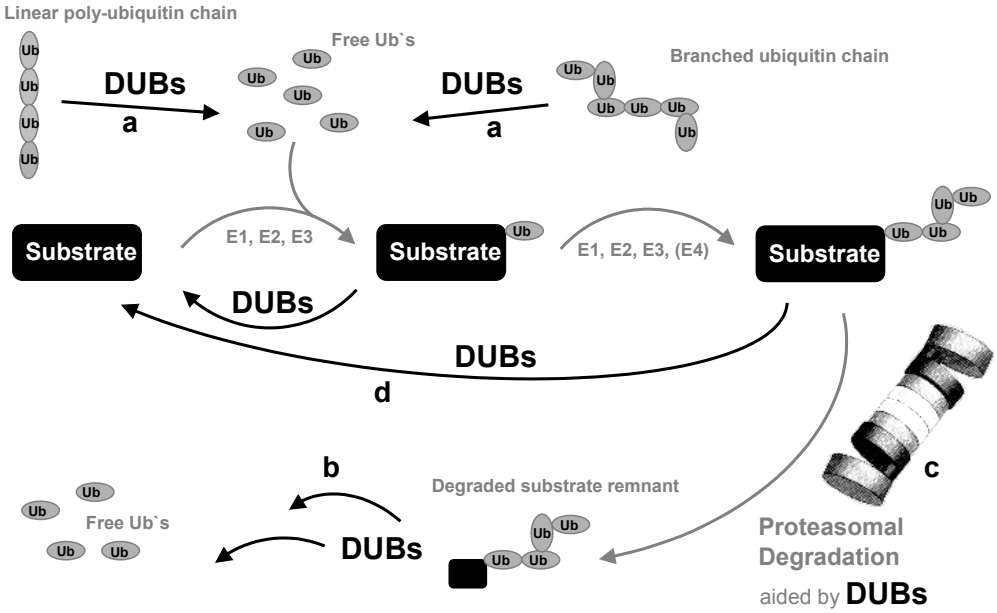


Fig. 1.9

The various functions of deubiquitinating enzymes as part of the ubiquitin/proteasome system. *DUBs are responsible for providing monoubiquitin units to the ubiquitination system by processing linear and branched chain polyubiquitin precursors (a) or by removing ubiquitin moieties still attached to protein remnants that result from the degradation of a protein at the proteasome (b). In addition, DUBs might be part of the proteasome multienzyme complex and thereby promote degradation of proteins by removing ubiquitin chains that otherwise would interfere with proteasome activity (c). Finally, DUBs supposedly possess specific regulatory functions by their ability to deubiquitinate distinct target proteins which thereby are rescued from degradation by the proteasome (d).*

1.2.3.2 Specific regulatory functions of DUBs

Whereas only a few different mammalian UCH enzymes are known, an increasing number (> 30) of mammalian USP family members has been identified, further supporting the assumption that USPs should play a role in specific biological processes by regulating the ubiquitination status and consequently the stability of discrete substrate proteins (also see Box 4.1).

An interesting example for the implication of USPs as regulatory enzymes in the response to growth factor signalling are DUB-1 and DUB-2. After addition of IL-3 to G_1 arrested hematopoietic cells, DUB-1 mRNA levels were reported to be rapidly induced, but to decline again before completion of G_1. Artificially prolonged expression of DUB-1 resulted in G_1 arrest, suggesting that DUB-1 expression must be tightly regulated and that DUB-1 may play an important role in cytokine-induced cell proliferation [Zhu, 1996]. Similar results were reported for the homologous DUB-2, which is induced by IL-2 in T lymphocytes, but no specific substrates of DUB-1 or DUB-2 have been identified so far [Zhu, 1997].

Several DUBs seem to have important roles in development, such as the product of the fat facets gene (*faf*) in Drosophila, which is required in the pathway of eye facets development [Huang, 1995]. In flies with defective *faf*, a signal that directs the death of certain cells in the facets precluster is not generated and the fly eyes consequently develop with additional photoreceptor cells. The *faf*$^{-}$ phenotype could be suppressed by proteasome mutants, suggesting that one function of faf is the deubiquitination of (a) short-lived regulatory molecule(s) which is essential for eye patterning [Wilkinson, 1997]. A recent report identified the liquid facets gene (*lqf*) as a specific substrate for faf-mediated deubiquitination. Lqf regulates endocytosis and such might be involved in the control of cell-cell comunication [Chen, 2002]. Interestingly, a faf homologue has been identified in mouse (Fam) that is expressed in rapidly dividing cells, among others in the apoptotic region between the digits [Wood, 1997]. Experiments indicated that Fam interacts with and might stabilize β-catenin by deubiquitination [Taya, 1999].

Suggesting a role of DUBs in oncogenesis, the ubiquitin-specific protease Unp has been discussed to act as a pre-oncogene when overexpressed and to be elevated in lung cancers [Gupta, 1994], although subsequent characterization of Unp could not confirm those initial results [Frederick, 1998]. In the meantime, Unp has been reported to physically interact with the retinoblastoma protein (RB) further suggesting a possible role for this isopeptidase in cancer [DeSalle, 2001].

Of major interest is the ubiquitin-specific protease HAUSP (herpes virus associated USP), which initially was described to be essential for Herpes virus infections [Everett, 1997], but recently was identified to specifically bind, deubiquitinate and stabilize p53 (**Fig. 4.1.1**). In overexpression experiments, HAUSP stabilized p53, enhanced p53-mediated cell death, and even rescued p53 from Mdm2-mediated inactivation. Moreover, prolonged overexpression of HAUSP in p53 positive but not p53 deficient carcinoma cell lines resulted in growth inhibition, suggesting that HAUSP might function as a tumor suppressor through the stabilization of p53 [Li, 2002]. Interestingly, HAUSP also appears to be processed in a caspase-3-dependent manner during thymocyte apoptosis, with the cleavage product still possessing enzymatic activity. This observation might be a possible link between the caspase and ubiquitin/proteasome proteolytic death pathways [Vugmeyster, 2002].

Another example for the implication of deubiquitinating enzymes in pathways that determine cell survival and cell death has been described recently. CYLD is a tumor-suppressor frequently mutated in cases of familial cylindromatosis, an autosomal dominant predisposition to tumors of skin appendages. CYLD was shown to interact with IKK-γ (NEMO) of the IKK complex and to possess deubiquitylating enzymatic activity towards the TNF receptor associated factor 2 (TRAF2). TRAF2 in its ubiquitylated status positively stimulates the phosphorylation of IκB which thereupon is ubiquitylated and degraded, allowing the activation of NF-κB survival signalling. CYLD removes ubiquitin from TRAF2 and therefore interferes with NF-κB activation. Interestingly, downregulation of the CYLD mRNA message results in enhanced activation of NF-κB and in a reduction of cell death induced by TNF-α [see **Fig. 4.1.2** and Brummelkamp, 2003; Kovalenko, 2003; Trompouki, 2003].

1.2.4 Involvement of the ubiquitin/proteasome system in apoptosis

1.2.4.1 The proteasome and its impact on survival and cell death

The ubiquitin/proteasome system appears to be required for both, survival and cell death processes, presumably depending on the cellular and physiological context. First indications for an apoptosis promoting function of the ubiquitin/proteasome system came from studies focusing on developmental cell death occuring in abdominal intersegmental muscles of the tobacco hawkmoth, *Manduca Sexta*. This apoptotic muscle regression is accompanied by a dramatic increase in ubiquitin expression and proteasomal proteolytic activity, as well as a change in proteasome subunit composition [Naujokat, 2002]. Proteasomal activity also might be required for the progression of apoptosis in non-proliferating, differentiated cells, since apoptotic cell death of resting thymocytes and differentiated neuronal cells was inhibited in the presence of proteasome inhibitors [Grimm, 1996a].

In contrast, rapidly proliferating cells, including most cancer cell lines in culture, appear to display abnormally high proteasomal expression and activity, suggesting that the 26S proteasome plays a pivotal role in maintaining survival and proliferation of abnormally growing cells [Ichihara, 1995; Ichihara, 1993]. Supporting this "survival hypothesis", proteasome inhibitors have been reported to induce apoptosis in neoplastic and rapidly growing mammalian cells of hematopoietic, neuronal, mesenchymal, and epithelial origin [Naujokat, 2002].

Underlying those observations, the proteasome was discovered as a target for cancer therapy, and selective proteasome inhibitors, such as PS-341, are currently evaluated as novel chemotherapeutic agents in cancer patients with advanced disease [Adams, 2000]. The role of proteasome inhibition in cancer therapy may ultimately lie in its ability to overcome chemoresistance, and indeed, proteasome inhibitors were shown to sensitize cancer cells to conventional cytotoxic drugs [Shah, 2001]. The molecular mechanisms responsible for the impact of proteasome inhibitors on apoptotic pathways are not fully understood but they certainly eventually lead to an interference with the delicate balance of apoptotic and survival signals which are under the control of the ubiquitin/proteasome system. A recent example is the observation that the proteasome inhibitor PS-341 sensitizes various cancer cells to TRAIL-induced apoptosis. This effect is likely due to the enhanced expression of the TRAIL receptors DR4 and DR5 [Johnson, 2003].

1.2.4.2 Apoptosis pathways regulated by ubiquitin/proteasome-dependent proteolysis

A manifold of apoptotic signalling factors are direct targets for ubiquitination with consequent degradation by the proteasome **(Fig. 1.10)**, and several survival and death signalling pathways are regulated at multiple points by the ubiquitin/proteasome system (**Fig. 1.11**). One of the most prominent examples is the regulation of the p53 signalling network which involves oncogene-regulated (e.g. Mdm2, E6) ubiquitination of p53 that is dependent on the p53 phosphorylation status and the interplay of p53 with regulatory factors such as ARF and the ubiquitin-specific protease HAUSP (**Fig. 1.7** and **Fig. 4.1.1**).

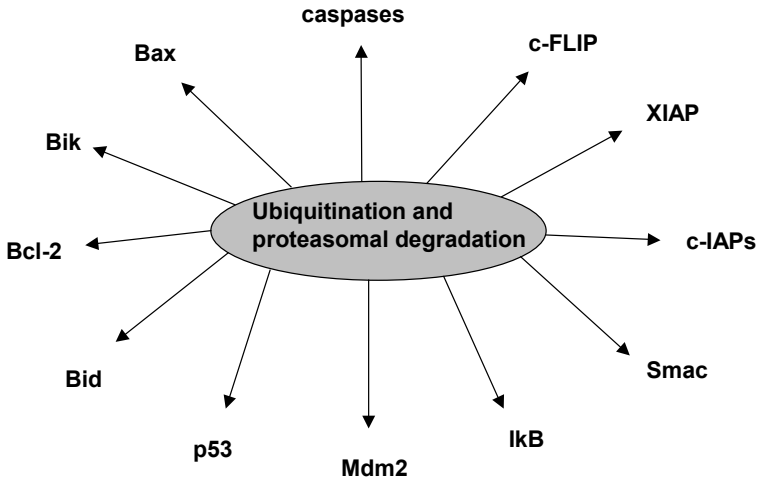


Fig. 1.10
Apoptosis factors as targets of ubiquitination and degradation by the proteasome. *Selected proteins which have been described to be ubiquitylated and degraded by the ubiquitin/ proteasome system [Yang, 2003; Naujokat, 2002; Jesenberger, 2002].*

Another example is NF-κB signalling which usually is regarded as a survival pathway that can suppress apoptosis by promoting the expression of antiapoptotic molecules such as Bcl-2, c-FLIP and IAPs. NF-κB is kept inactive when sequestered in the cytoplasm by its inhibitor IκB. In response to survival signals (e.g. activation of Akt kinase or TNF-α survival pathways), IκB is phosphorylated, ubiquitylated and degraded by the proteasome, resulting in the translocation of NF-κB into the nucleus where it is active as a transcription factor. Dysregulated proteasomal degradation of IκB can result in abnormal high levels of NF-κB activity in neoplastic cells [Guzman, 2001; Izban, 2001], and it is the stabilization of IκB and the consequent blockade of NF-κB signalling that might be one major mode of action of proteasome inhibitors in the sensitization of tumor cells to cell death induction [Adams, 2000]. Target genes of NF-κB such as IAPs and Bcl-2 are themselves targets for ubiquitination and

proteasomal degradation in response to apoptosis induction [Naujokat, 2002], with IAPs possessing RING-mediated ubiquitin ligase activity, resulting in their auto-ubiquitination as well as ubiquitination of caspases.

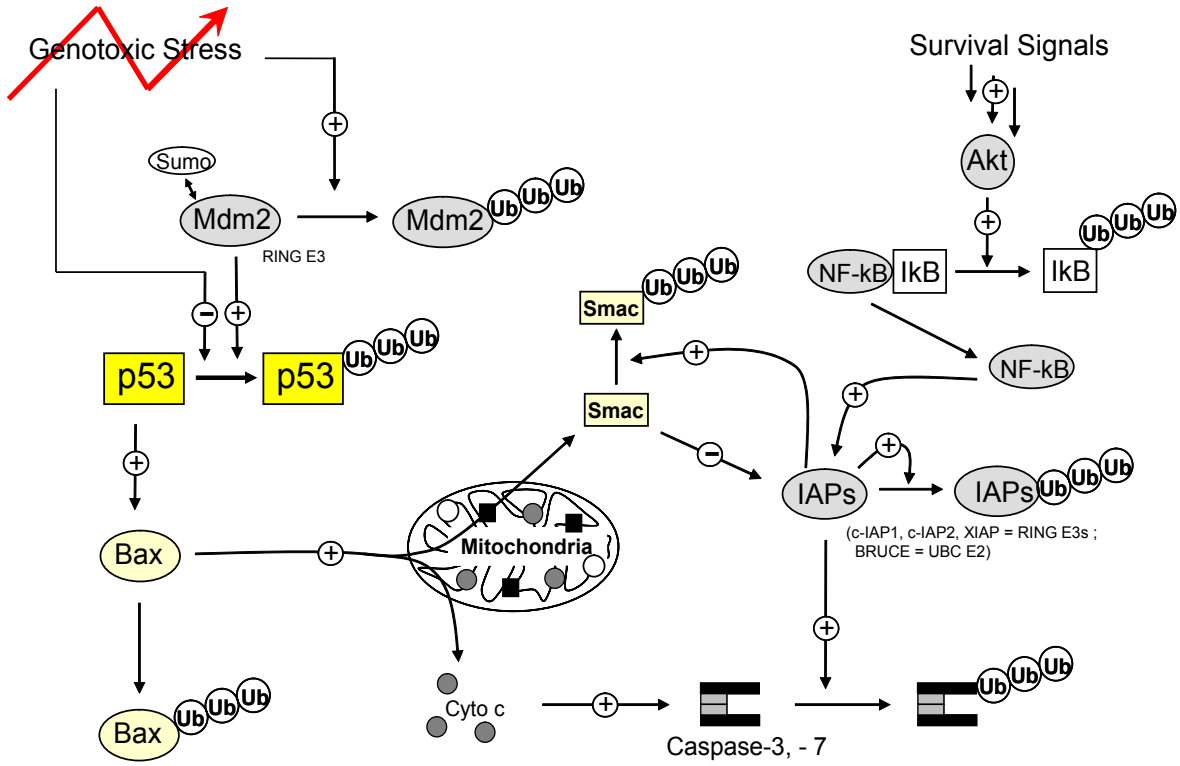


Fig. 1.11
Apoptosis and survival pathways are regulated at multiple points by the ubiquitination/ proteasome system. *Several factors of the p53 signalling network are targets for ubiquitination, i.e. p53 itself but also its central regulator Mdm2 which is a E3 ubiquitin ligase. Auto-ubiquitination of Mdm-2 can be influenced by modification with the ubiquitin-like protein SUMO. Also the stability of the central apoptosis-inducers Bax and of caspases-3 and -7 is dependent on the ubiquitin/proteasome system. Moreover, the activation and mediation of survival signalling pathways is affected by ubiquitination at several points, particularly on the level of NF-κB activation. The inhibitors of apoptosis (IAPs) possess E3 ubiquitin ligase activity and mediate auto-ubiquitination as well as ubiquitination of caspases [Jesenberger, 2002; Yang, 2003].*

1.3 Lysosomes and their possible involvement in apoptosis

1.3.1 Lysosomes are 'suicide bags'

Lysosomes are the major compartment for cellular catabolism and contain about 40 types of hydrolytic enzymes mostly active at acidic pH, e.g. proteases, nucleases, lipases, and glycosidases [deDuve, 1966]. Christian de Duve, soon after his discovery of these organelles, nicknamed them 'suicide bags' and suggested that an uncontrolled leakage of their enzymes to the cytoplasm might be lethal for cells [deDuve, 1969]. For many years, only non-selective degradation of long-lived intracellular and extracellular proteins was attributed to lysosomal proteases, but accumulating evidence suggests an implication of lysosomes in more selective types of protein degradation contributing to various biological processes [Turk, 2000]. The largest group of lysosomal proteases are the papain-like cysteine proteases, the cathepsins, which besides being part of the general proteolytic machinery inside the lysosome, are also involved in more specific processes such as the maturation of MHC class II molecules (cathepsins L, S, and V), bone remodeling (cathepsin K), or specifically processing protein substrates [Reinheckel, 2001; Turk, 2000].

1.3.2 Evidence for a role of lysosomes and cathepsins in cell death

Numerous observations suggest that cathepsins may also act as mediators of programmed cell death, since in response to various apoptotic stimuli, cathepsins appear to translocate from the lysosomes to the cytosol [Antunes, 2001; Brunk, 2001; Kessel, 2000; Neuzil, 2002]. This translocation is believed to be the result of a more or less severe damage to the lysosomal membrane, and it is not likely that distinct enzymes (such as cathepsins), are selectively released from the damaged lysosomes but that most lysosomal hydrolytic enzymes should be able to diffuse into the cytosol [Brunk, 2001; Turk, 2002]. It has been proposed that there might be a quantitative relationship between the extent of lysosomal rupture and the resulting mode of cell death. According to this model, a partial rupture of the lysosomal membrane would cause a limited release of lysosomal content resulting in apoptosis, whereas a complete rupture would lead to necrosis [Leist, 2001b]. Although cathepsins are actually expected to be largely inactive at neutral cytoplasmic pH, cathepsins (as reported for cathepsin B) might still display endopeptidase activity under cytosolic conditions [Leist, 2001b]. Another obstacle for cathepsins being proteolytically active in the cytoplasm would be the presence of intracellular protease inhibitors, the stefins. Interestingly, stefin B knockout resulted in substantial cerebellar apoptosis, further supporting a possible involvement of cathepsins in neuronal cell death. Also in

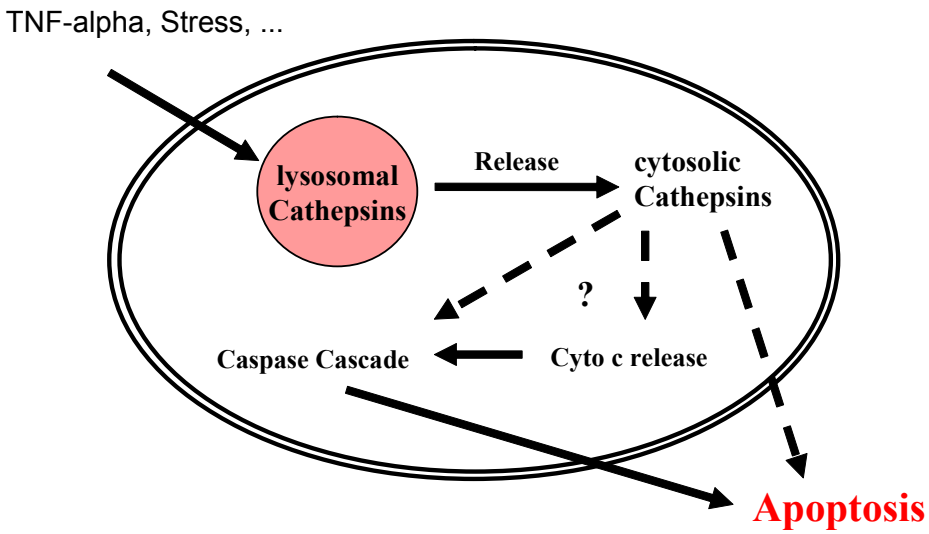


Fig. 1.12
Cathepsins as putative mediators of cell death signals. *Lysosomes have been proposed to be an integral part of certain apoptosis pathways. Apoptosis-inducing signals such as TNF-α or oxidative stress were reported to result in a (partial) rupture of the lysosomes and in the release of their hydrolytic content into the cytosol. Therefore, lysosomal cysteine proteases, the cathepsins, might mediate proteolysis-dependent activation of cytosolic processes, such as the processing of the Bcl-2 member Bid with subsequent cytochrome c release from the mitochondria, or even direct activation of the caspase cascade. Thus far, the mechanisms by which cathepsins might act in apoptosis signalling have not been elucidated in detail.*

agreement with an active role of cathepsins in apoptosis, inhibition of cathepsin activity by e.g. overexpression of stefin A or by synthetic cathepsin inhibitors, was reported to block cell death induced by a variety of stimuli [Guicciardi, 2001; Hishita, 2001; Kagedal, 2001b; Kiso, 2001; Mathiasen, 2001; Turk, 2002].

1.3.3 Cathepsins - proteases with proapoptotic activity

The mechanisms by which cathepsins, once released to the cytoplasm, might contribute to apoptosis pathways are still not completely understood, but might include the cleavage and activation of Bid and the consequent release of cytochrome c from mitochondria to the cytosol, whereas the direct activation of caspases by cathepsins remains questionable [**Fig. 1.12** and Guicciardi, 2000; Stoka, 2001].

Overexpression as well as microinjection of the aspartic lysosomal protease cathepsin D (cath-D) were reported to result in apoptosis induction [Deiss, 1996; Roberg, 2002], and ceramide, which possibly acts as an apoptosis-inducing second messenger, was reported to trigger the autocatalytic proteolysis and activation of cath-D [Gewies, 2000a; Heinrich, 1999]. Moreover, there are reports that suggest a

role for cath-D in the mediation of extrinsic and intrinsic apoptosis pathways, for example by initiating mitochondrial apoptotic events such as activation of Bax [Bidere, 2003; Demoz, 2002; Kagedal, 2001a; Roberg, 2001].

Accumulating evidence appears to implicate cathepsin B (Cath-B) in the mediation of proapoptotic signalling what might prove to be important in terms of a possible connection between tumorigenesis, apoptosis, and its exploitation for novel therapeutic approaches. Cath-B expression levels, but also those of Cath-L, are frequently strongly elevated in tumors of diverse origin and in their secreted form are thought to contribute to cancer progression and metastasis either by directly degrading the extracellular matrix or by proteolytically activating other extracellular proteases [Turk, 2000; Chauhan, 1991; Duffy, 1996; Konduri, 2001; Kos, 1998; Sloane, 1990; Yan, 1998]. Therefore, Cath-B and Cath-L are usually regarded as tumor markers correlated with bad prognosis. On the other hand, in recent years there has been increasing evidence that particularly Cath-B might be involved in the mediation of apoptotic cell death triggered by various stimuli. Most importantly, cells deficient or downregulated in Cath-B appear to be more resistant to TNF-α-mediated apoptosis [Guicciardi, 2000;Foghsgaard, 2001]. Inhibitor experiments further supported the view that CathB might act as a dominant executioner protease in TNF-α-induced apoptosis pathways [Foghsgaard, 2001]. Caspase-8, which is the immediate initiator caspase in the TNF-alpha death pathway, might be able to trigger lysosomal release of CathB, and it has been shown *in vitro* that Cath-B triggers cytochrome c release from the mitochondria into the cytosol [Guicciardi, 2000], although the latter observation seems to be somewhat controversial [Stoka, 2001]. In conflict with a direct proapoptotic activity of Cath-B is a recent report according to which the microinjection of Cath-D but not of Cath-B does trigger apoptosis [Roberg, 2002]. Considering the possible proapoptotic activity of cathepsins, it has been even hypothesized that e.g. Cath-B-like proteases, despite their implication as bad prognosis factors in the promotion of metastasis and tumor invasion, may prove useful in selectively targeting tumor cells for apoptosis-induction, exploiting the fact that tumors frequently express high levels of cathepsins [Foghsgaard, 2001]. Nevertheless, it should be kept in mind that the mechanisms by which cathepsins possibly contribute to apoptosis and the significance of cathepsin-mediated cell death under certain physiological or pathological conditions remain somewhat elusive and require further investigation.

1.4 Specific Aims of this Thesis

Even though the previous chapters primarily were intended to give an insight into some of the basic concepts of what is currently known about apoptosis, this introduction also should have raised awareness about the multitude and complexity of signalling pathways and factors involved in the induction, mediation, and execution of programmed cell death as illustrated in **Fig. 1.5**. Currently, about 10.000 scientific reports are published in the field of apoptosis research every year, and presumably several hundreds of factors have already been recognized to contribute to mechanisms that control apoptosis [Melino, 2001]. Nevertheless, our understanding of the network of interactions between the various factors that determine whether a cell is committed to survive or to die, remains limited. It also must be assumed that there are still proteins which, up to now, have not been recognized to play a role in the induction or regulation of the apoptotic machinery, and which eventually might prove to be missing links in the effort to find a complete description of the pathways involved. In this context, the main goal of this doctoral thesis was the isolation, identification, and characterization of genes which induce apoptosis upon overexpression by the application of a genetic expression screening procedure as described by Grimm and Leder [Grimm, 1997]. Several well-described apoptosis factors, e.g. p53, FADD, Bax, and caspases, are known to possess dominant apoptosis-inducing activity when overexpressed [Chinnaiyan, 1995; Fernandes-Alnemri, 1994; Li, 2002; Li, 2001b; Pastorino, 1998], and therefore it is justified to assume that application of the genetic expression screen is an appropriate approach for the identification of further proteins with proapoptotic activity.

This thesis describes the characterization of two cDNAs which during the screening procedure were identified to induce apoptosis-like morphological changes when overexpressed in 293T human kidney cells. One cDNA was determined to code for the ubiquitin-specific protease UBP41, the other for the lysosomal cysteine protease cathepsin-L, i.e. both cDNAs encode enzymes that are part of the cellular apparatus for the proteolytic destruction of proteins. Importantly, UBP41 and cathepsin-L are non-caspase cysteine proteases, and recently the importance of alternative proteolytic pathways in the mediation of caspase-independent apoptosis signalling has been discussed intensely [Jaattela, 2003]. During the last few years there has been accumulating evidence that both, the ubiquitin/proteasome system, to which UBP41 belongs, as well as the lysosomes, to which cathepsin-L localizes, might have significant impact on the mediation and regulation of programmed cell death (see chapters 1.2 and 1.3, respectively). The two projects described here contribute to the expanding knowledge about the possible connections that exist between protein degradation and apoptosis.

2 Materials and Methods

2.1 Materials

2.1.1 Fine Chemicals and Biochemicals

If not indicated different in following listing, all fine chemicals were from Sigma (Taufkirchen). Restriction enzymes were from MBI Fermentas (St.Leon-Rot), and further chemicals and enzymes used but not listed here are indicated in the text.

Acetic acid (Merck, Darmstadt); Aceton (Merck, Darmstadt); Acrylamide – 30 % Acrylamide, 0,8 % **B**isacrylamide, 37.5:1 (Roth, Karlsruhe); Agarose (Biomol, Hamburg); Bacto Agar (Difco, Detroit, USA); Boric acid (Merck, Darmstadt); Bromphenol blue (Merck, Darmstadt); **C**alcium chloride (Merck, Darmstadt); Chloroform (Merck, Darmstadt); Chloroform/Isoamylalkohol - 24:1 (Roth, Karlsruhe); **D**TT - Dithiothreitol (BioTech, St. Leon-Rot); Dry Milk Powder (Milupa, Friedrichsdorf); **E**GTA - Ethylene glycol-bis-(2-aminoethyl)-N,N,N', N'-tetraacetic acid (Biomol, Hamburg); Ethanol (Riedel-de Haen, Seelze); **F**ormaldehyde (Merck, Darmstadt); D(+)-**G**lucose (Merck, Darmstadt); L-Glutamine (Gibco BRL, Eggenstein); Glycerin (Merck, Darmstadt); Glycine (Merck, Darmstadt); **H**EPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Biomol, Hamburg); **I**sopropanol (Merck, Darmstadt); **M**agnesium chloride (Merck, Darmstadt); Magnesium sulfate (Merck, Darmstadt); Mercaptoethanol (Merck, Darmstadt); Methanol (Merck, Darmstadt); **P**henol/ Chloroform/Isoamylalkohol - 25:24:1, pH 8 (Roth, Karlsruhe); Potassium acetate (Riedel-de Haen, Seelze); Potassium chloride (Merck, Darmstadt); 2-Propanol (Merck, Darmstadt); **S**odium acetate (Merck, Darmstadt); Sodium chloride (Merck, Darmstadt); Sodium dihydrogenphosphate (Merck, Darmstadt); Sodium dodecylsulfate – SDS (Roth, Karlsruhe); Sodium hydrogenphosphate (Merck, Darmstadt); di-Sodium hydrogenphosphat (Merck, Darmstadt); Sodium hydroxid (Merck, Darmstadt); Sodium pyruvate (Gibco BRL, Eggenstein); Saccharose = Sucrose (Merck, Darmstadt); **T**NF-α, human, recombinant (Biomol, Hamburg or Bachem, Weil am Rhein); Triton X-100 (Roth, Karlsruhe); **U**rea (Merck, Darmstadt); **Y**east extract (Difco, Detroit, USA); **z**VAD-FMK (Enzyme Systems, Livermore, USA)

2.1.2 Reagents for Cell Culture

Dulbecco modified Eagle-Medium - DMEM (Sigma, Taufkirchen); **F**BS - Fetal Bovine Serum (Sigma, München); L-**G**lutamine - 100 x (LifeTechnologies, Karlsruhe); **P**enicillin/Streptomycin - 100 x (Life Technologies, Karlsruhe); **R**PMI-1640 medium (Sigma, Taufkirchen); **S**odium pyruvate - 100 x (Life Technologies, Karlsruhe); **T**rypsine/EDTA - 10 x (Life Technologies, Karlsruhe).

2.1.3 Laboratory Equipment

Centrifuges 5417R, 4K15, and RC 5B Plus (Eppendorf, Sigma, Sorvall); **E**lectrophoresis chambers (own construction by MPI workshop); **F**low Cytometer FACS-Calibur (Becton Dickinson, Heidelberg); Fluorescence Plate Reader (Fluoroskan Ascent FL Labsystems); **I**ncubators (Heraeus, Hanau); **P**ower-Supplies EPS 600 (Pharmacia Biotech); **M**icroscope Axiovert 25 (Zeiss, Oberkochen); Microscope Axiophot IM-35 (Zeiss, Oberkochen); Microscope Axioscop 2 (Zeiss, Oberkochen); Micropipets (Gilson, Middleton, USA); **P**CR MasterCycler Personal / Gradient (Eppendorf, Hamburg); **S**haking Incubator (own construction by MPI workshop); **S**peedVac Concentrator 5301 (Eppendorf, Hamburg); **S**pectrophotometer DU 530 (Beckman, Fullerton, USA); Sonicator Cell Disruptor B15 (Branson); **T**able top Centrifuges (Eppendorf, Hamburg); **U**V Illuminator UVT-14L - Eagle Eye II Herolab (Stratagene, La Jolla, USA)

2.1.4 Kits and Ready-to-Use Reagents

Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Weiterstadt); BioRad Protein Microassay (BioRad, München); **C**aspase-3 Activity Assay (Roche, Mannheim); **E**xpand Long Template PCR Kit (Roche, Mannheim); **P**rotease-Inhibitor-Complete Cocktail (Roche, Mannheim); **Q**IAquick Gel Extraction Kit (QIAGEN, Hilden); Effectene Transfection reagent (QIAGEN, Hilden); QIAGEN Plasmid Kit - Mini, Midi, Maxi (QIAGEN, Hilden); QIAquick Spin Gel Extraction Kit (QIAGEN, Hilden); **R**Neasy Mini Kit - RNA-Isolation (QIAGEN, Hilden); **S**uper Signal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, USA)

2.1.5 Standard Buffers and Stock Solutions

Coomassie-staining solution:	50% (v/v) Methanol, 10% Acetic acid, 0.1% (w/v) SERVA Blue R
Denhardt's Reagent:	50x1% Ficoll 400, 1% polyvinolpyrrolidon, 1% BSA fraction V in H_2O
DNA sample buffer, 6 x:	0.25% bromophenol blue, 15% Ficoll
Hypotonic Lysis Buffer - PI FACS buffer:	20 µg/ml Propidium iodide, 0.1% (w/v) Na_3Citrate, 0.1% Triton X-100 in PBS
PBS:	140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7,4
Ponceau staining solution:	0,1% Ponceau S, 5% (v/v) Acetic Acid
SDS-Gel Running buffer:	25 mM Tris, 192 mM Glycine, 0,1% (w/v) SDS
SDS Sample buffer, 2x:	100 mM Tris-HCl pH 6.8, 4% SDS, 10% mercaptoethanol, 20% glycerol, 0.05% bromophenol blue
SDS stacking gel buffer:	1.5 M Tris/HCl, pH 6,8
SDS separation gel buffer:	1,0 M Tris/HCl, pH 8,8

SSC, 20 x:	0,3 M Trinatriumcitrate, 3 M NaCl, pH 7,0 adjusted with HCl
TAE buffer:	40 mM Tris acetate, 2 mM EDTA pH 8.0
TBS-Tween buffer:	150 mM NaCl, 10 mM Tris/HCl, pH 8,0, 0,05% Tween 20
TE Buffer:	10 mM Tris/HCl, 1 mM EDTA, pH 8,0
Transfer Buffer:	47,9 mM Tris, 38,6 mM Glycin, 0,04% (w/v) SDS, 20% (v/v) Methanol
Triton-X lysis buffer:	50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% TritonX-100

2.1.6 Antibodies

Anti-ß-**A**ctin, mouse (Sigma); Anti-**C**aspase-3 (CPP32), mouse (Pharmingen - Transduction Laboratories); Anti-**C**athepsin B, mouse (Oncogene, Ab-2); Anti-Cathepsin D, mouse (Oncogene, Ab-1); Anti-**C**athepsin-L, mouse (Pharmingen - Transduction Laboratories); Anti-Cyclin-B1, mouse (Promega); Anti-**H**A High affinity, rat (Roche, Basel); Anti-**M**ouse IgG, HRP-gekoppelt, goat (Amersham-Pharmacia); Anti-**p**21, mouse (Promega); Anti-p27, mouse (Promega); Anti-**R**abbit IgG, HRP-gekoppelt, goat (Amersham-Pharmacia); Anti-Rat IgG, HRP-gekoppelt, goat (Amersham-Pharmacia); Anti-**T**im-23, mouse (Pharmingen - Transduction Laboratories); Anti-**U**biquitin, rabbit (Sigma);

2.1.7 Oligonucleotides

For RT PCR cloning of human ubiquitin-specific proteases (USPs):

Outer primers (for first round of "nested PCR"):

hUBP41 Fwd outer:	ATgCTCAACAAAgCCAAgAATT
hUBP41 Rev outer:	gAAAggAAAAAAgggACgTg
hUSP18 Fwd outer:	CTgggggTTTTggAgTgATC
hUSP18 Rev outer:	CCAggAACggAAATggAAAA
hUSP21 Fwd outer:	CCCTgTTCAgCATACggACA
hUSP21 Rev outer:	TAAAgggCTTCACAggTgCC

Inner primers (containing restriction site linkers and Kozak sequence for cloning into pHA vector) :

hUBP41-HA Fwd:	ATAT AAgCTT CCACC ATgCTCAACAAAgCCAAgAATT
hUBP41-HA Rev:	ATTT gcggccgc A CATTCgggAgggCgggC
hUSP18-HA Fwd:	ATAT ggTACC CCACC ATGAGCAAGGCGTTTGGGC
hUSP18-HA Rev:	ATTT gcggccgc A GCACTCCATCTTCATGTAA
hUSP21-HA Fwd:	ATAT ggTACC CCACC ATGATATCCGCCCGGTCCTC
hUSP21-HA Rev:	ATTT gcggccgc A CAGGCACCGgggTggcT

For site-directed mutagenesis of the active site of the USPs:

hUBP41 C24A Fwd:	gggAACACggCCTTCATgAACT
hUBP41 C24A Rev:	AgTTCATgAAggCCgTgTTCCC
hUSP18 C64A Fwd:	ggACAgACCgCCTgCCTTAAC
hUSP18 C64A Rev:	gTTAAggCAggCggTCTgTCC
hUSP21 C37A Fwd:	gAAACACggCCTTCCTgAATg
hUSP21 C37A Rev:	CATTCAggAAggCCgTgTTTC

For the ImmunoPeptide (IP-B) fragment for insertion into pGex-4T-1:

IP-B Fwd:	ATATggATCCAACgAggTgAACCgAgTg
IP-B Rev:	ATTTgCggCCgCTTTTCTCCACATCTgTCg

For RT PCR cloning of human ubiquitin from human poly-ubiquitin gene cDNA:

hUbiquitin Fwd:	ATAT ggTACC T CCACC ATgCAgATCTTCgTgAAgAC
hUbiquitin Rev:	ATTT gCggCCgC CTACCCACCTCTgAgACggAgT

For site-directed mutagenesis of human ubiquitin:

hUbiquitin K48R Fwd:	CTTTgCCggAAgACAgCTgg
hUbiquitin K48R Rev:	CCAgCTgTCTTCCggCAAAg
hUbiquitin G76A Rev:	ATTT gCggCCgC CTACgCACCTCTgAgACggAgT

For RT PCR cloning of human cathepsins:

hCath-L Fwd:	ATAT ggATCC AgATATgAATCCTACACTCATC
hCath-L Rev:	ATTT CTCgAg gTCAAgTCCTTCCTCATCAC
hCath-B Fwd:	ATAT ggATCC CCAACATgTggCAgCTCTgg
hCath-B Rev:	ATTT CTCgAg ACAggCCCACggCAgATTAg
hCath-D Fwd:	ATAT AAgCTT CgCCATgCAgCCCTCCAgC
hCath-D Rev:	ATTT CTCgAg gCCTTgggAACTAgAggCgg

For RNAi experiments using the pSUPER vector:

pSuper-hUBP41-#1 sense: gATCTCCCACTCgggAgTTgAgAgATTTCAAgA…
…gAATCTCTCAACTCCCgAgTgTTTTTggAAA

pSuper-hUBP41-#1 antisense: AgCTTTTCCAAAAACACTCgggAgTTgAgAgATT…
…CTCTTgAAATCTCTCAACTCCCgAgTgggA

pSuper-hUBP41-#2 sense: gATCTCCCCTCgATCATCTTCCTgATTTCAAgAgAA…
…TCAggAAgATgATCgAggTTTTTggAAA

pSuper-hUBP41-#2 antisense: AgCTTTTCCAAAAACCTCgATCATCTTCCTgATTCTCTT…
… gAAATCAggAAgATgATCgAggggA

pSuper-hCath-L sense: gATCTCCgAATTgCCTCAgCTACTCTTTCAAgAgAA…
… gAgTAgCTgAggCAATTCTTTTTggAAA

pSuper-hCath-L antisense: AgCTTTTCCAAAAAgAATTgCCTCAgCTACTCTTCTC…
… TTgAAAgAgTAgCTgAggCAATTCggA

pSuper-hCath-B sense: gATCTCCCgTgTCggATgAgCTggTCTTCAAgAgAgA…
…CCAgCTCATCCgACACgTTTTTggAAA

pSuper-hCath-B antisense: AgCTTTTCCAAAAACgTgTCggATgAgCTggTCTCTC…
…TTgAAgACCAgCTCATCCgACACgggA

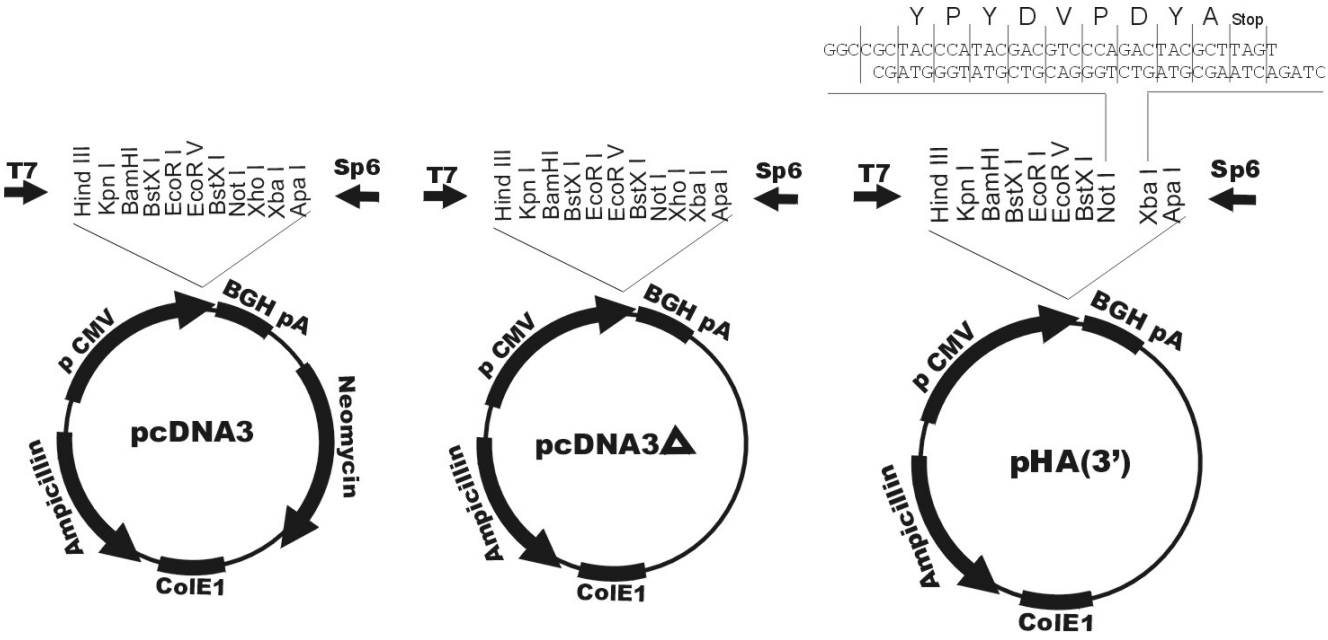
2.1.8 Plasmid Clones

For cloning, following standard plasmids were used:

pcDNA3 (Invitrogen, eukaryotic expression vector with CMV promoter), **pcDNA3Δ** (pcDNA3 with neomycin resistance gene deleted), **pHA(3')** (based on pcDNA3Δ for C-terminal HA-tagging), **pEYFP(3')** (based on pcDNA3Δ with C-terminal EYFP-tag inserted between the XhoI, XbaI restriction sites with reading frame being compatible to that of the pHA(3') vector when using the NotI site for cloning), and **pGex-4T-1** (Amersham, for N-terminal GST-tagging). For RNAi experiments, annealed oligonucleotides for the target sequence were ligated into the **pSuper** vector (Oligoengine).

Clone	in vector	5' cloning site	3' cloning site
hUBP41-HA	pHA(3')	Hind III	Not I
hUBP41-C24A-HA	pHA(3')	Hind III	Not I
hUSP18-HA	pHA(3')	Kpn I	Not I
hUSP18-C64A-HA	pHA(3')	Kpn I	Not I
hUSP21-HA	pHA(3')	Kpn I	Not I

hUSP21-C37A-HA	pHA(3')	Kpn I	Not I
hUBP41-EYFP	pEYFP(3')	Hind III	Not I
hUBP41-C24A-EYFP	pEYFP(3')	Hind III	Not I
hUSP18-EYFP	pEYFP(3')	Kpn I	Not I
hUSP18-C64A-EYFP	pEYFP(3')	Kpn I	Not I
hUSP21-EYFP	pEYFP(3')	Kpn I	Not I
hUSP21-C37A-EYFP	pEYFP(3')	Kpn I	Not I
GST-IP-B	pGex-4T-1	BamH I	Not I
hUbiquitin-HA	pHA(3')	Kpn I	Not I
hUbiquitin-K48R,G76A-HA	pHA(3')	Kpn I	Not I
hCathepsin-L	pcDNA3Δ	BamH I	Xho I
hCathepsin-B	pcDNA3Δ	BamH I	Xho I
hCathepsin-D	pcDNA3Δ	Hindd III	Xho I



2.2 Methods

2.2.1 Prokaryotic Cell Culture

E.coli DH5α [Hanahan, 1983] and *E.coli* Sure cells (Stratagene, La Jolla, USA) were used for transformation and extraction of plasmid DNA, whereas protease deficient *E.coli* BL21 cells were used for recombinant protein expression. All *E.coli* straines were grown in liquid LB medium (LB Broth, Sigma) in a shaking incubator at 37°C or on LB-Agar (Sigma) at 37°C.

2.2.2 Transformation of E.coli

Chemo-competent E.coli cells were used for transformation. For generation of chemo-competent cells, a pre-culture of E.coli was grown in 100 ml LB medium containing 50 µg/ml tetracyclin up to an OD (600 nm) of 1. Cells were then cooled down on ice, spun down (10 min, 4000 rpm, 4°C) and resuspended in 20 ml of icecold 0.1 M $CaCl_2$. After centrifugation the pellet was resuspended in 4 ml of 0.1 M $CaCl_2$, then 830 µl of glycerol (87%) were added and aliquots were frozen at -80°C. For transformation, aliquots of chemo-competent E.coli were thawn on ice, the corresponding plasmid DNA was added to the cells. After 15 min of incubation on ice, the cells were treated by heat shock (45 s at 42°C) with subsequent quick chill on ice (1 min). Then 1 ml of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2.5 mM KCl, 20 mM glucose) was added to the transformed cells for incubation at 37°C for 30 min in order to give the cells time for resistance gene expression. Afterwards, transformed E.coli were either given into LB medium containing the appropriate antibiotic (usually 100 µg/ml ampicillin) or were plated onto LB agar containing the appropriate antibiotic (usually 100 µg/ml ampicillin) and cultured over night.

2.2.3 Extraction of plasmid DNA from E.coli

For extraction of plasmid DNA from transformed E.coli, the transformed bacteria were grown in a shaking incubator at 37°C over night in a volume of 3 ml LB (miniprep) or 100 ml LB (maxiprep). The plasmid DNA was extracted from the cells using the QIAprep Spin Miniprep (Qiagen) or the QIAGEN Plasmid Maxi Prep (Qiagen) according to the manufacturers instructions.

2.2.4 Eukaryotic cell culture

Eukaryotic cell culture was performed under a laminar flow hood (Heraeus, Hanau) using sterile glass pipets and disposable plastic cell culture dishes (Greiner, Solingen, Nunc, Wiesbaden). Cells were maintained in DMEM (HeLa, 293T, McA RH 7777 cells) or RPMI (PC3 cells) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1 mM Na-pyruvate, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were kept at 5% CO_2 and 37°C in an incubator (Heraeus, Hanau). Cells were harvested by washing the cells once with PBS and subsequent incubation with 0,25% trypsine solution for approximately 5 min at 37°C. The detached cells were transferred into a sterile tube containing 5 ml of complete medium (for inactivation of trypsine). After centrifugation for 2 min at 250g the supernatant was removed by aspiration and the cell pellet was resuspended in fresh medium and distributed to culture dishes of the desired format. Cells were splitted and reseeded every 2-3 days. For long-term storage, eukaryotic cells were harvested as described and resuspended at a concentration of about 10^7 cells per ml medium. Then an equal volume of 2x Freezing Medium (appropriate culture medium containing 20% FCS and 20% DMSO) was added and aliquots of 1.8 ml were immediately transferred into cryo-vials and cooled down to –80°C. Subsequently cells were transferred into liquid nitrogen. For bringing frozen cells back to culture, cells were rapidly thawn, washed in culture medium and seeded in 10 cm culture dishes.

2.2.5 Transfection of eukaryotic cells

2.2.5.1 Transfection of 293T cells

293T cells were transfected by the calcium phosphate transfection method. The cells were seeded one day prior to transfection so that on the day of transfection the cell density was about 70%. About 30 min prior to transfection, chloroquine was added to the cells at a final concentration of 40 µM. For the 6 well format, 2 µg plasmid DNA (plus 0.2 µg of pEGFP vector as transfection control) was diluted to 90 µl final volume in water. 10 µl $CaCl_2$ (2.5 M) were added to the DNA and subsequently 100 µl of 2xHBS buffer (280mM NaCl,1.5mM Na2HPO4, 50mM Hepes) at a pH between 6.9 and 7.1 (empirically to be determined). After 10-15 min of incubation at RT the DNA precipitate was added to the cells and incubated for 6 h under culture conditions whereafter medium was changed. Transfection efficiencies as determined by the percentage of EGFP-positive cells (FACS analysis) were usually approximately 50 to 60 percent. Up- and downscaling of the protocol was possible taking into account the size of the well or culture dish used.

2.2.5.2 Transfection of HeLa cells

HeLa cells were transfected using the Effectene transfection reagent (Qiagen). The cells were seeded one day prior to transfection so that on the day of transfection the cell density was about 70%. For the 10 cm culture dish format, 0.8 µg plasmid DNA was diluted in 400 µl of EC-Buffer together with 0.2 µg pEGFP vector (Clontech). 6.4 µl Enhancer was added, thoroughly mixed, incubated for 5 min, and then 8.0 µl Effectene were added with subsequent incubation of 15 min at RT. During incubation times, the cells were prepared for transfection by washing with PBS and addition of 10 ml fresh RPMI (10% FCS) medium. The DNA-Effectene complex was added to the prepared cells and was kept in the medium for 6 to 10 hours. Then the cells were washed once with PBS and supplied with fresh medium. Transfection efficiencies as determined by the percentage of EGFP-positive cells (FACS analysis) were approximately 70 to 80 percent. If even higher eficiencies or stronger expression was required, the described transfection protocol was changed by using double the amounts of DNA, Enhancer and Effectene in the same volume of EC-Buffer (400 µl). Up- and downscaling of the protocol was possible taking into account the size of the well or culture dish used.

2.2.5.3 Transfection of PC3 and McA RH 7777 cells

PC3 and McA RH 7777 cells were transfected using the Effectene transfection reagent (Qiagen) according to the protocol described for HeLa cell transfection using 1.6 µg DNA, 12.8 µl Enhancer and 16 µl Effectene in 400 µl EC-Buffer for the 10 cm culture dish format. Transfection efficiencies as determined by the percentage of EGFP-positive cells (FACS analysis) were approximately 40 to 50 percent. Up- and downscaling of the protocol was possible taking into account the size of the well or culture dish used.

2.2.5.4 Transfection for generation of stable pool clones

Cells were transfected with the pcDNA3 vector containing either the coding sequence of cathepsin-L or cathepsin-B. Since the pcDNA3 vector carries a neomycin resistance gene which codes for an aminoglycoside-phosphotransferase, transfected cells could be selected by treatment with the antibiotic geneticin (G418) at a concentration of 400 µM. Surviving cells were continously kept in presence of G418 and checked for stable integration of the expression plasmid by immunoblot and cathepsin enzymatic assay.

2.2.6 The Screen for dominant apoptosis-inducing genes

The screen was performed as described by Grimm and Leder [Grimm, 1997]. For the generation of a normalized cDNA library , mRNA was extracted from kidneys of 4-6 week-old FVB mice. Normalization of this mRNA was achieved by its hybridization with first strand cDNA during which highly abundant mRNA species associate much more rapidly than rare mRNA species. After two rounds of hybridization, the non-associated, normalized mRNA was used as template for cDNA synthesis using a cDNA synthesis kit (Gibco BRL) and the obtained cDNA was ligated into the pcDNA3.1 vector lacking the neomycin resistance cassette, designated pcDNA3d, via a BstXI adaptor and NotI digest. The plasmids were then transformed into *E.coli* SURE cells thus delivering the desired cDNA library. Transformed bacteria were diluted and subsequently seeded into 96 deep-well plates in a way that on the average a pool of only a few different *E.coli* clones (corresponding to about 5 different cDNAs per pool) were raised within each single well. Plasmid DNA was prepared from the 96-well bacteria cultures and transfected into 293T human kidney cells using the calcium phosphate method in a 24 well format. The bacteria pool whose plasmid DNA caused morphological signs of apoptosis in the transfected 293T cells was identified and spread on plates. Plasmid DNAs from individual bacteria colonies were again transfected into 293T cells to isolate the active clone.

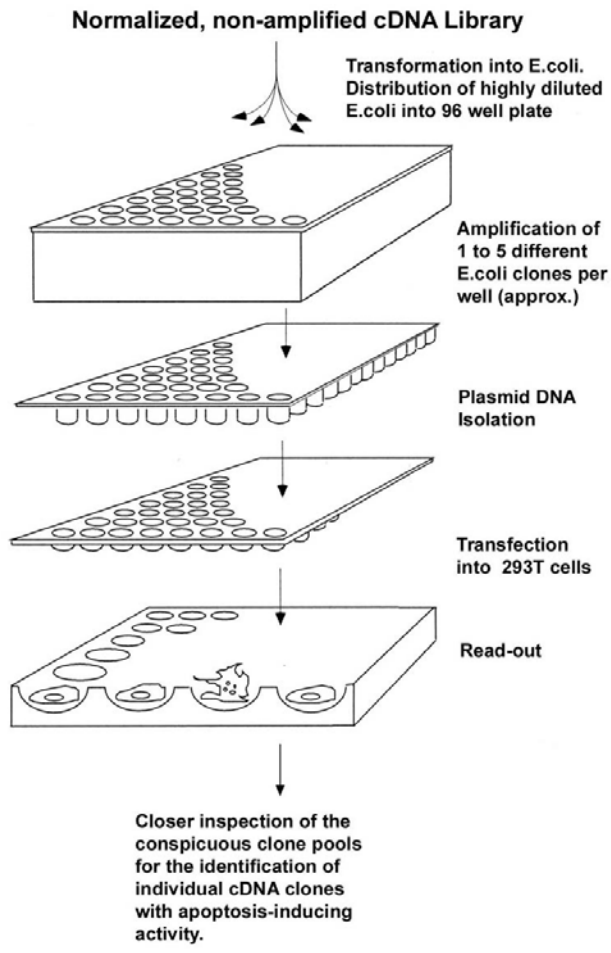


Fig. 3.1
The screening procedure for dominant apoptosis-inducing genes. *For details see the text in paragraph 2.2.6 and paragraph 3.1.1.*

2.2.7 Apoptosis and Cell Death Assays

2.2.7.1 DNA ladder

Floating cells and adherent cells were combined by harvesting with a cell scraper. After centrifugation at 800g for 2 min, the cells were taken up in 1 ml PBS and transfered into a microfuge tube and spun down at 800g for 2 min. The pellet was resuspended in Hypotonic Lysis Buffer B (10 mM Tris-HCl, 10 mM EDTA, 0.2% Triton X-100, pH 7.2) and incubated on ice for 10 min. After centrifugation for 10 min at 14000 rpm at 4 degrees celsius in a microfuge, the supernatant was transfered into a new microfuge tube, and 1 volume equilibrated phenol solution (Roth) was added and mixed by vortexing. Then the samples were spun for 10 min at 14000 rpm at RT: the liquid upper phase was transfered into a new microfuge tube and mixed with 1 volume of phenol/chloroform (1:1) solution (Roth). After centrifugation for 10 min at 14000 rpm at RT the liquid upper phase was transfered into a new tube and first 45 µl of 5 M NaCl and then 1.5 ml icecold ethanol were added following incubation for at least 2h at -20 degrees celsius. The sample was spun at maximum speed in a microfuge at 4 degrees celsius and the DNA pellet was air dried. The pellet was redissolved in 15 µl TE buffer by incubation at 50 degrees celsius for at least 1 h. Then 3 µl of RNase A (10 mg/ml, Qiagen) were added and incubation at 37 degrees celsius for 90 min followed. The sample was mixed with loading buffer (15% Ficoll type 400, 0.05 % bromphenolblue) and loaded onto a 2% agarose gel (Biomol) containing 0.5 µg/ml ethidiumbromide. The gel was run at 60 V for about 2.5 h, and the gel monitored with the Eagle Eye II system (Stratagen).

2.2.7.2 PI FACS analysis of HeLa cells and McA RH 7777 cells

Cells were harvested by trypsination, resuspended in PBS and taken up in 3 volumes of hypotonic PI-buffer (20 µg/ml propidium iodide, 0.1% w/v Na-citrate, 0.1% Triton X-100 in PBS). The resulting cell nuclei were analysed by flow cytometry for sub-G1 DNA content using the FACScalibur (BD) machine in FL-2 and subsequent evaluation using the CellQuest Software allowing to calculate the percentage of cells containing fragmented DNA. If not indicated different, the apoptotic cell population was calculated by taking into account the percentage of transfected, i.e. GFP-positive cells which was determined in parallel by FACS analysis in FL-1 using an aliquot of the corresponding cell sample. Each datapoint was usually represented by triplicates and the result was confirmed in several independent experiments.

2.2.7.3 PI FACS analysis of 293T cells

DNA fragmentation in 293T cells by PI FACS analysis was determined applying a more sensitive protocol including a citrate-phosphate buffer extraction step essentially as described by Gong et al [Gong, 1994]. In brief, the cells were harvested by trypsination, resuspended in Hanks buffered saline and fixed by addition of 10 volumes of 70% ethanol and subsequent storage for several hours at -20 degrees celsius. The cells were spun down at 800g for 5 min and the ethanol thoroughly removed. Then the cells were resuspended in 500 µl of phosphate-citrate buffer consisting of 192 parts of 0.2 M Na_2HPO_4 and 8 parts of 0.1 M citric acid (pH 7.8), and incubated at RT for at least 30 min. After centrifugation at 1000g for 5 min, the supernatant was removed, and the pellet resuspended in PBS containing 20 µg/ml propidium-iodide. The cells were then analysed by FACS as described above for PI FACS analysis of HeLa cells.

2.2.7.4 Caspase-3 Activity Assay

The caspase-3 activity assay was purchased from Roche and was used as recommended. Each datapoint was measured at least in triplicates and was confirmed by several independent experiments. Alternatively to the expensive Roche Caspase-3 Activity Assay kit, a procedure was developed that is similar to the Roche Kit protocol but uses own reagent components. The cells were harvested, washed twice with PBS, and then lysed 5 min on ice in Caspase-3 Activity Buffer (CAB: 10 mM HEPES pH 7.2, 50 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 0.5% Igepal) containing freshly added aprotinin, leupeptin, pepstatin at a final concentration of each 2 µg/ml as well as 1mM PMSF and 10 mM DTT. After centrifugation (10,000 rpm, 8 min, 4°C) the supernatants were transferred into a new microtube and the protein concentration was measured using the Bradford assay. The extracts were preferentially directly used for the subsequent caspase assay (without being frozen), and thus samples were kept on ice until used. For the caspase-3 activity assay, a 96 well fluorescence microtiter plate (FluoroNUNC C96 white MaxiSorp, NUNC) was coated with 2.5 µg/ml of the anti-CPP32 monoclonal antibody (Transduction Laboratories, clone 19) in 100 µl of Antibody Coating Buffer (50 mM Na2CO3, pH 9.6) for 1 hour at 37°C. Then the plate was blocked with 3 mg/ml BSA in PBS for 30 min at RT. After washing the wells of the plate 3 times with CAB buffer, 100 µg/well protein extract in a total volume of 100 µl (adjusted with CAB buffer) were incubated for 1 hour at 37°C. Then the wells were washed 3 times with CAB buffer, and finally 50 µM zDEVD-AMC caspase-3 substrate in 100 µl CAB buffer (freshly supplemented with 10 mM DTT) were added to the wells. After incubation in the dark for 2 hours at 37°C, AMC fluorescence was measured with a Fluoroskan Ascent FL fluorecence plate reader (Labsystems) at a wavelength of 380 nm for excitation and 450 nm for emission.

2.2.7.5 MTT proliferation / viability assay

Cells were transfected in 10 cm petri dishes as described, 24h later cells were harvested and reseeded in equal numbers (approximately 7500 cells/well) in the wells of 96 well plates. 24h after reseeding, cells were treated as indicated in a total volume of 100 µl DME-Medium. At time of analysis, 10 µl of a MTT stock solution (5 mg/ml in PBS) was added per well and cells were incubated for 4h at 37°C and 5% CO_2. Then 100 µl of 0.04 N HCl in 2-propanol was added to the wells and mixed vigorously with a multichannel pipet. Within an hour, formazan absorbance was measured on an ELISA plate reader (Dynatech MR7000) at 590 nm and a reference wavelength of 750 nm. The blank value (MTT in absence of cells) was substracted from all sample values and results were calculated in terms of "loss of viability, %" according to following equation: (U – S) / U * 100, where U = absorption value of untreated control, S = absorption value of sample.

2.2.8 Protein- and Immunochemical Methods

2.2.8.1 Immuno Blotting (Western Blotting)

For detecting protein expression, cells were harvested by trypsinization, washed with PBS, and lysed in TritonX buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% TritonX-100) 10 min on ice. After centrifugation at 14,000xg in a microfuge, supernatants were obtained as cytoplasmic extracts which were quantified for protein content using the Bradford reagent (BioRad). Equal amounts of protein were loaded after boiling with 1 volume of 2x sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 10% mercaptoethanol, 20% glycerol, 0.05% bromophenolblue) on a 12% SDS-polyacrylamide gel (stacking gel: 4.8% PA, 125 mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% APS, 0,1% TEMED; separating gel 10-15% PA, 375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% APS, 0,05% TEMED), separated in an electric field of approximately 20 V/cm in Running Buffer (25 mM Tris, 192 mM Glycin, 0,1% (w/v) SDS), and transferred to a PVDF membrane in a semidry blotting device using Transfer Buffer (47,9 mM Tris, 38,6 mM Glycin, 0,037% (w/v) SDS, 20% (v/v) Methanol) and an electric field corresponding to about 4 mA/cm2 of PVDF membrane with a blotting time of 1 h. The membrane was blocked for 1h with 5% drymilk powder in TBS-Tween (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween) and probed overnight at 4 degrees celsius with primary antibodies at appropriate dilution (usually about 1 µg/ml). Membranes were washed three times with milk, and then were probed for 1h at RT with secondary HRP-conjugated antibodies (usually at 1:5000 dilution, Roche). After washing four times with TBS-tween, the blot was developed using Super Signal enhanced chemiluminescence reagents (Pierce) and by exposition to BioMax MR films (Kodak).

2.2.8.2 Coomassie and Ponceau staining

Proteins in polyacrylamid gels were stained using Coomassie Brilliant Blue staining solution (Coomassie Blue R250 in 10% acetic acid, 50% methanol, and 40% H2O), and destained with 10% acetic acid, 50% methanol, and 40% H2O. Proteins on PVDF blotting membranes were visualized using reversible Ponceau staining (0.1% Ponceau S in 5% acetic acid).

2.2.8.3 Immunofluorescence

Cells were seeded on cover glasses and 24h later were incubated for 16h in absence or presence of NDI-HCl, CHX, or TNF-α/CHX. Cells were then fixed with methanol for 5 min at RT. Then cells were incubated for at least 30 min in blocking buffer (PBS supplemented with 10% FCS and 0.1% Triton-X). Then cells were incubated with anti-Cath-L mAb (1:100, Transduction Laboratories, cat #78820) in blocking buffer for 45 min. After 3 times washing with blocking buffer, cells were incubated with a FITC-conjugated polyclonal Ab (1:500, Pharmingen, cat #12064D) for 45 min. Cells were then washed again several times in blocking buffer and finally placed on object slides in embedding buffer (0.5 M Tris-HCl pH 8.2, 0.02 g/ml DTT, 0.04 g/ml polyvinyl alcohol, 40% glycerol). Fluorescence was observed under a fluorescence microscope (Axioscop 2, Zeiss, Jena, Germany) and pictures taken with a digital camera (Visitron Systems, Puchheim, Germany) using the IPLab imaging software (Spectra Services, NY, USA).

2.2.8.4 Generation of polyclonal antibodies

2.2.8.4.1 Expression of GST-fusion proteins

The antigenic UBP41 peptide for the immunization of a rabbit was expressed as a GST-fusion protein. The DNA fragment encoding the amino acids residues 119-150 of hUBP41 was generated by PCR and was cloned in frame with GST into the pGEX.4T1 (?) expression vector. The resulting GST-IP-B (IP-B stands for Immuno Peptide B) construct was transformed into *E.coli* BL21 cells which were seeded for an overnight preculture in 50 ml LB medium containing 100 µg/ml ampicillin. 4 ml of the preculture were then transferred into 500 ml LB medium containing 100 µg/ml ampicillin and were further incubated. When the cell density reached an OD (600 nm) of 0.5, IPTG was added to a final concentration of 1 mM (1:1000 of a 1M IPTG stock solution) and the bacteria were incubated for another 3 hours. The bacteria were harvested by centrifugation and the pellet could be stored at –80°C

or it was immediately resuspended thoroughly in PBS whereafter lysozyme was added to a concentration of 1 mg/ml with subsequent incubation on ice for 30 min. The bacteria were then lysed by sonification for 10 min in a pulse modus (sonificator) and the cell extract was obtained after centrifugation (20,000g, 4°C, 10 min) as the supernatant. Since the extracts were frequently still cloudy, they were usually cleared by ultracentrifugation (100,000g for 30 min at 4°C) using ultracentrifuge model.

2.2.8.4.2 Purification of GST-fusion proteins

Glutathione Sepharose beads suspension (Amersham) were washed 3 times in an equal volume of PBS supplemented with 500 mM NaCl and 1 mM EDTA. Beads were intermediately kept on a rotating wheel and were pelleted at 500g. Washed beads were incubated with GST-fusion protein (e.g. GST-IP-B) for 1 hour on the rotating wheel at 4°C (50 ml extract + approximately 1.5 ml bead bed volume). The beads were then given onto a disposable plastic column (BioRad) and washed with 100 x bed volumes PBS supplemented with 500 mM NaCl + 1 mM EDTA + 1 mM DTT (icecold). After washing, the GST-fusion protein was eluted in two 5 ml fractions of PBS containing 50 mM glutathione. Successful expression and purification of the GST-peptides was checked by SDS PAGE and subsequent Coomassie staining of the gel. Following purification on the GSH-sepharose affinity column, the obtained eluate was further purified on a small Sephadex G200 gel filtration column (Pharmacia). 0.5 ml fractions were collected and the fractions containing the desired protein were identified by SDS PAGE / coomassie staining.

2.2.8.4.3 Immunization of rabbit against hUBP41

Rabbits were immunized using an antigenic peptide consisting of amino acids 119-150 of hUBP41 as a fusion protein with GST (GST-IP-B) for appropriate expression and purification as described above. For initiation of the immunization, 100 µg of GST-IP-B in a volume of 500 µl PBS were emulsified with 500 µl Titer Max Gold adjuvant (Sigma) using two syringes and a double hub emulsifier needle system. The emulsion was then injected intramuscularly using a 18 gauge needle. On day 30, the animal was boosted with another 100 µg of antigen (500 µl) which this time was emulsified in an equal volume of Freund Adjuvant (Sigma). On day 43, a small blood sample (3 ml) was drawn from the animal and serum prepared as follows: blood was stirred for 1 min with a glass pasteur pipet, then the blood was kept at 4°C over night, after repeated stirring with a pasteur pipet the blood was centrifuged at 5000g for 30 min and the supernatant taken as serum. The titer of the serum was tested by immunoblot analysis using the serum at a dilution of 1:5000. The serum was able to recognize overexpressed hUBP41-HA in HeLa extracts as well as 50 ng of the GST-IP-B antigen itself. The

animal was boosted one more time using 200 µg of GST-IP-B in Freund Adjuvant on day 44 and was killed by bleeding on day 70 giving approximately 100 ml blood. Serum was prepared on the larger scale as descibed above.

2.2.8.4.4 Affinity purification of the polyclonal anti-hUBP41 antibodies

For affinity purification, GST as well as the GST-linked hUBP41 immunopeptide GST-IP-B were expressed and purified over a GSH column from 2 l cultures of BL21 *E.coli*. Yield was 30 µg protein in 10 ml PBS containing 10 mM glutathione and protein inhibitor cocktail (Roche). 3 ml of the GSH-purified protein eluate were dialysed against 5 l of Dialysis Buffer (200 mM NaCl + 0.1 M $NaHCO_3$, pH8.3) over night at 4°C using a 6-8 kDa membrane to remove GSH and protein inhibitors. Dialysis buffer was exchanged the next morning and dialysis continued for another 3 h. After dialysis the protein concentration was still 3 µg/ml as measured by Bradford assay with a total volume of about 3 ml. 1.5 g of freeze-dried CNBr-activated sepharose 4B (Amersham) was prepared by swelling in 50 ml of 1 mM HCl and subsequent washing twice in 50 ml 1 mM HCl and once in 50 ml Coupling Buffer (0.1M $NaHCO_3$ + 500 mM NaCl, pH 8.3). For coupling, 3 ml of dialysed GST (approximately 10 mg) and 1 ml of dialysed GST-IP-B (about 3 mg) were added to approximately 3 ml swollen beads of the prepared CNBr-activated sepharose 4B in a total volume of 13 ml adjusted with Coupling Buffer and kept on a rotator at 4°C over night. Then the beads were spun down at 1500xg for 2 min and in the supernatants no protein could be detected anymore. For blocking of remaining CNBr active sites, beads were incubated for 2 h at 4°C with 25 ml of 0.1 M Tris-HCl, pH 8.0. Then beads were washed in three cycles of first 5 volumes of Acetate Buffer (0.1 M acetate, pH 4.0), then 5 volumes of 0.1 M Tris-HCl, pH 8.0 + 500 mM NaCl. For immunodepleting anti-GST antibodies from the GST-IP-B-immunized rabbit antiserum, 20 ml of the serum were incubated on a rotating wheel overnight at 4°C with the GST-coupled beads in a final volume of about 50 ml by filling up with PBS. Beads were spun down (from which anti-GST antibodies could be eluted as described below for anti-hUBP41 antibodies), and the supernatant was transferred to the GST-IP-B-coupled sepharose beads with subsequent incubation over night at 4°C on a rotating wheel. The beads were then washed several times with Washing Buffer (PBS + 500 mM NaCl) until no protein was detectable in the Washing Buffer as determined by Ponceau staining of samples spotted on nitrocellulose membranes. The beads were then loaded into a glass wool plugged pasteur pipet and washed again with 10 ml of Washing Buffer. Bound antibodies were eluted with Elution Buffer (0.2 M acetic acid, pH 2.7 + 500 mM NaCl) in about 400 µl fractions which were immediately neutralized by prelaid 100 µl of 1 M Tris base. Fractions were tested by spotting of 2 µl onto nitrocellulose and Ponceau staining. Protein was present in fractions 4 to 8 which were combined, aliquoted and frozen at –80°C. 1:5000 dilutions of the eluate were able to detect 50 ng of purified GST-IP-B as well as hUBP41-HA overexpressed in 293T cells.

2.2.9 DNA and RNA techniques

2.2.9.1 Polymerase Chain Reaction (PCR)

For the amplification of specific DNA fragments, the PCR technology was used which originally was developed by Kary B. Mullis in 1987 [Mullis, 1987]. Reactions were usually performed in a total volume of 50 µl containing 1 µl of DNA template (cDNA or plasmid DNA), 1 µl of 10 mM forward and reverse oliginucleotide primers (final concentration 200 nM of each primer), 1 µl of 10 mM dNTP mix (final concentration 200 µM of each nucleotide), 5 µl of 10x Reaction Buffer, and 1 µl polymerase. In most cases the Expand Long Template PCR System (Roche) was used. The reaction cycle profile was usually as follows: 30 s at 95°C for denaturation, 40 s at the appropriate annealing temperature, 90 s at 68°C for elongation, 28 cycles.

2.2.9.2 Agarose gel electrophoresis of DNA

Agarose gels were prepared at concentrations of 0.8% up to 1.5 % by boiling of the agarose in TAE Buffer. The dissolved agarose was poured into the electrophoresis chamber with a slot comb inserted into the forming gel which after solidification was covered with TAE Buffer. Up to 500 ng of a DNA sample was mixed with 1/5 volume of 6x Sample Buffer (0.25% Bromophenol blue + 15% Ficoll) and loaded into the gel slots together with size markers as a reference (Eurogentec). The gel was run at about 100 V and the separated DNA bands were visualized by staining of the gel in ethidium bromide solution (10 µg/ml in TAE Buffer), subsequent destaining in TAE Buffer and eventual illumination with UV light on a transilluminator with the Eagle-Eye II imaging system (Stratagene).

2.2.9.3 Isolation of DNA from agarose gels

For the isolation of DNA from agarose gels the Qui Quick Spin System (Qiagen) was used.

2.2.9.4 Restriction reactions

For specific cleavage of DNA fragments from plasmid vectors or generation of restiction sites from linker sequences, about 1 µg DNA was incubated for at least 2 h in presence of each 10 U of the respective restriction enzymes (usually in double digests) under the appropriate buffer conditions in usually 50 µl total volume.

2.2.9.5 Ligation reactions

For ligation of a DNA fragment into a linearized vector, the appropriately restricted DNA fragments (about 50 ng of vector DNA and a 5-fold molar excess of insert fragment) were incubated over night at 4°C with 10 U of T4 DNA ligase (New England Biolabs) in presence of Ligation Buffer in a total volume of 20 µl. 5 µl of the ligation reaction were usually taken for transformation of chemo-competent *E.coli*.

2.2.9.6 Dephosphorylation of vector DNA

In order to prevent religation of incompletely cut vector DNA, the digested vector DNA was occasionally (when necessary) dephosphorylated by incubating 1 µg cut plasmid with 1 U of calf-intestine alkaline phosphatase (CIAP) in presence of the appropriate buffer in a total volume of 20 µl. After 30 min incubation at 37°C, CIAP was inactivated by adding an equal volume of equilibrated phenol (Roth). After mixing for 5 min, phases were separated by centrifugation at 5000 rpm in a microcentrifuge for 5 min. The upper aqueous phase was transferred into a new microvial and incubated with an equal volume of a 1:1 mixture of equilibrated phenol/chloroform (Roth) as described for the phenol step. Finally, the aqueous phase was equilibrated with choroform as well. Then the aqueous phase was transferred into a new vial and precipitated by adding 1:10 volumes of 7 M ammonium acetate and then 2 volumes of ice-cold ethanol for at least 30 min at –20°C. Afterwards, precipitated DNA was spun down at maximum speed in a microcentrifuge at 4°C and washed once with ice-cold 70% ethanol. After centrifugation, the (invisible) pellet was air-dried and dissolved in usually 20 µl sterile water.

2.2.9.7 DNA Sequencing

For DNA sequencing the Big Dye Terminator Cycle Sequencing RR-Mix (Applied Biosystems) was used based on the chain termination dideoxynucleotide method by Sanger [Sanger, 1977]. 0.5 µg template DNA were mixed with 0.8 pmol of a sequencing primer (usually T7 or BGH primer) and 4 µl of Big Dye Mix in a total volume of 20 µl. After 2 min of denaturation at 96°C, following 40 cycle profile was run: 10 s at 96°C for denaturation, 5 s at 50°C for annealing, 4 min at 60°C for elongation. The analysis by sequence gel electrophoresis was done by a commercial provider (SeqLab, Göttingen).

2.2.9.8 RNA isolation and generation of cDNA by RT PCR

For isolation of total RNA from cultured cell lines, the RNeasy Kit (Qiagen) was used. For synthesis of cDNA from extracted total RNA, the Superscript II system (Gibco BRL) was applied according to the manufactorer's recommendations.

2.2.9.9 Northern Hybridization

For the Multiple Tissue Northern (MTN) blot and the Matched Tumor/Normal Expression Array (both from Clontech), the coding sequence of hUBP41 was excised from the expression plasmid and approximately 25 ng were labelled with 5'-[α^{32}P]-dCTP (3000 Ci/mmol, Amersham) using the RediPrime random prime labelling kit (Amersham). Excessive radioactive dCTP and random prime oligonucleotides were removed using the Nucleotide Removal Kit (Qiagen). Labelled probes were eluted in 200 µl TE and 2 µl of this eluate were tested for sufficient incorporation by measuring Cherenkov-radiation in a scintillation counter.
The blots were prehybridized for 3h at 65 degrees celsius in 40 ml of Hybridization buffer (6x SSC, 5x Denhardt's reagent, 0.5% SDS, sheared and freshly denatured 0.1 mg/ml salmon sperm DNA), then 10 ml of a 50% dextrane sulfate solution was added, and finally the denatured, labbelled DNA probe (approximately 5x10^7 cpm) was added to the Hybridization solution and incubated at 62 degrees celsius for 16h. The blot was washed with Wash buffer 1 (4x SSC, 0.5% SDS) for 1h at 62 degrees celsius and then for 5 min with Wash buffer 2 (2x SSC, 0.5% SDS). The blot was exposed to a Fuji BAS 2500 phosphoimager screen and the signals analyzed and quantified using the Image Gauge V3.01 software.

2.2.9.10 RNA_i

For targeted downregulation of gene expression, the RNA_i technology was applied by using the pSuper vector system (Oligoengine) which is a mammalian expression vector that directs intracellular synthesis of siRNA-like transcripts. The vector uses the polymerase-III H1-RNA gene promoter, as it produces a small RNA transcript lacking a polyadenosine tail and has a well-defined start of transcription and a termination signal consisting of five thymidines in a row. Most important, the cleavage of the transcript at the termination site is after the second uridine, yielding a transcript resembling the ends of synthetic siRNAs, which also contain two 3' overhanging T or U nucleotides. The pSuper knockdown constructs were obtained by inserting 64-mer synthetic DNA oligonucleotides, of which complementary sequences were annealed in a way that overhanging restriction sites were generated, into the Hind III / Bgl II restriction sites of pSuper.

The 64-nt oligonucleotides contained a 19-nt sequence which by databank analysis was determined to be unique for a portion of the target gene. Within the 64-nt oligos, the 19-nt target appears in both sense and antisense orientation, separated by a 9-nt spacer sequence. The resulting transcript is predicted to fold back on itself to form a 19–base pair stem-loop structure. Within the cell, the stem-loop precursor transcript is quickly cleaved to produce a functional siRNA because the hairpin had been designed to be an optimal substrate for the enzyme Dicer, which cleaves the hairpins to generate the short dsRNA, both strands having the two-uridine 3' overhang. This processed construct is an efficient effector of RNA_i and complies with the design principles established for synthetic short interfering RNAs by the work of Tuschl and colleagues [Elbashir, 2001].

pSuper cloning procedure:

For annealing, each 1 µl of the sense and antisense oligonucleotide (3 µg/µl) were incubated in 48 µl of Annealing Buffer (100 mM KOAc + 30 mM HEPES-KOH pH 7.4 + 2 mM MgOAc) for 4 min at 95°C, then for 10 min at 70°C with subsequent slow cool down to 4°C. The annealed oligos were phosphorylated by mixing 2 µl of the annealing reaction, 1 µl T4 PNK buffer, 1 µl ATP (10 mM), 1 µl T4 PNK, and 5 µl H_2O. Following to incubation at 37°C for 30 min, the T4 PNK was inactivated by incubation at 70°C for 10 min. 2 µl of the phosphorylation reaction were ligated into the pSuper vector (which prior to its use in ligation was treated with calf intestine alkaline phosphatase (CIAP), subsequently purified with phenol/chloroform and precipitated with ethanol).

2.2.10 Cathepsin zFR-AMC Enzymatic Assay

Cells were left untreated or were transfected with pcDNA3d expression vector containing the ORFs of cathL, or cathB. The cells were harvested by trypsination, spun down at 400xg at 4°C for 3 min, washed 2x PBS, and the were lysed for 5 min on ice in SDS buffer A (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5 % NP-40, 0.05% SDS) in absence of proteinase inhibitors. Lysates were spun at maximum speed in a microfuge (14.000 rpm) at 4°C for 5 min and the supernatant was transferred into a new microtube. Extracts were preferentially immediately used for the enzymatic assay since freezing at -80°C appears to result in a partial loss of enzymatic activity. Protein concentration of the samples were determined by the Bradford assay (BioRad), and 100 µg total protein (in a maximum of 50 µl lysate) were pipetted into 200 µl of Urea Buffer (20 mM NaOAc pH 5.0, 4 mM EDTA, 0.4 M urea), freshly substituted with 10 mM DTT and 400 µM zFR-AMC (i.e. 4 µl of a 20 mM stock solution of zFR-AMC in DMSO into 200 µl Urea Buffer, Enzyme Systems Products). Samples were incubated approximately for 1h at 37°C, and AMC fluorescence was measured with a Fluoroskan Ascent FL fluorescence plate reader (Labsystems) at a wavelength of 380 nm for excitation and 450 nm for emission.

2.2.11 Cell-free activation of caspases for inhibitor experiments with E64d

Cytoplasmic extracts were prepared and activated by addition of cytochrome c and dATP in the absence or presence of various concentrations of E64d and were analysed by a caspase enzymatic assay essentially as described [Gewies, 2000b].

3 Results

3.1 Identification and characterization of UBP41 as a dominant apoptosis-inducing factor

3.1.1 Isolation of *ubp41* in the genetic screening procedure for apoptosis-inducing genes

For the identification of dominant apoptosis-inducing factors, the direct functional genetic screening procedure was applied as described by Grimm and Leder [Grimm, 1997]. In this screen, a normalized cDNA library was prepared from murine kidney tissue and was transformed into *E.coli* cells. The transformed E.coli cells were diluted and distributed into 96 well plates, in a way that per well only about 1 to 5 different cDNA clones were amplified. Plasmid DNA was prepared from those small bacteria pool clones and was transfected into human embryonic kidney 293T cells which subsequently were inspected microscopically for an apoptotic phenotype. The plasmid DNA pools which upon transfection were identified to induce signs of apoptosis in 293T cells, were retransformed into E.coli and plasmid DNA from single colonies was extracted in order to identify the single cDNA clone that was responsible for apoptosis induction (for more details see Materials and Methods 2.2.6).

The large-scale application of this screen in our laboratory led to the isolation of several cDNAs which dominantly induce apoptosis upon transient transfection into 293T cells. Some of those cDNAs were identified to be genes which were already known to be involved in apoptotic pathways and have the capacity to dominantly induce apoptosis upon overexpression such as FADD, ANT-1, CIDE-A and -B or ZIP kinase and which serve as positive controls for the functionality of the screen [Bauer, 1999; Chinnaiyan, 1995; Inohara, 1998; Kawai, 1998]. The adenine-nucleotide translocator 1, ANT-1, serves as an instructive example for the specificity by which the described screening procedure allows the identification of distinct apoptosis-inducing factors of potential physiological relevance: whereas overexpression of ANT-1 results in the induction of cell death, its highly conserved homologue ANT-2 does not induce apoptosis. This observation indicates that the observed cell death is not unspecifically induced by an inappropriate accumulation of ANT-1 and concurrent misfolding of the ANT-1 protein within the inner mitochondrial membrane, because – if this were true – the same effect would have to be expected in case of the almost identical ANT-2 protein. Thus, ANT-1 appears to specifically exert its proapoptotic activity by acting as a component of the permeability transition pore complex which is known to play an essential role in the mediation of apoptosis signals at the mitochondria [Bauer, 1999].

One of the cDNA clones that was selected from the genetic screen (by myself in collaboration with Ulla Cramer) was designated UI64, which not only induced the typical apoptotic morphological changes in 293T cells but also produced oligonucleosomal DNA fragmentation as a hallmark event of apoptosis **(Fig 3.1.1)**.

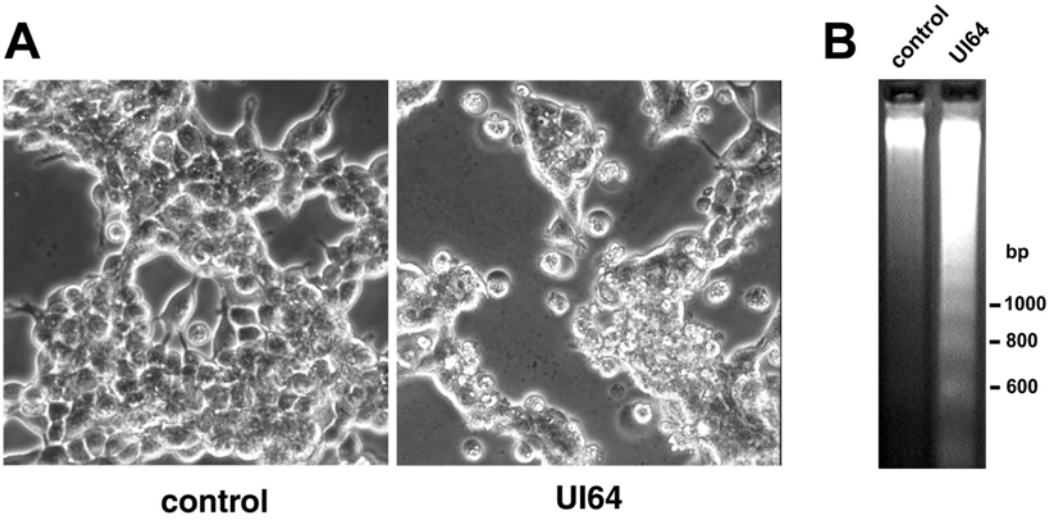


Fig. 3.1.1
The cDNA clone UI64 causes cell death when transiently transfected into 293T cells. *(A) 293T cells were transfected with a control plasmid (left)or with the conpicuous plasmid clone UI64 from the screen for dominant apoptosis inducing genes (right). 48h post transfection, the phenotype was observed by phase contrast microscopy. (B) 293T cells were transfected with a control plasmid or with UI64. 48h post transfection the cells were harvested, DNA was extracted and separated on a 2% agarose gel, stained with ethidiumbromide and visualized on a transilluminator.*

DNA sequencing of the UI64 cDNA clone and sequence analysis **(Fig 3.1.2)** using BLAST [Altschul, 1997] identified UI64 as the mouse orthologue (gi:7949157) of the ubiquitin-specific protease UBP41 (also denominated USP2) which recently had been isolated and characterized in chicken skeletal muscle [Baek, 1997].

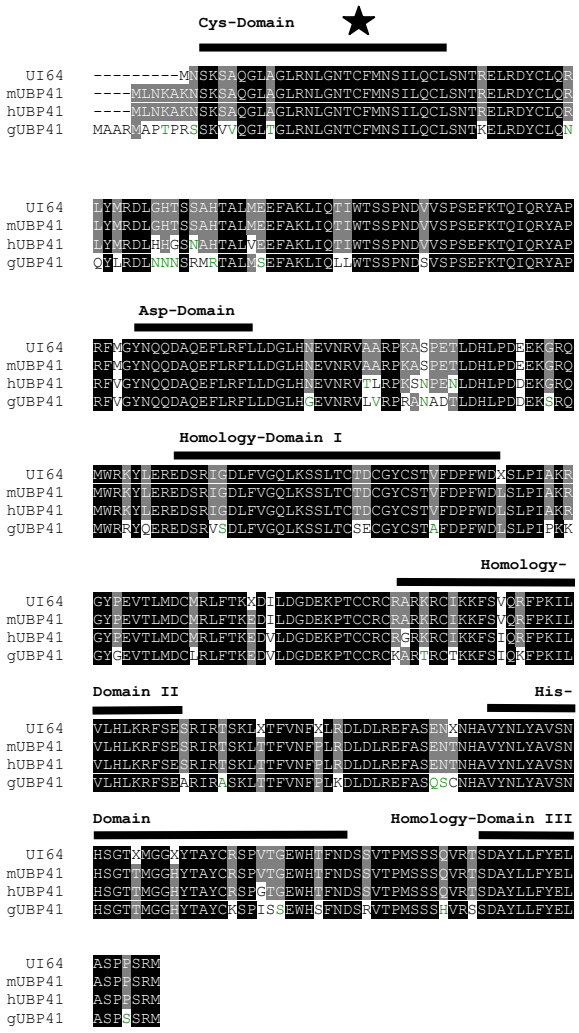


Fig. 3.1.2
The cDNA clone UI64 is identified as the mouse orthologue of UBP41. *BLAST sequence analysis revealed that the isolated apoptosis-inducing clone UI64 encodes the mouse orthologue of the ubiquitin-specific protease UBP41. The UBP41 protein sequence is highly conserved between homo sapiens (hUBP41), mus musculus (mUBP41), and gallus gallus (gUBP41). Identical residues are marked by blocks. Homology domains characteristic for ubiquitin-specific proteases are indicated, such as the cysteine, aspartate and histidine domain, as well as three additional homology domains. The catalytic cysteine is marked by an asteriks.*

For the isolation of the human orthologue of UBP41, PCR primers were designed based on the Genbank entry for hUBP41 (gi: 4759291) with the forward primer containing the start ATG and the reverse primer representing a region downstream of the stop codon. The obtained PCR product confirmed the published sequence with the exception of a few residues at the extreme carboxy-terminus which was determined to be identical to the mouse amino acid sequence. Therefore, the PCR product was regarded to represent human UBP41, subsequently designated hUBP41. A carboxy-terminal HA-tag sequence was added to hUBP41, resulting in the HA-fusion protein hUBP41-HA for convenient detection in immunoblot analysis using anti-HA antibodies. Interestingly, the alignment of its human, murine, and chicken amino acid sequences **(Fig. 3.1.2)** indicates that UBP41 is highly conserved not only within the catalytic or homology domains but also in the regions in between those domains.

3.1.2 Characterization of hUBP41-induced apoptosis

For being able to address the question whether the enzymatic activity of hUBP41 is essential for its proapoptotic activity, site-directed mutagenesis was performed to generate the enzymatically inactive hUBP41-C24A-HA variant by replacing the active site cysteine with alanine [Baek, 1997]. Transfection of 293T cells with hUBP41-HA resulted in apoptosis induction as judged by the apoptotic phenotype when observed under the microscope as well as by oligonucleosomal DNA fragmentation **(Fig. 3.1.3 A,B)**. In contrast, control transfected 293T cells or cells expressing the inactive site mutant hUBP41-C24A-HA did not show significant signs of apoptosis, although hUBP41-C24A-HA expression was comparable to that of hUBP41-HA **(Fig. 3.1.3 A)**.

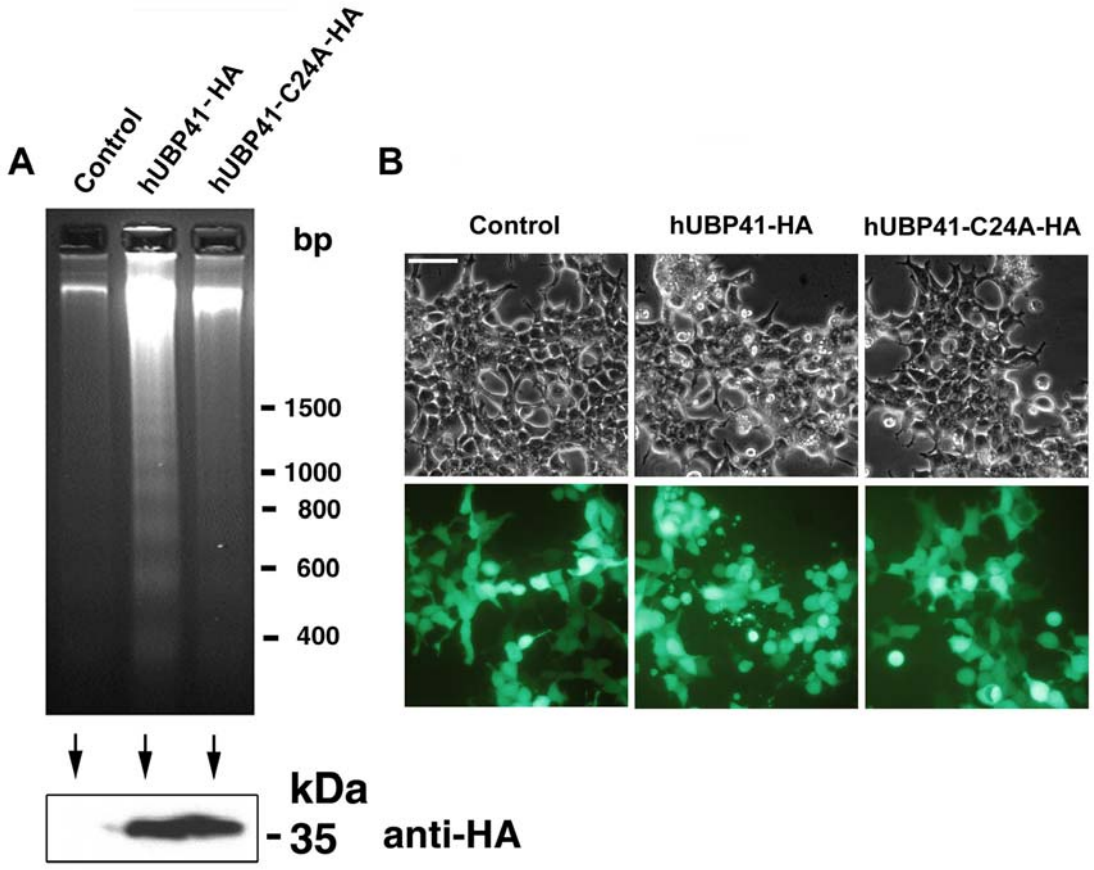


Fig. 3.1.3
Characterization of cell death induced by human UBP41 (hUBP41). *(A) Human UBP41 induces cell death that is dependent on the integrity of the catalytic center of the protease. 293T cells were transfected with a control vector, with wild type hUBP41-HA, or with the active-site mutant hUBP41-C24A-HA in which the catalytic cysteine had been replaced by an alanine residue. Degradation of cellular DNA was analysed 48h post transfection (upper panel). Equal expression of hUBP41-HA and hUBP41-C24A-HA was confirmed by an anti-HA immunoblot yielding bands of the expected size (lower panel). (B) Only the wild type UBP41 construct induces the characteristic phenotype of apoptosis in 293T cells. 293T cells were transfected with hUBP41-HA or hUBP41-C24A-HA together with an expression vector forEGFP. The upper panel shows phase contrast pictures 48h post transfection, whereas the lower panel presents EGFP fluorescence microscopical images by which apoptosis frequently can be easier observed.*

UBP41 belongs to the large family of ubiquitin-specific proteases (USPs). Therefore it was of interest whether also other USPs would be able to induce apoptosis upon overexpression. To this end, we isolated the two most homologous human family members of hUBP41, i.e. hUSP18 (gi:32313609; [Schwer, 2000]), and hUSP21 (gi: 6693823; [Gong, 2000]) with 25% or 45% identity to hUBP41, respectively **(Fig. 3.1.4)**.

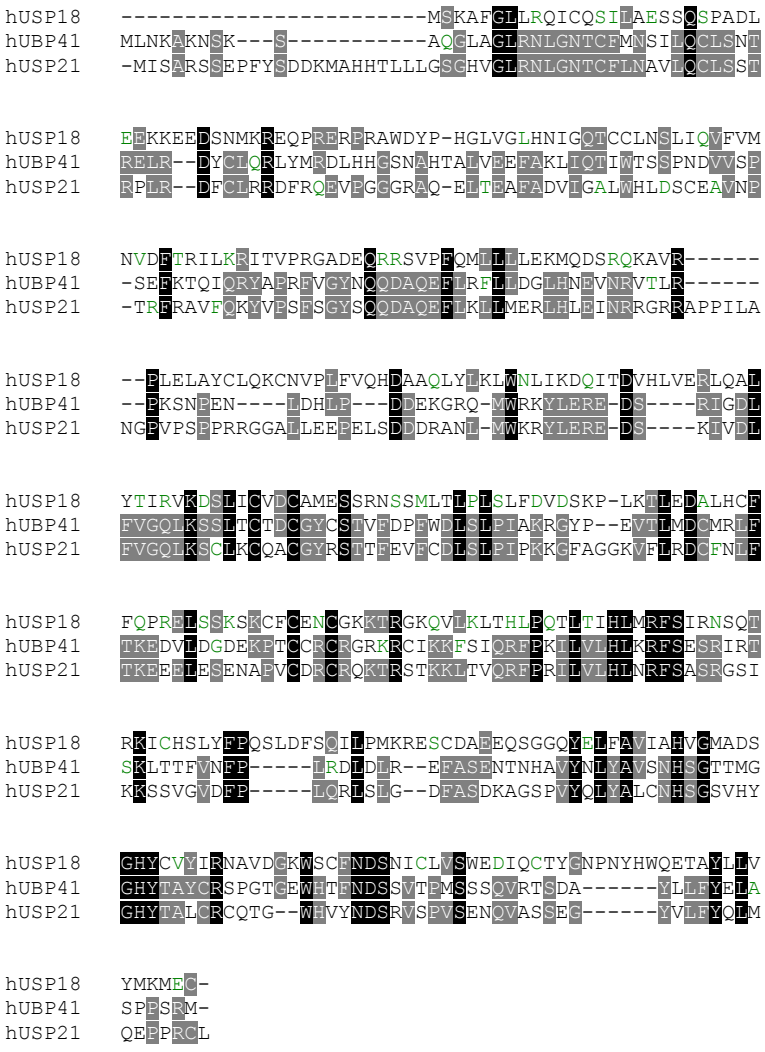


Fig. 3.1.4
Sequence alignment of human USP members most homologous to hUBP41. *hUSP21 (Genbank Accession AF134213; gi: 6693823) shares 45% sequence identity with hUBP41, whereas hUSP18 (Genbank Accession NM_017414; gi:32313609) is to about 25% identical to hUBP41. hUSP18 and hUSP21 additionally were the only USP members found that were of comparable size as hUBP41. The catalytic-site cysteine residues are C24, C37, and C64 for hUBP41, hUSP21, and hUSP18, respectively.*

hUSP18 and hUSP21 were generated as the carboxy-terminally HA-tagged proteins hUSP18-HA and hUSP21-HA, as well as their corresponding active site mutants hUSP18-C64A-HA and hUSP21-C37A-HA. Whereas overexpression of hUBP41-HA in 293T cells resulted in apoptotic DNA fragmentation as measured by propidium iodide FACS analysis and in the induction of caspase-3 enzymatic activity, hUSP18-HA and hUSP21-HA did not show any significant signs of apoptosis in 293T cells **(Fig. 3.1.5 A,B)**.

In those experiments, expression levels of hUSP18-HA and hUSP21-HA were usually clearly lower than those of hUBP41-HA and could not reproducibly be adjusted to the same level **(Fig. 3.1.5 C)**.

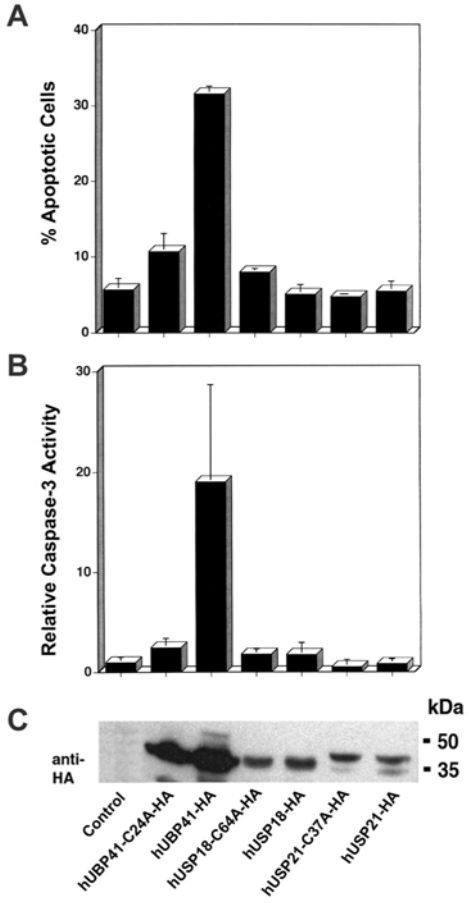


Fig. 3.1.5
hUBP41 but not hUSP18 or hUSP21 induce apoptosis in 293T cells. *(A) 293T cells were transfected with expression constructs for hUBP41, hUSP18, and hUSP21, as well as with the corresponding active-site mutants. The cells were harvested and stained with propidium iodide (PI) in Hypotonic Lysis Buffer. Cells with with sub-G1 DNA content were detected by FACS analysis. Shown are the means and the standard deviations of three values measured in parallel. (B) 293T cells were transfected as given in (A), harvested, washed with PBS, and lysed for detection of caspase-3 activity. Each data point was accessed at least in triplicate. (C) Expression of the HA-constructs was confirmed by anti-HA immunoblot analysis.*

To test whether hUBP41 overexpression also induces cell death in other cell systems, the prostatic carcinoma cell line PC3 was transfected. As shown in **Fig. 3.1.6**, only hUBP41-HA triggered clear signs of cell death in the transfected cells.

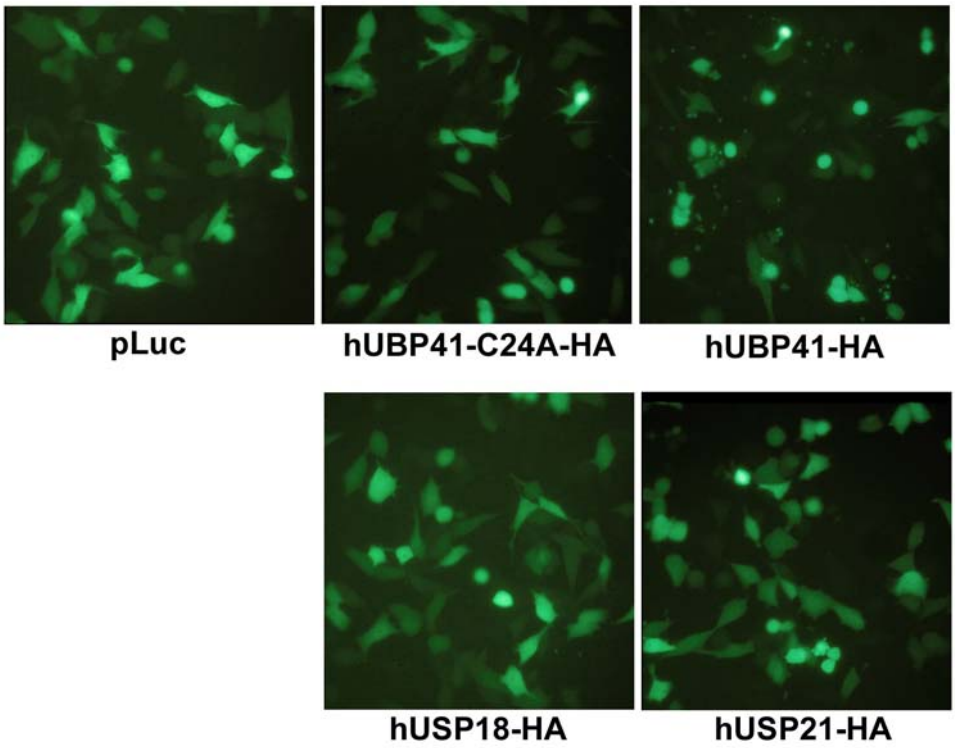


Fig. 3.1.6
hUBP41 also induces apoptosis when transfected into PC3 cells. *Transfection of hUBP41-HA into the prostatic carcinoma cell line PC3 cells resulted in cell death as observed by its phenotype. Here the fluorescence microscopic pictures are shown after cotransfection with hUBP41 and pEGFP for better detection sensitivity. Transfection of the enzymatic inactive form hUBP41-C24A-HA or hUSP18-HA and hUSP21-HA did not show clear signs of cell death. Pictures were taken at 200-fold magnification.*

The most reproducible and the highest transfection efficiencies could be obtained in HeLa cells using the Effectene transfection reagent (Qiagen). Therefore, the HeLa cell system was used to further investigate the UBP41-mediated apoptosis induction. Overexpression of hUBP41-HA in HeLa cells resulted in strong cell death induction displaying typical membrane blebbing already 24 to 30 hours post transfection **(Fig. 3.1.7 D)**, whereas in 293T cells clear signs of apoptosis were usually observed earliest about 40 hours post transfection. In contrast to the response in 293T cells, in HeLa cells also overexpression of the active site mutant hUBP41-C24A-HA **(Fig. 3.1.7 C)** and hUSP21-HA **(Fig. 3.1.7 H)** resulted in significant signs of apoptosis. This observation also could be confirmed by measuring DNA fragmentation by FACS analysis **(Fig. 3.1.8)**.

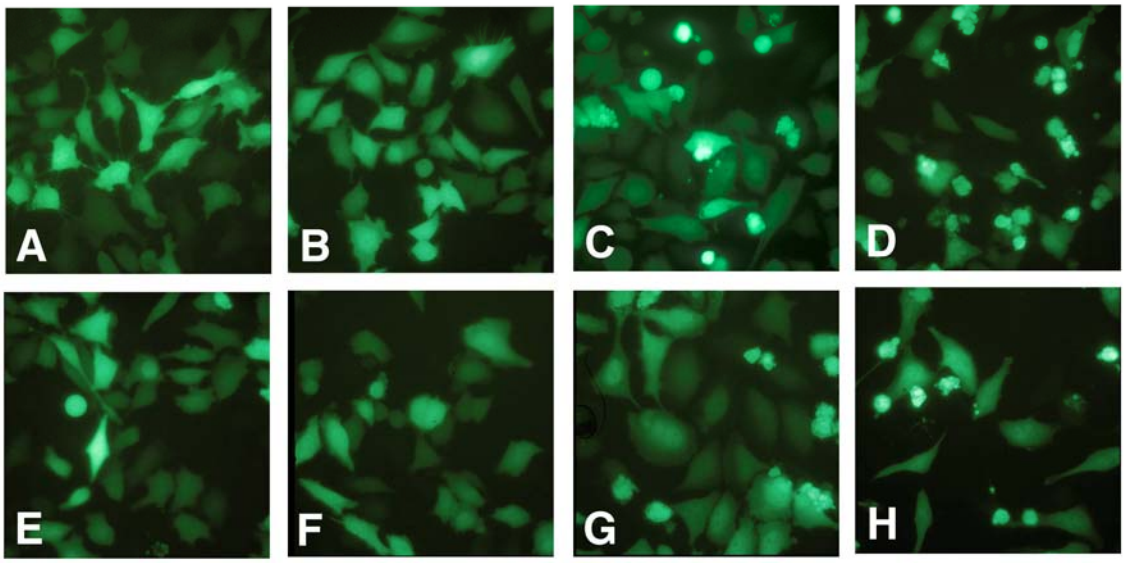


Fig. 3.1.7
Overexpression of various USPs in HeLa cells. *HeLa cells were transfected with expression vectors for (A) Luciferase as a negative control, (B) the hUBP41 sequence with its start ATG deleted, (C) the active-site mutant hUBP41-C24A-HA, (D) wildtype hUBP41-HA, (E) the active-site mutant hUSP18-C64A-HA, (F) wildtype hUSP18-HA, (G) the active-site-mutant hUSP21-C37A-HA, and (H) wildtype hUSP21-HA. All constructs were cotransfected with pEGFP for better visualization of transfected cells. The pictures were taken about 30 h post transfection. Cells are shown at 200-fold magnification.*

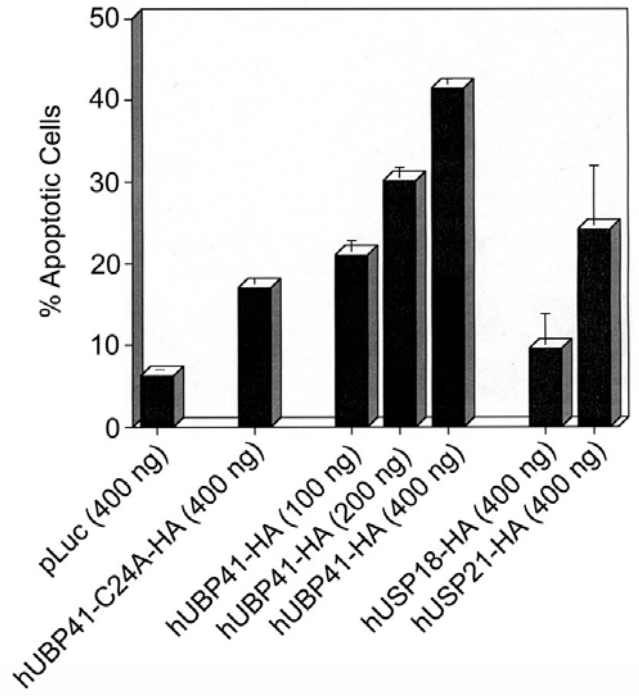


Fig. 3.1.8
Apoptosis induction by USP overexpression in HeLa cells as measured by PI FACS analysis. *USP expression plasmids were cotransfected with pEGFP. In case of hUBP41 different amounts of plasmid DNA were transfected. Approximately 36 h post transfection cells were analysed in PI FACS analysis for DNA content. The percentage of cells with hypoploid DNA content ("sub-G1 population") was considered to be a measure for the percentage of apoptotic cells. Values given were calculated by taking into acount the transfection efficiency as measure by EGFP-positive cells. Each data point was measured in triplicate.*

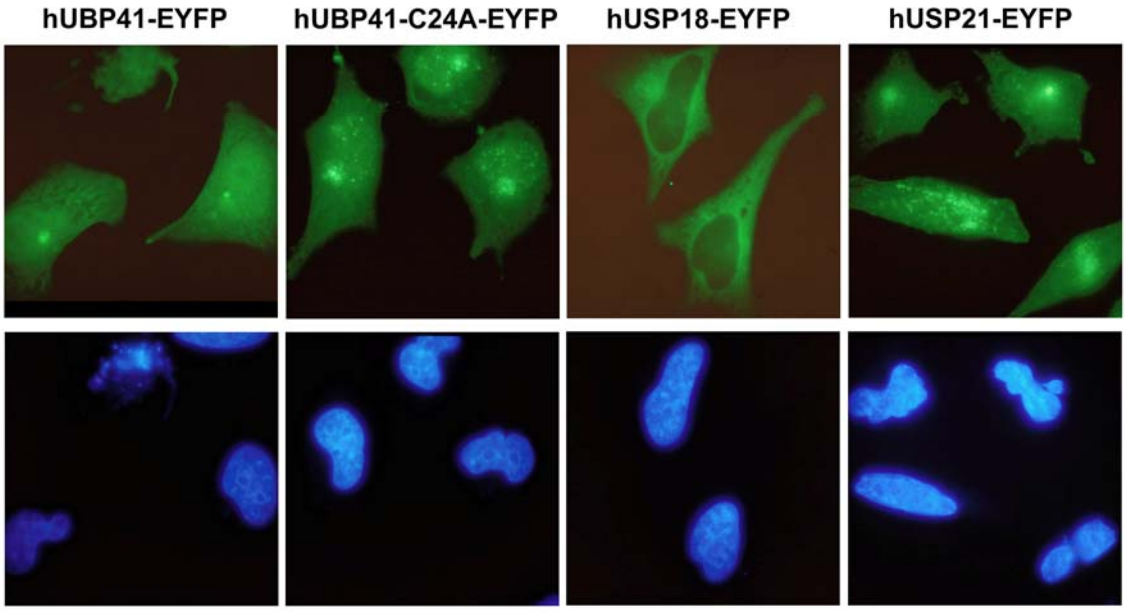


Fig. 3.1.9
Cellular localization of overexpressed USP proteins tagged with EGFP. *HeLa cells adherent to a cover glass were transfected with EYFP fusion constructs of hUBP41, hUBP41-C24A, hUSP18, and hUSP21. About 24 h post transfection, cells were stained with DAPI and observed by fluorescence microscopy displaying EYFP fluorescence (upper panels) and DAPI fluorescence (lower panels). Magnification is 600-fold.*

Since HeLa cells spread tightly on the surface of cell culture dishes or on glass cover slips, they could be used for studying the intracellular localization of the various USPs by fluorescence microscopy. Determination of the intracellular distribution of hUBP41 was of interest because it might have allowed to gain some information about the subcellular location at which hUBP41 overexpression unfolds its proapoptotic action. hUBP41, hUBP41-C24A, hUSP18, and hUSP21 were subcloned into a vector with a carboxy-terminal EYFP tag, the resulting EYFP fusion proteins were expressed in HeLa cells, and the intracellular distribution of the proteins was observed under the fluorescence microscope **(Fig. 3.1.9)**. hUBP41-EYFP usually displayed a uniform distribution within the whole cell, whereas the hUBP41-C24A-EYFP fusion protein in addition displayed larger spots of higher fluorescence intensity, possibly representing protein aggregates. hUSP21-EYFP was evenly distributed in the cells, albeit occasionally displaying spots of higher fluorescence intensity, whereas hUSP18-EYFP was excluded from the nucleus **(Fig. 3.1.9)**.

In order to further characterize the mode of cell death induced by overexpression of hUBP41, HeLa cells, transfected with hUBP41-HA, were cultivated in absence or presence of the pan-caspase inhibitor zVAD-fmk. Propidium iodide FACS analysis and visual inspection of the cells indicated a suppression of cell death in the presence of zVAD-fmk, suggesting a caspase-dependent, i.e. apoptotic mode of cell death **(Fig. 3.1.10 A,B)**.

Also the co-transfection of hUBP41-HA with the antiapoptotic Bcl-2 family member Bcl-X_L resulted

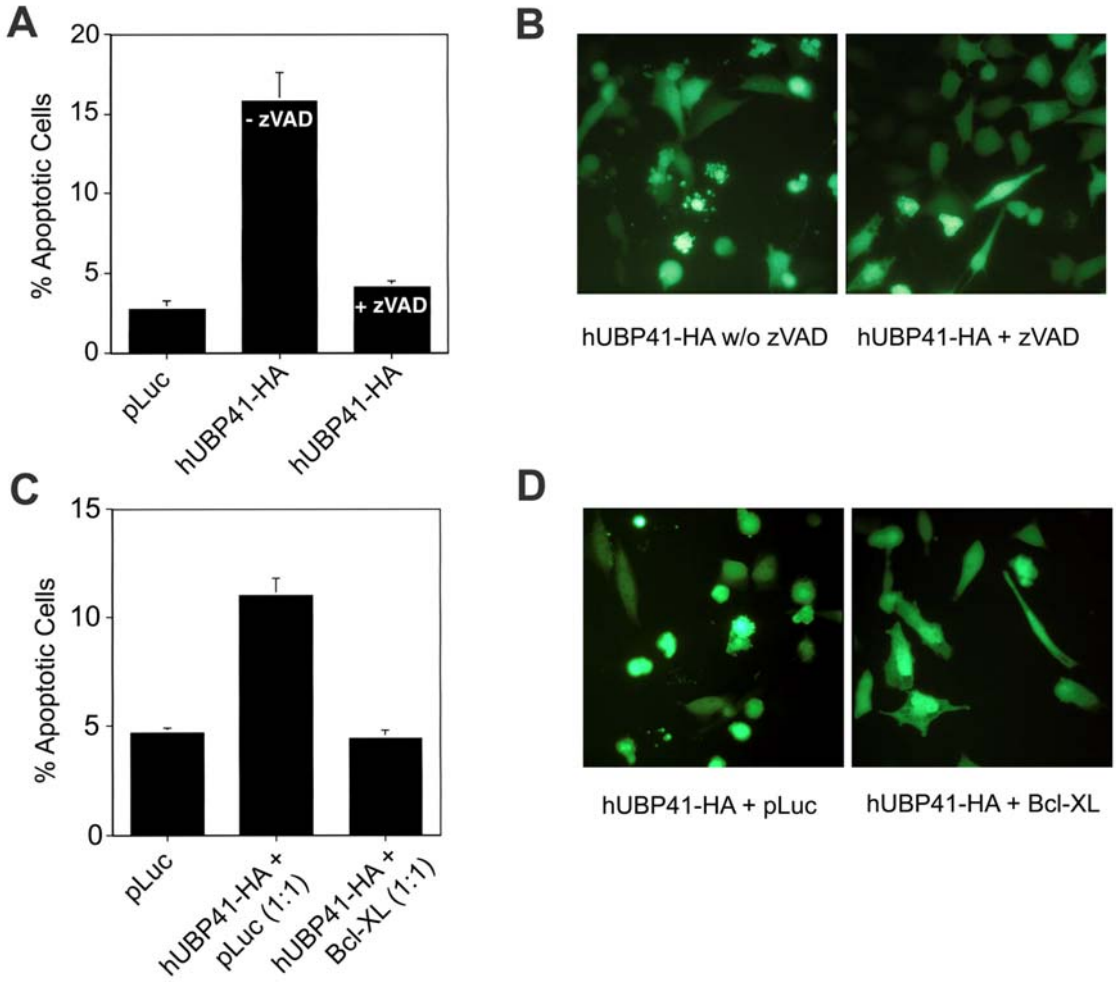


Fig. 3.1.10
Apoptosis induced by overexpression of UBP41 in HeLa cells can be suppressed by the pan-caspase inhibitor zVAD-fmk and by coexpression of the antiapoptotic Bcl-2 member Bcl-X_L.
(A,B) HeLa cells were transfected with the hUBP41-HA expression vector in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 µM) which was added to the cells at the time point of media change during the transfection procedure. About 30 h post transfection the percentage of apoptotic cells was measured in terms of DNA fragmentation by PI FACS analysis (A) or cells were investigated by fluorescence microscopy at 200-fold magnification (B). (C,D) HeLa cells were transfected with the hUBP41-HA expression vector in absence or presence of additional Bcl-X_L co-expression, and the cells were analysed as indicated under (A,B).

in the inhibition of hUBP41-induced cell death, further indicating that major apoptotic signalling events such as those regulated by the Bcl-2 family are involved in hUBP41-induced cell death **(Fig. 3.1.10 C,D)**. It might be worth noting that while zVAD-fmk and Bcl-X_L were able to inhibit typical features of apoptosis, i.e. the typical morphological changes as well as DNA fragmentation, some of the cells nevertheless displayed an unusual phenotype by forming long, extended shapes or by containing large open, vacuole-like structures **(Fig. 3.1.10 B,D)**.

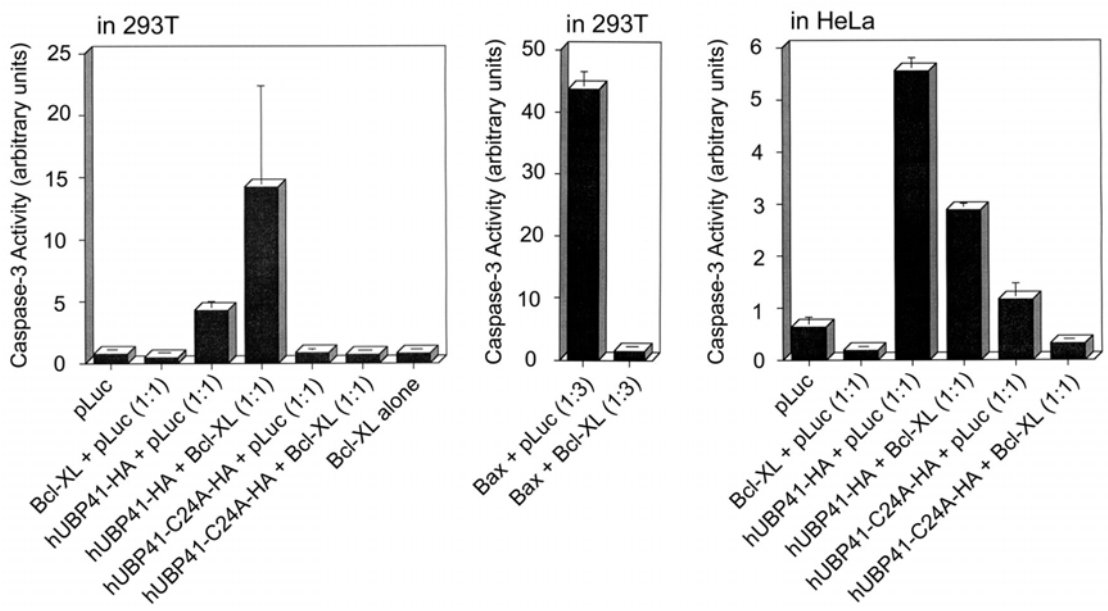


Fig. 3.1.11
In 293T cells, Bcl-X_L unexpectedly does not inhibit UBP41-induced apoptosis but even enhances the killing effect of UBP41 overexpression. *(A) 293T cells were co-transfected with the hUBP41 expression vector and a luciferase expression vector (pLuc) or with a Bcl-X_L expression vector using equal DNA amounts for transfection. As a control, the same was done with the active-site-mutant hUBP41-C24A-HA. Moreover, Bcl-X_L was transfected on its own or co-transfected with the pLuc vector. Cells were harvested for a caspase-3 activity assay about 40 h post transfection. (B) 293T cells were co-transfected with an expression vector for Bax together with pLuc or Bcl-X_L using a DNA ratio of 1:3. Cells were harvested for the caspase-3 activity assay about 24 h post transfection. (C) The same experiment described under (A) was performed in HeLa cells.*

Surprisingly, in contrast to the situation in HeLa cells, hUBP41-induced apoptosis in 293T cells could not be suppressed but was enhanced by co-transfection of Bcl-X_L. As expected, Bcl-X_L could almost completely block Bax-induced apoptosis under the same experimental settings. This effect could be observed reproducibly by microscopical inspection (not shown) as well as by caspase-3 enzymatic activity assay which for the 293T cell system proved to be more sensitive than PI FACS analysis in case of hUBP41-mediated cell death **(Fig. 3.1.11)**.

3.1.3 Evaluation of possible modes of action for the UBP41-mediated proapoptotic effect

In the attempt to find possible explanations for the observed proapoptotic effect of UBP41 overexpression, various scenarios were taken into consideration and subsequently were tested experimentally for their potential significance. The most obvious issue was to test the impact of

UBP41 overexpression on the ubiquitination status of proteins. As a protein with deubiquitylating activity, UBP41 was expected to be able to remove ubiquitin from multi-ubiquitylated protein substrates but it remained to be determined whether UBP41 acts on a selected set of proteins or on a broad range of proteins. Another question was whether it is the deubiquitination of proteins or rather the possibly concomitant accumulation of the mono-ubiquitin pool that triggers the induction of apoptosis.

To first answer the question whether overexpression of UBP41 has an impact on the total ubiquitination status of endogenous proteins, 293T cells were transfected with pLuc as a control, hUBP41-HA, its enzymatically inactive mutant hUBP41-C24A-HA, or its homologues hUSP18-HA and hUSP21-HA. About 42h post transfection, total protein extracts were prepared from the transfected cells, equal amounts of protein were separated in SDS gel electrophoresis, and the subsequent immunoblot was probed with anti-ubiquitin antibodies. As can be seen in **Fig. 3.1.12**, overexpression of hUBP41-HA resulted in a dramatic deubiquitination of a wide range of substrates, whereas all other transfected constructs did not show any significant effect, although in some experiments the ubiquitin-signal in hUSP21-HA transfected 293T also appeared to be slightly diminished. The same results were obtained in transfection experiments using HeLa cells (data not shown).

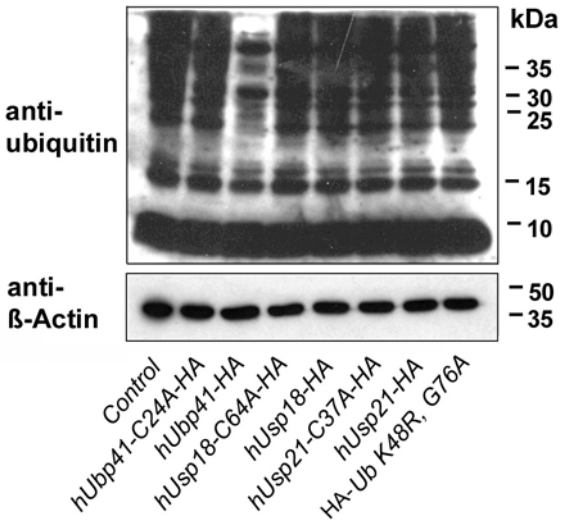


Fig. 3.1.12
UBP41 overexpression results in the deubiquitination of a broad range of proteins. *293T cells were transfected with a negative control vector, the various USP gene constructs, as well as with a vector encoding the HA-tagged ubiquitin double mutant Ub K48R, G76A. 42 hours post transfection cells were harvested and lysed. 100 µg of total protein were loaded in each lane of a 15% SDS PAGE gel for immunoblot analysis using anti-ubiquitin polyclonal antibodies. Equal loading was confirmed by an anti-ß-actin control blot.*

As a consequence of the dramatic deubiquitylating activity mediated by hUBP41-HA overexpression **(Fig. 3.1.12)**, free monomeric ubiquitin could be expected to accumulate within the cell. An inappropriately increased ubiquitin pool then might interfere with cellular processes, e.g. by its putative chaotropic action towards other proteins as described by Wenzel and Baumeister

[Wenzel, 1993]. To test this hypothesis, wildtype ubiquitin was overexpressed in HeLa cells and 293T cells, but no effect concerning cell death induction could be observed (data not shown). In this context it also should be noted that in anti-ubiquitin immunoblots very high basal levels of monoubiquitin were observed in control transfected 293T as well as in HeLa cells which did not recognizably change upon hUBP41-HA overexpression (see the intense band at < 10 kDa in **Fig. 3.1.12**).

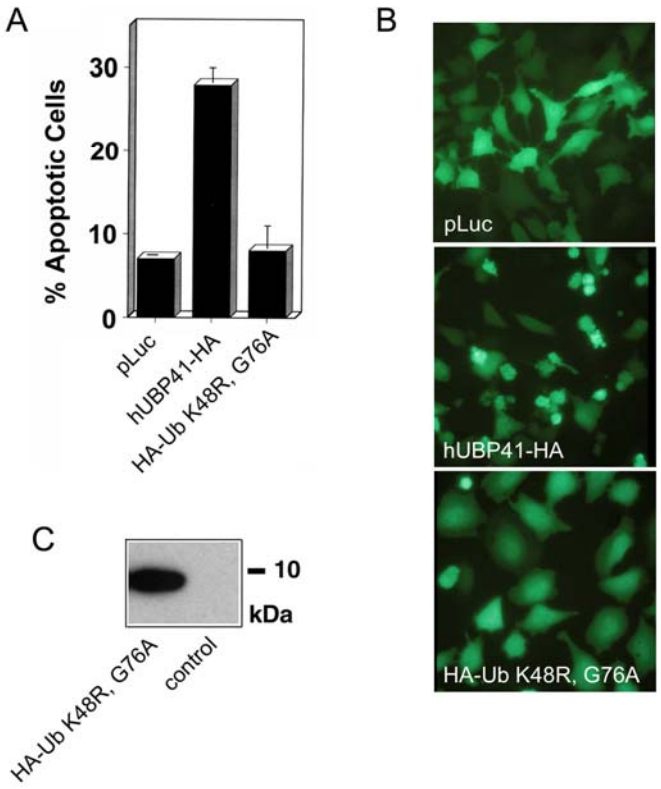


Fig. 3.1.13
Overexpression of the ubiquitin double mutant Ub K48R, G76A does not result in apoptosis induction. *An expression vector for hUBP41-HA or for the aminoterminally HA-tagged ubiquitin double mutant HA-Ub K48R, G76A was transfected into HeLa cells (pEGFP was co-transfected). (A) The percentage of cells with fragmented DNA was analysed by PI FACS analysis about 35 h post transfection. (B) The same cells that were analysed in (A) are shown here under the fluorescence microscope showing the transfected, i.e. EGFP-positive cells. (C) Expression of the ubiquitin double mutant HA-Ub K48R, G76A was confirmed by anti-HA immunoblot analysis.*

In the attempt to interfere with the ubiquitination system in an alternative and potentially similar way as does the overexpression of hUBP41, a HA-tagged double mutant of ubiquitin, HA-Ub K48R,G76A, was generated and overexpressed in HeLa cells. Overexpression of HA-Ub K48R,G76A is expected to result in, on the average, shorter ubiquitin chain lengths of ubiquitin-modified proteins, since the K48R mutation lacks the common lysine residue for chain elongation, whereas the G76A mutation still allows conjugation to a growing ubiquitin chain but is more resistant to deubiquitination [Finley, 1994]. As depicted in **Fig. 3.1.13**, overexpression of HA-Ub K48R,G76A was confirmed by anti-HA immunoblot analysis, but in contrast to hUBP41 overexpression did not result in any signs of apoptosis.

The next experiments focused on the question whether the observed deubiquitylating activity of UBP41 affects the stability of proteins that normally are polyubiquitylated and degraded by the proteasome. In presence of elevated hUBP41 levels those proteins should be expected to be

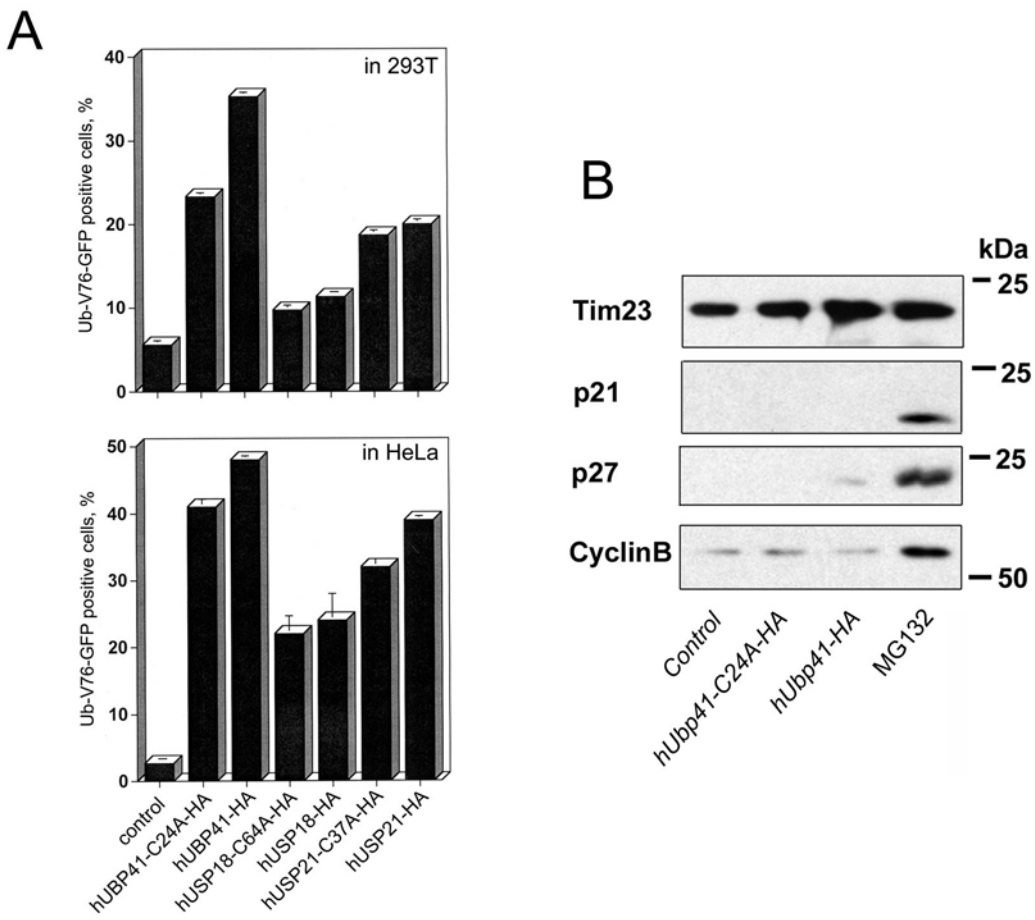


Fig. 3.1.14
The artificial test substrate Ub-G76V-GFP is stabilized by UBP41 overexpression but the tested endogenous candidate substrates are not. *(A) 10 ng of an expression vector encoding the test substrate Ub-G76V-GFP were co-transfected with the various USP expression constructs either in 293T cells (upper graph) or in HeLa cells (lower graph). As a control, Ub-G76V-GFP was expressed together with an arbitrary HA-tagged protein. The percentage of GFP-positive cells was determined by FACS analysis about 24 h post transfection. (B) UBP41 does not stabilize various known endogenous substrates of the proteasome. HeLa cells were transfected with a control vector, with hUBP41-HA or its active-site mutant hUBP41-C24A-HA. Alternatively, the cells were treated with 1 µM of the proteasome inhibitor MG312. Extracts were prepared 25 hours after transfection or start of treatment. Equal amounts of extracts were used for immunoblot analysis, probing with antibodies to the indicated proteins.*

deubiquitylated and thus saved from degradation. To test this hypothesis, the destabilized ubiquitin-GFP fusion protein Ub-G76V-GFP was employed as an artificial test substrate [Dantuma, 2000]. When Ub-G76V-GFP was co-expressed with an arbitrary HA-tagged protein as a control, only a small fraction of transfected cells displayed GFP fluorescence, likely due to immediate degradation of the ubiquitin-labelled GFP. In contrast, co-expression of hUBP41-HA strongly increased the number of GFP-positive cells, indicating a stabilizing effect of hUBP41-HA on the test substrate **(Fig. 3.1.14 A)**

in both, HeLa and 293T cells. Unexpectedly, also co-expression of the active site mutant hUBP41-C24A-HA resulted in stabilization of the GFP test substrate, almost as efficient as hUBP41-HA itself, and co-expression of hUSP18-HA, hUSP21-HA and their corresponding active-site mutants also led to a significant extent of stabilization. Irrespective of those unexpected observations, the stabilizing effect of hUBP41 on the artificial substrate Ub-G76V-GFP still allowed to assume that ubiquitylated proteins might be stabilized by the deubiquitinating property of hUBP41.

Therefore, the influence of hUBP41-HA on the stability of endogenous target proteins was examined. Potential candidate target proteins were the cell cycle proteins $p21^{Cip1/Waf1}$, $p27^{Kip1}$, cyclin B1, and p53, which are known to be regulated by ubiquitination and proteasomal degradation [Elledge, 1998; Zhang, 1998]. As a positive control, HeLa cells were treated with the peptide aldehyde proteasome inhibitor MG132 which has been described to potently block the degradation of $p21^{Cip1/Waf1}$, $p27^{Kip1}$, and cyclin B1 [Chang, 1998; Fan, 2001; Maki, 1996; Nahreini, 2001; Naujokat, 2000]. In the HeLa cell system investigated in this study, the protein levels of $p21^{Cip1/Waf1}$, $p27^{Kip1}$ and cyclin B1 increased markedly in presence of MG132 **(Fig. 3.1.14 B)**. In contrast to treatment with MG132, overexpression of hUBP41-HA obviously did not cause any detectable stabilization of $p21^{Cip1/Waf1}$, $p27^{Kip1}$ or cyclin B1. Therefore, even though a broad range of proteins is deubiquitylated upon hUBP41 overexpression **(Fig. 3.1.12)**, apparently not all those proteins can be expected to be stabilized as a consequence of deubiquitination **(Fig.4.1.14 B)**.

Consistent with its stabilizing effect on cell cycle inhibitors such as $p21^{Cip1/Waf1}$ and $p27^{Kip1}$, the proteasome inhibitor MG132 is known to induce cell cycle arrest, eventually leading to the activation of apoptosis pathways [Emanuele, 2002; Lee, 1998]. In order to compare the drug MG132 with hUBP41 overexpression regarding their respective impact on the cell cycle and concurrent apoptosis induction, HeLa cells were either treated with MG132 or transfected with hUBP41-HA. At various times, cellular DNA content was evaluated by propidium iodide FACS analysis, allowing the quantification of hypoploid apoptotic cells as well as the assessment of the cell cycle distribution of the non-apoptotic cell population.

As shown in **Fig. 3.1.15**, MG132 primarily induced cell cycle arrest in G_2/M and only moderately triggered apoptosis induction, possibly as a consequence of the cell cycle arrest. In contrast, hUBP41-HA did not arrest the cells in G_2/M but obviously was driving cells directly into apoptosis. These data indictate that hUBP41-mediated cellular changes are different from those caused by a general block of proteasomal activity as caused by the proteasome inhibitor MG132.

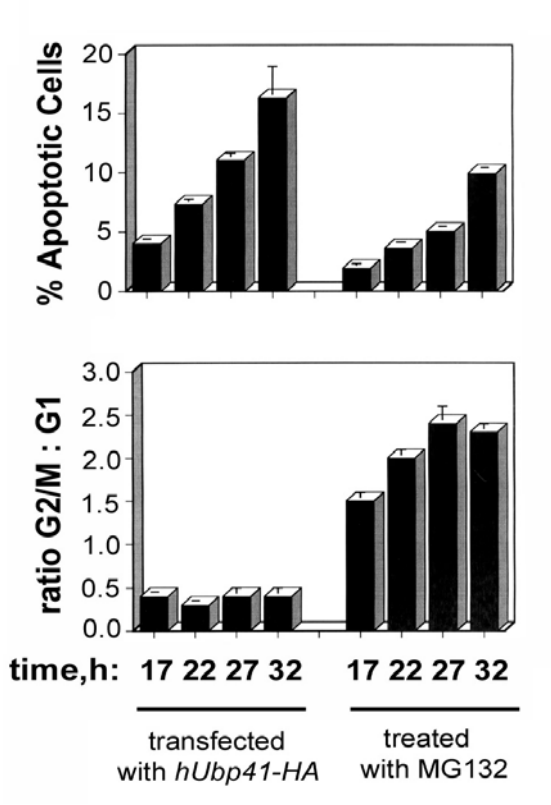


Fig. 3.1.15
Comparison of the effects induced by hUBP41 overexpression and treatment with the proteasome inhibitor MG-132. *The proteasome inhibitor MG132 primarily results in a G_2/M arrest and only moderately induces apoptosis, whereas overexpression of hUBP41 triggers apoptosis without prior G_2/M arrest. HeLa cells were transfected with hUbp41-HA or treated with the proteasome inhibitor drug MG-132 (1 μM) for the indicated times. Apoptosis (upper panel) and cell cycle arrest (lower panel) was quantified by PI staining and FACS analysis. Each data point was measured in triplicate.*

3.1.4 Generation and characterization of a polyclonal anti-hUBP41 antibody

The data presented thus far resulted from experiments that were based on the overexpression of hUBP41 in various cell lines. In order to gain more insight into the biochemical properties of hUBP41 and its physiological roles in cell biology as well as its possible involvement in pathological conditions such as cancer, we decided to investigate expression of endogenous hUBP41. To this end, a specific polyclonal anti-hUBP41 antibody was raised in rabbit: a peptide (GST-IP-B) comprising the amino acids 119-150 of hUBP41 as the antigenic determinant was expressed as a GST-fusion protein using the pGEX vector system in the E.coli strain BL21, purified on a GSH-sepharose column and subsequently on a sephadex 200 column, and eventually was used for immunization.

The crude serum obtained after final bleeding indeed recognized the immunogenic peptide as well as overexpressed hUBP41 but certainly also contained anti-GST antibodies **(Fig. 3.1.16 A).** For this reason and to eliminate as many cross reactivity as possible, the crude serum was affinity purified in a two-step protocol first depleting anti-GST antibodies by incubation with GST-linked cyanogen bromide sepharose and in the second step by binding to and eventually eluting from GST-IP-B-coupled cyanogen bromide sepharose. The affinity purified eluate indeed still recognized the immunopeptide GST-IP-B as well as overexpressed hUBP41 but did not crossreact with GST **(Fig. 3.1.16 B)**.

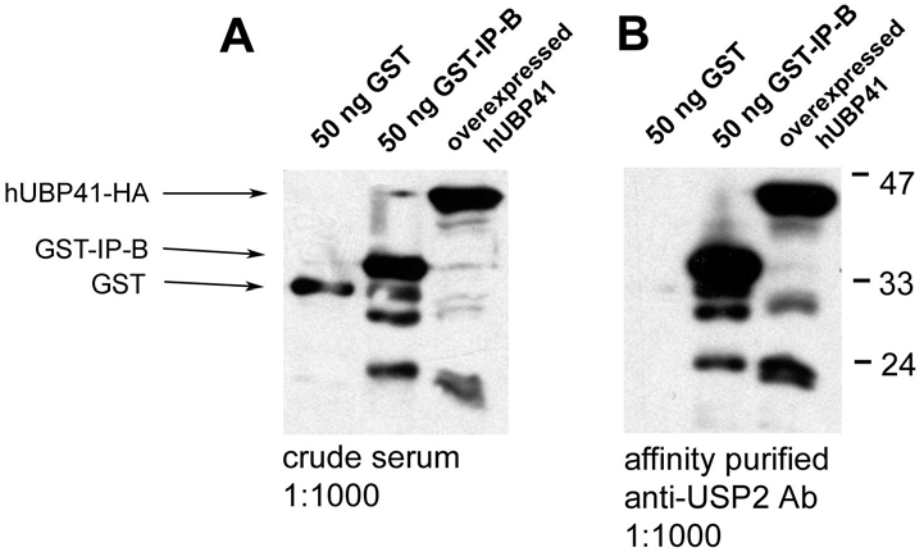


Fig. 3.1.16
Evaluation and affinity purification of the polyclonal anti-hUBP41 antibodies.
(A) The crude serum of the immunized rabbit recognized the immunopeptide (GST-IP-B) against it was raised. It also recognized hUBP41 in extracts of hUBP41-transfected cells. Since the immunopeptide was a GST fusion peptide, the crude serum also crossreacted with GST. (B) After affinity purification, the obtained antibody eluate still recognized GST-IP-B and overexpressed hUBP41 but did not significantly crossreact with GST.

The affinity purified anti-hUBP41 antibodies were then applied to determine the endogenous expression status of hUBP41. In extracts from HeLa, PC3, 293T and PC3 cells a pattern of up to five protein bands was detected of which no band did correspond to the expected size of overexpressed hUBP41, indicating that hUBP41 might be expressed as several alternative isoforms with the 41 kDa form not being detectable in the cell lines investigated **(Fig. 3.1.17)**. Indeed, UBP41 is known to be a member of the USP2 family of isoforms, and in chicken, for example, four isoforms have been described [Baek, 1998].

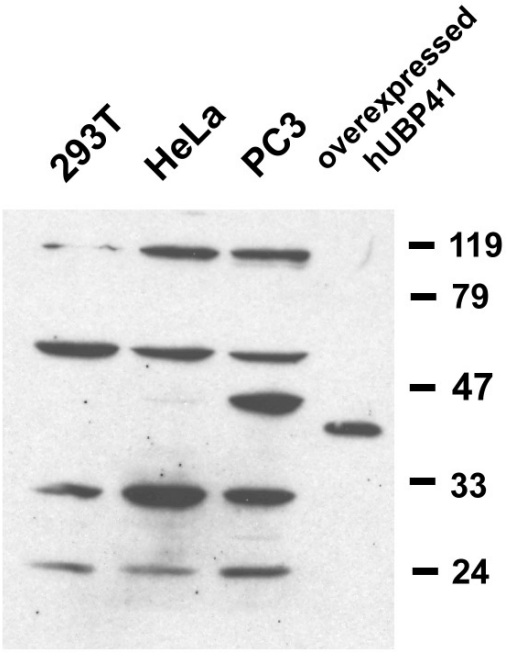


Fig. 3.1.17
Immunoblot analysis of cell extracts using the anti-UBP41 polyclonal antibodies. *Cell extracts (100 µg protein) from untreated 293T, HeLa, and PC3 cells were analysed by western blot, probing the membrane with affinity purified anti-UBP41 polyclonal antibodies (1:1000). Extracts from hUBP41-transfected cells were loaded as a positive control (last lane). Several bands were detected in the extracts from untreated cells which might represent isoforms of the USP2 protein family. No band of the size expected for hUBP41 was observed.*

In the attempt to confirm the identity of some of the endogenous bands shown in **Fig. 3.1.17** as a member of the USP2 isoform family, an approach using the RNAi technology was chosen [Elbashir, 2001]. Two target sequences unique to the human USP2 sequence were selected and were inserted into the pSuper vector system. To test the capability of those RNAi target sequences in suppressing the expression of the hUBP41 message, the hUBP41 expression plasmid was co-expressed with either the pSuper-hUBP41 #1 or pSuper-hUBP41 #2 RNAi construct, respectively. Only the pSuper-hUBP41 #1 construct could reproducibly diminish hUBP41 expression, whereas the pSuper-hUBP41 #2 construct as well as another unrelated RNAi construct did not alter hUBP41 expression **(Fig. 3.1.18)**. Expression of the SAIP-HA protein - whose expression vector was additionally co-transfected as an internal control - was not affected by the pSuper-hUBP41 #1 RNA_i construct suggesting its specific activity towards the hUBP41 message.

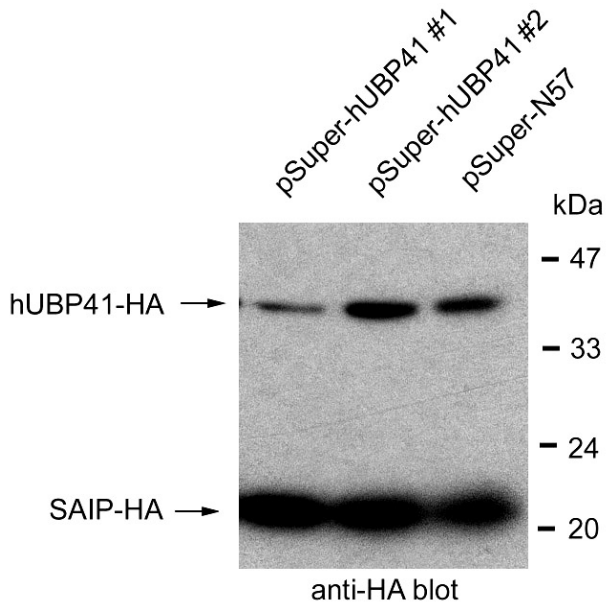


Fig. 3.1.18
Evaluation of candidate RNAi constructs for the downregulation of hUBP41 in a co-transfection experiment. *In the HeLa cell system, the hUBP41-HA expression plasmid was co-transfected with both, an expression plasmid for the SAIP-HA protein, as well as with one of the RNAi constructs pSuper-hUBP41 #1, pSuper-hUBP41 #2, or the unrelated pSuper-N57 construct. 20 hours post transfection, the cells were harvested, lysed and and equal amounts of the extracts were loaded on a SDS-PAGE gel for immunoblot analysis using an anti-HA antibody. hUBP41 expression was clearly decreased in the presence of the pSuper-hUBP41 #1 RNAi construct but not in presence of pSuper-hUBP41 #2 or pSuper-N57. Expression of SAIP-HA was not affected by pSuper-hUBP41 #1.*

Even though the RNAi construct pSuper-hUBP41 #1 could successfully suppress hUBP41-HA expression in a co-transfection experiment (**Fig. 3.1.18**), transfection of pSuper-hUBP41 #1 into HeLa cells did not alter the signal intensities of any of the putative endogenous USP2 bands that are detected by the anti-hUBP41 polyclonal antiserum (not shown).

In conclusion, the generated polyclonal anti-hUBP41 antibody does recognize the overexpressed hUBP41 protein (**Fig. 3.1.16**) but in extracts of untreated cells a corresponding endogenous hUBP41 protein of the expected molecular size could not be detected when using this antibody in immunoblot analyses, possibly because hUBP41 is not strongly expressed enough in the cell lines investigated. Instead, the antibody recognized a number of endogenous proteins of different size (**Fig. 3.1.17**) which might correspond to the hUSP2 isoforms of the hUBP41 protein. The attempt to clearly identify those proteins bands as isoforms of hUBP41 by using RNAi technology was not successful. Therefore, the polyclonal anti-hUBP41 antibody could not be further used for expression analysis of endogenous hUBP41 or its hUSP2 isoforms, respectively. Instead, northern hybridization experiments were performed to study hUBP41/hUSP2 expression.

3.1.5 Analysis of hUBP41 / hUSP2 expression by northern hybridization

For northern hybridization, the radioactively labelled hUBP41 cDNA was used as a probe. This probe was expected to recognize the human UBP41 transcript but also its described longer USP2 isoforms. The design of a sequence-specific hybridization probe for discriminating between hUBP41 and its isoforms was not possible because the hUBP41 cDNA sequence is virtually identical to the entire 3' terminal end of its two isoforms. Therefore, the subsequently shown hybridization experiments represent the expression profile of the hUSP2 isoform family rather than that of hUBP41 alone.

First of all, the tissue distribution of hUBP41 expression was examined by hybridization of a multiple tissue northern blot (Clontech) using the radioactive hUBP41 cDNA probe. As shown in **Fig. 3.1.19**, the hUBP41 cDNA probe hybridized with two bands of approximately 3.5 and 4.0 kb, respectively, suggesting the presence of two alternative transcripts, which could be detected most strongly in skeletal muscle and kidney, but also in brain, heart and liver.

Subsequently, the same hUBP41 cDNA probe was used to hybridize a Cancer Profiling Array blot provided by Clontech which allowed the comparison of expression levels in normal tissue and tumor tissue from the same patient (**Fig. 3.1.20**). Analysis of the signal intensities as measured by a phosphoimager revealed a decrease of the hUBP41 hybridization signal in tumor specimen of the kidney when compared to normal kidney tissue whereas between samples of other organ origin no significant differences could be detected (**Fig. 3.1.21**). This would suggest a possible correlation between tumorigenesis of the kidney and a decrease of hUSP2 transcript expression.

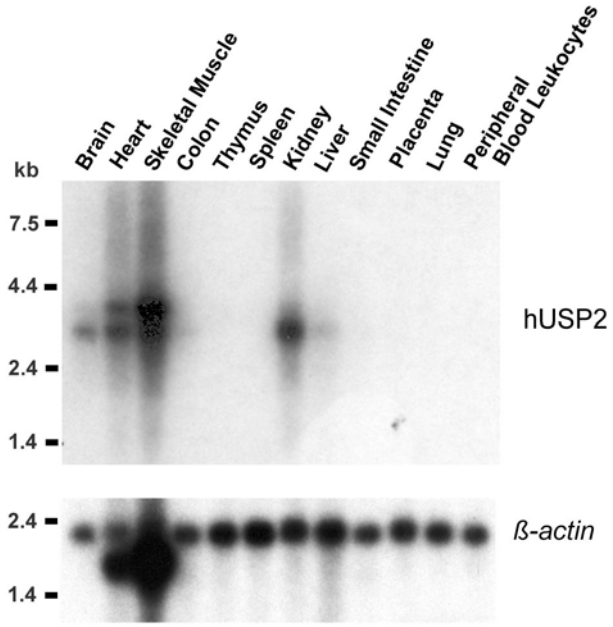


Fig. 3.1.19
Tissue expression pattern of human USP2 isoforms. *A multiple tissue northern blot (Clontech) was hybridized with the human UBP41 cDNA probe which can hybridize to transcripts of the USP2 isoform family (upper panel). The same blot was probed with β-actin probe as a loading control (lower panel). The hUBP41 cDNA probe recognizes two main transcripts which are most strongly expressed in kidney, skeletal muscle, heart and brain.*

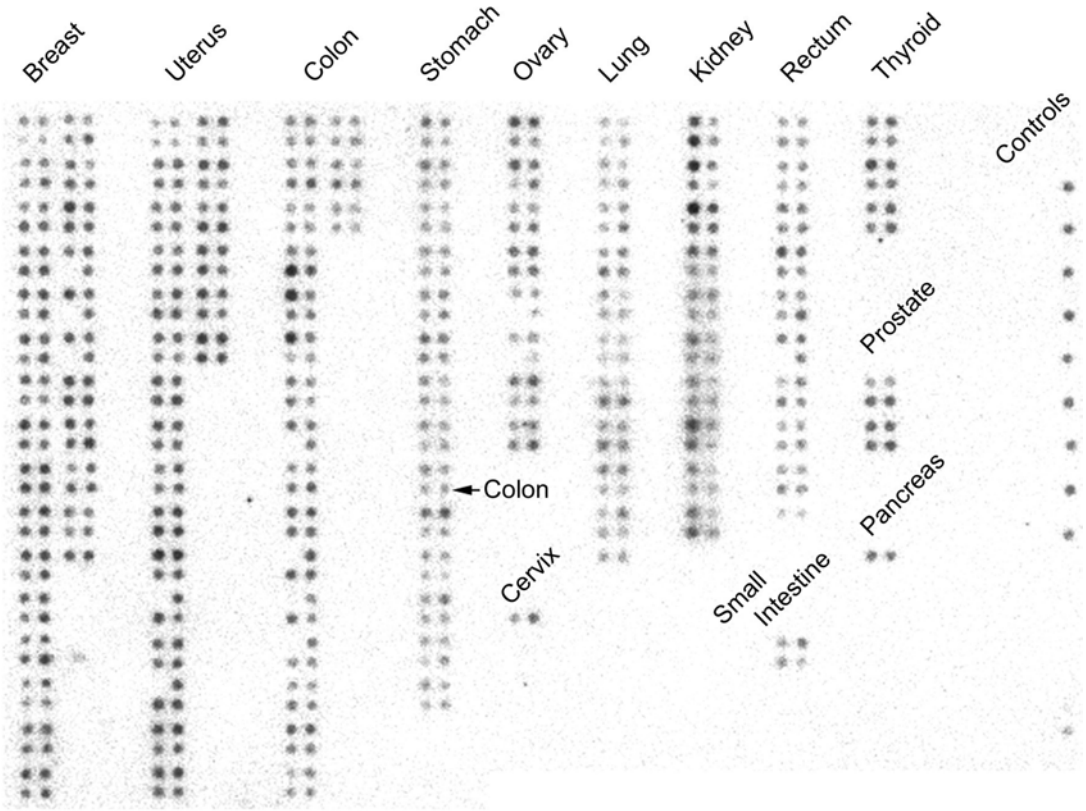


Fig. 3.1.20
Hybridization of a hUBP41 cDNA probe with the Cancer Profiling Array (Clontech). *Hybridization of a radioactive hUBP41 cDNA probe with the Cancer Profiling Array blot from Clontech. The Cancer Profiling Array contains pairs of cDNA which were generated from tumor and corresponding normal tissue samples from individual patients, spotted side by side on a nylon membrane (Normal at the left/ tumor at the right). This blot revealed equal expression of the hUBP41 mRNA message in all normal/tumor tissues except in tumors of the kidney which in many cases apparently display a decrease of the hUBP41 signal in tumor samples when compared to that in normal tissue.*

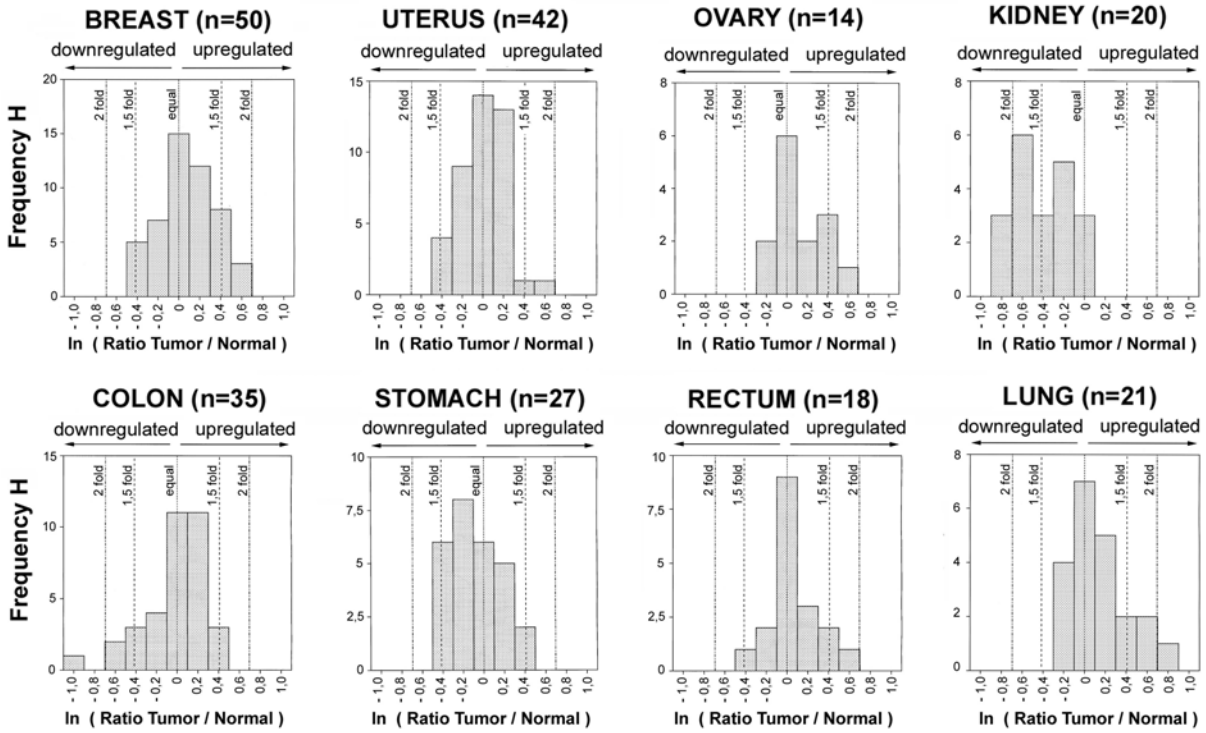


Fig. 3.1.21
Evaluation of the Cancer Profiling Array hybridized with the hUBP41 cDNA probe. *The Cancer Profiling Array (Fig. 3.1.17) was evaluated for normal/tumor pairs of tissues for which a sufficient number of samples (n > 14) were available. For evaluation the signal intensities were used that were measured and processed with the Fuji BAS 2500 phosphoimager and the Image Gauge V3.01 software. For representation of the data in this figure, the ratio of the corresponding tumor:normal signal intensity pair was calculated and from this the percentage of deviance from an intensity ratio of 1 (if the signals were the same for tumor and normal sample) was deduced. The number of samples belonging to a certain range of deviance were combined to groups representing a bar in this histogram. A negative value of deviance corresponds to a decrease of signal intensity in tumor compared to its normal counterpart, therefore suggesting a downregulation (down) of the hUSP2 transcript expression in tumor. A positive deviance value describes an increase in signal intensity in tumor compared to its normal counterpart, suggesting an upregulation (up) of the hUSP2 message in tumor.*

3.2 Investigation of a possible involvement of cathepsin-L and cathepsin-B in apoptosis induction

3.2.1 A mouse cathepsin-L cDNA was identified to cause morphological changes in 293T cells when overexpressed

The cDNA for murine cathepsin-L was identified by our group as one of the cDNA clones that became conspicuous during the screening procedure for apoptosis-inducing genes according to the method described by Grimm and Leder [Grimm, 1997]. The original photo from the screen (**Fig. 3.2.1)** shows 293T cells which were transfected with the cDNA clone that was later identified to code for cathepsin-L: a significant number of cells display a detached, rounded and sometimes fragmented phenotype.

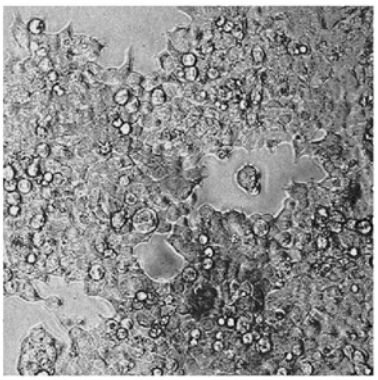


Fig. 3.2.1
293T cells transfected with a cDNA clone from the screen encoding cathepsin-L. *This is an original photo of 293T cells transfected with a cDNA clone that was identified by our group to encode cathepsin-L.*

3.2.2 Transient overexpression of cathepsin-D but not of cathepsin-L and cathepsin-B results in significant induction of apoptosis

Towards a further investigation of the initial observation from the screen that suggests a possible apoptosis-inducing effect of cathepsin-L overexpression, the human cathepsin-L coding sequence (hCath-L) was isolated by RT-PCR and cloned into the pcDNA3 expression vector lacking the neomycin resistance cassette. For comparison, the human coding sequences of the related lysosomal cysteine protease cathepsin-B (hCath-B) and that of the lysosomal aspartate protease cathepsin-D (hCath-D) were also cloned. Successful overexpression of the cathepsins upon transient transfection into HeLa cells could be confirmed by western blot analysis **(Fig. 3.2.2 A)** as well as by the measurement of hCath-L and hCath-B enzymatic activity using zFR-AMC as a substrate **(Fig. 3.2.2 B)**.

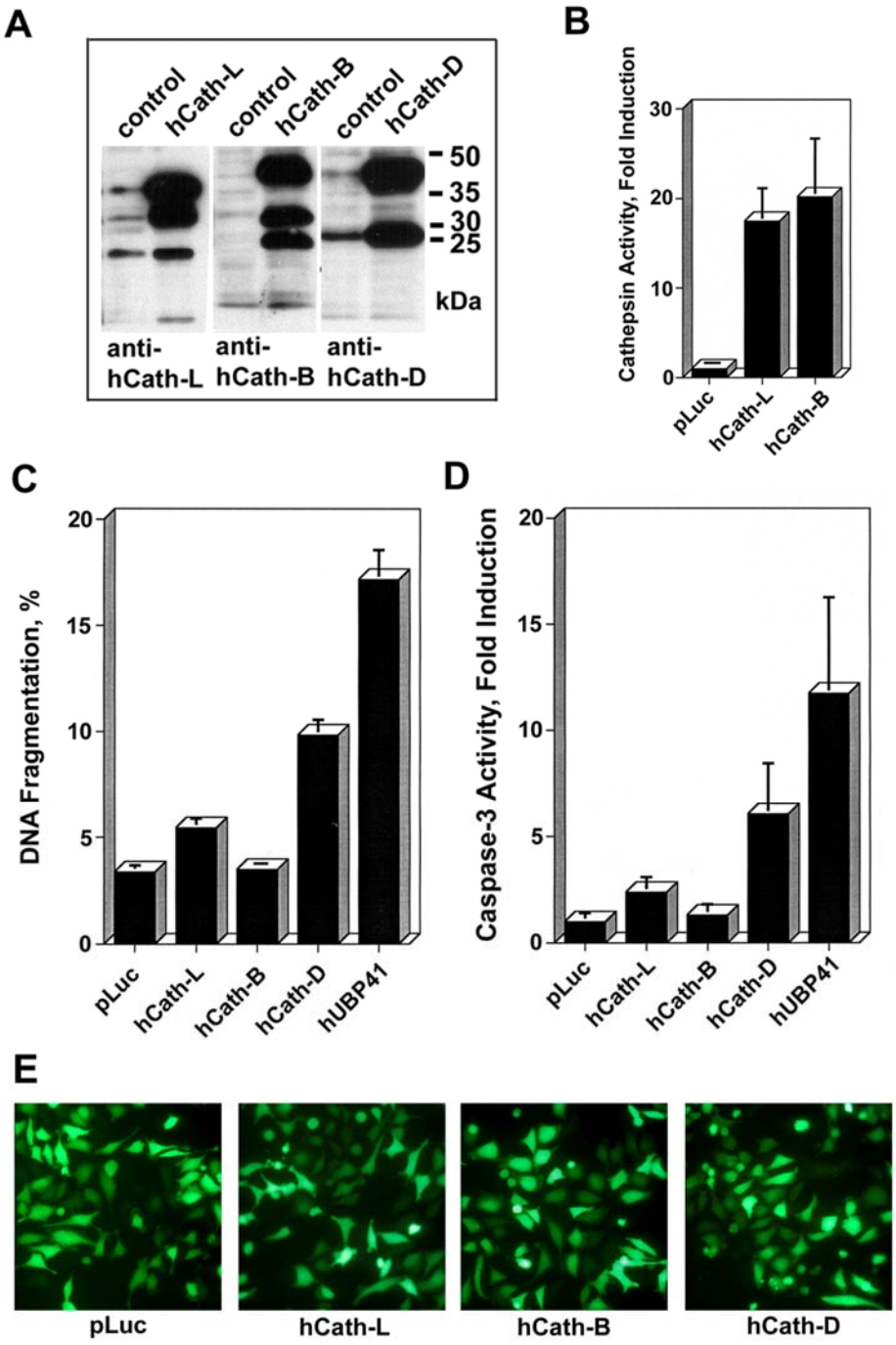


Fig. 3.2.2
Effect of high level overexpression of cathepsin-L, -B, and –D in HeLa cells. *(A) HeLa cells were transfected with expression plasmids for human cathepsin-L (hCath-L), cathepsin-B (hCath-B), and cathepsin-D (hCath-D). Expression was checked by western blot analysis about 24h post transfection. (B) Overexpression and activity of hCath-L and hCath-B was controlled by performing a cathepsin enzymatic activity assay using the zFR-AMC substrate using extracts from transfected HeLa cells. (C) For an apoptosis assay, DNA fragmentation (PI FACS analysis) was measured in HeLa cells transfected with the cathepsin expression plasmids as well as with the hUBP41-HA expression plasmid as a positive control about 30h post transfection. (D) HeLa cells were treated as described under (C) and caspase-3 activity was measured. (E) The phenotype of HeLa cells as observed by fluorescence microscopy when co-transfected with cathepsin espression plasmids and the pEGFP vector.*

As expected from a previous publication [Deiss, 1996], transient overexpression of hCath-D in HeLa cells resulted in cell death induction as observed by DNA fragmentation, caspase-3 enzymatic activity and cell morphology **(Figs. 3.2.2 C,D,E)**, and thus served as a positive control in this overexpression experiment. Overexpression of the ubiquitin-specific protease hUBP41-HA also triggered apoptosis and therefore was regarded as an additional positive control. In contrast, overexpression of hCath-L and hCath-B did not result in significant cell death, even though in case of hCath-L overexpression a slight increase in DNA fragmentation values and caspase-3 activity could occasionally be detected **(Figs. 3.2.2 C,D)**, concomittant with an increased number of round and detached cells **(Fig. 3.2.2 E)**. As a conclusion, the directed overexpression of the cathepsin-L coding sequence in HeLa cells (but also in 293T cells; data not shown) resulted in only minor detectable changes that resembled the phenotypical features of apoptosis. However, the effect of cathepsin-L overexpression was not strong enough to be unequivocally attributed to apoptosis since the available apoptosis assays were not sufficiently sensitive to detect and quantify the small fraction of affected cells in a reproducible and significant manner. The initial observation from the expression screen which suspected cathepsin-L to be a putative dominant apoptosis-inducing factor therefore could not be reliably confirmed.

3.2.3 The cysteine protease inhibitors E64d and CA074Me suppress TNF-mediated apoptosis in HeLa cells

Although overexpression of hCath-L and hCath-B did not by itself result in sufficient induction of apoptosis to be studied on its own (see paragraph 3.2.2), it nevertheless appeared worthwhile to further study a possible correlation between hCath-L and hCath-B expression levels and the sensitivity of cells to apoptosis induction. Major interest in this topic was justified in light of several studies during recent years which provided evidence for a potential involvement of cathepsins in the apoptotic cell death process. For many different cell types it was reported that apoptosis induced by various stimuli could be decreased in the presence of cathepsin inhibitors [Foghsgaard, 2001; Ishisaka, 1999; Kagedal, 2001b; Katz, 2001; Kingham, 2001; Kiso, 2001; Mathiasen, 2001], and several publications focused on the possible contribution of Cath-B in the mediation of the TNF-α mediated cell death signal [Foghsgaard, 2001; Guicciardi, 2000; Guicciardi, 2001].

In this context, it was investigated whether lysosomal cysteine proteases might be involved in apoptosis induced by TNF-α in the cervical carcinoma cell line HeLa which was supposed to serve as a model cell system. Cell death was induced by co-treatment of HeLa cells with TNF-α and cycloheximide (CHX) since TNF-α alone was not sufficient to induce apoptosis in this cell system. The pan-caspase inhibitor zVAD-fmk could completely prevent TNF-α/CHX-triggered DNA fragmentation indicating an apoptotic mode of cell death **(Fig. 3.2.3 A)**. Cell death as assessed by

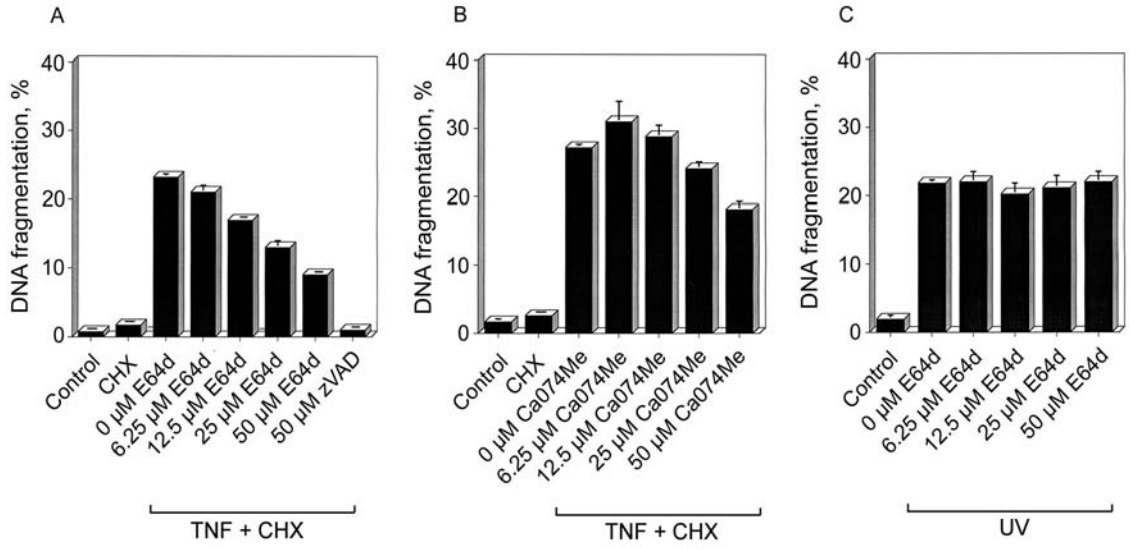


Fig. 3.2.3
Effect of cathepsin inhibitors on TNF-α or UV-induced apoptosis in HeLa cells. *HeLa cells were preincubated for 3h with the indicated amounts of E64d or CA074Me and then cotreated with 10 ng/ml TNF-α and 5 µg/ml CHX (A, B) or irradiated with 40 mJ/cm2 UV light (C). During treatment or after irradiation, respectively, the cells were further kept in the presence of the indicated concentrations of inhibitor and were analysed 12h (TNF-α) or 20h (UV) later by propidium iodide FACS analysis. The values for every datapoint were acquired in triplicate and are given as the percentage (mean +/ s.d.) of apoptotic cells with sub-G_1 chromosomal DNA content. The data shown are representative for several independent experiments.*

flow cytometric measurement of DNA fragmentation could also be inhibited in presence of the broad spectrum cysteine protease inhibitor E64d in a dose-dependent manner, albeit not as efficiently as with zVAD-fmk **(Fig. 3.2.3 A)**. When CA074Me was used, a described specific Cath-B inhibitor, a likewise decrease of TNF-induced DNA fragmentation could be measured **(Fig. 3.2.3 B)**. In order to confirm the inhibitory effect of cathepsin-inhibitors on TNF-α-induced apoptosis in a further cell system, the prostatic carcinoma cell line PC3 was treated with TNF-α/CHX in absence or presence of E64d, CA074Me, and additionally of the cathepsin inhibitor zFA-FMK [Rasnick, 1985]. Also in PC3 cells the inhibitors could reduce TNF-α/CHX-induced DNA fragmentation (**Fig. 3.2.4**).

To address the question whether the observed inhibitory effect of E64d on apoptotic DNA fragmentation can be generally observed upon cell death induction, the impact of E64d on UV-mediated cell death was tested in HeLa cells. As shown in **Fig. 3.2.3 C**, E64d did not suppress DNA fragmentation induced by UV irradiation under the conditions used.

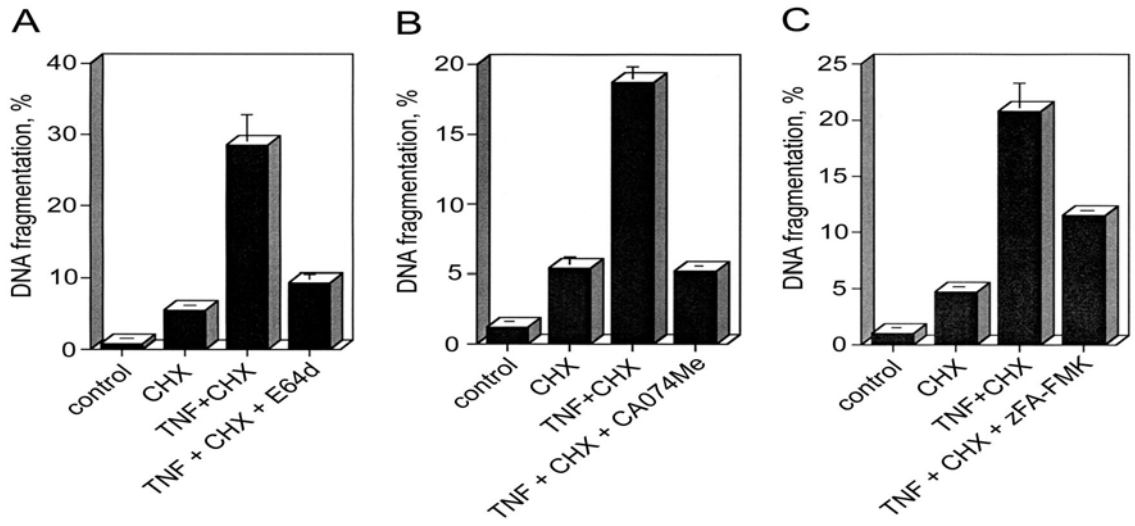


Fig. 3.2.4
Effect of cathepsin inhibitors on TNF-induced apoptosis in PC3 cells. *PC3 cells were preincubated for 3h with 50 µM of E64d (A), CA074Me (B) or zFA-FMK (C) and then were cotreated with 10 ng/ml TNF and 2 µg/ml CHX. During treatment, the cells were further kept in the presence of inhibitor and were analysed 20 h later by propidium iodide FACS analysis. The values for every datapoint were acquired in triplicate and are given as the percentage (mean +/ s.d.) of apoptotic cells with sub-G1 chromosomal DNA content. The data shown are representative for several independent experiments.*

3.2.4 Cathepsin-B-like cysteine proteases are responsible for mediating the E64d- and CA074Me-sensitive TNF signal

The observation that the Cath-B inhibitor CA074Me is able to reduce TNF-induced DNA fragmentation in HeLa cells **(Fig. 3.2.3 B)** suggested an involvement of Cath-B in this cell death process. Consequently, the specificity of CA074Me on Cath-B activity was examined. To this end, hCath-B or hCath-L were transiently overexpressed in HeLa cells for subsequent inhibitor studies. Cathepsin activity was measured in extracts of Cath-B- or Cath-L-transfected cells which were grown for 3 hours in the presence or absence of 25 µM E64d or 25 µM CA074Me, respectively. Concentrations in the range of 25 µM had been used in recent reports [Foghsgaard, 2001; Varghese, 2001] and in this study were necessary to elicit a clear inhibitory effect as seen in **Fig. 3.2.3**.

As expected E64d could strongly inhibit both, hCath-L and hCath-B enzymatic activity **(Fig. 3.2.5)**. Unexpectedly, the supposedly specific Cath-B inhibitor CA074Me not only inhibited the enzymatic activity of the overexpressed hCath-B but also that of the overexpressed hCath-L. However, this observation is in line with a recent report [Montaser, 2002] which revealed that prior to cleavage by cellular esterases the methyl ester CA074Me cannot be regarded as a specific Cath-B inhibitor but also affects other lysosomal cysteine proteases such as cathepsin-L. The methyl group of CA074Me is necessary for its ability to permeate the cell membrane. Additionally, the effect of of E64d on caspase

enzymatic activity was tested *in vitro*: caspase activity induced by addition of cytochrome c and dATP to cell extracts in a cell-free system was not significantly reduced in presence of 50 µM E64d in the extract (data not shown), therefore excluding the possibility that the effect of E64d on TNF-induced apoptosis is due to concurrent inhibition of caspases. In conclusion, Cath-B but also related lysosomal cysteine proteases such as Cath-L should be responsible for mediating the E64d- and CA074Me-sensitive TNF signal.

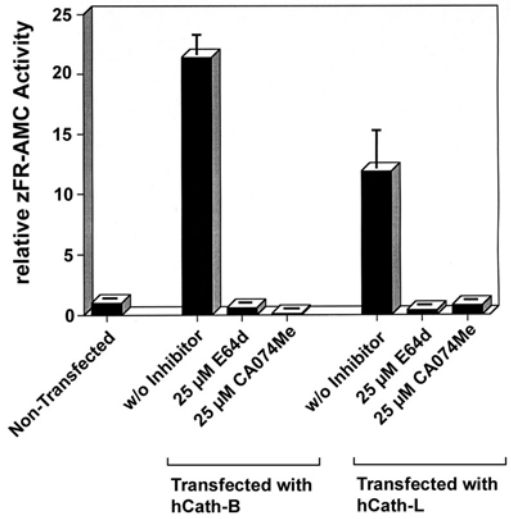


Fig. 3.2.5
Test of the specificity of E64d and CA074Me as inhibitors of cathepsin-B and cathepsin-L.
Expression vectors containing the cDNAs of hCath-L or hCath-B were transfected into HeLa cells. 24h post transfection, cells were incubated for 3 h in absence or presence of the indicated inhibitor, harvested, washed thoroughly in PBS, lysed and the resulting extracts were assayed for cathepsin zFR-AMC enzymatic activity by monitoring AMC fluorescence with a fluorescence plate reader. AMC fluorescence values were used to calculate relative enzymatic activity by normalization to values measured in extracts from non-transfected cells. Therefore, relative activity values represent the fold activity in comparison to endogenous cathepsin enzymatic activity levels. Each data point was measured in triplicate.

3.2.5 Augmented Levels of hCath-L or hCath-B fail to sensitize HeLa or McA RH 7777 cells to TNF-induced cell death

Since inhibitors of cathepsin enzymatic activity could suppress TNF-α-mediated apoptotic DNA fragmentation **(Fig. 3.2.3 and Fig. 3.2.4)**, we speculated that elevated cathepsin expression levels might influence the sensitivity of HeLa cells to the apoptosis inducing signal triggered by TNF-α. As shown in **Fig. 3.2.2**, overexpression of hCath-B and hCath-L could be obtained by transient transfection of HeLa cells in terms of both, protein levels detected in immunoblots **(Fig. 3.2.2 A)** as well as cathepsin activities **(Fig. 3.2.2 B)**. These experiments usually indicated an increase of cathepsin activity by at least 6- to 10-fold for hCath-L or by 10- to 20-fold for hCath-B when compared to endogenous cathepsin activity. This extent of increased enzymatic activity is comparable

to the situation found in malignant human tumor cells for which elevated cathepsin-expression and -activity levels have been described [Chauhan, 1991; Lah, 1998; Yan, 1998].

As shown in **Fig. 3.2.6 A**, HeLa cells transiently transfected with hCath-L or hCath-B did not show any increased apoptosis when compared to control transfected cells. In addition, HeLa cell pools were generated that stably overexpressed hCath-L and hCath-B at levels of 2-fold or 4-fold enzymatic activity, respectively, compared to control cells. Also those stable expression pool clones of HeLa cells did not exhibit an enhanced response to TNF-induced apoptosis **(Fig. 3.2.6 B)**. Since Cath-B has been reported to play a major role in apoptosis of hepatocytes [Guicciardi, 2000; Guicciardi, 2001; Roberts, 1999], hCath-L or hCath-B were transiently overexpressed in the rat hepatoma cell line McA RH 7777. Treatment of these McA RH 7777 cells with TNF did not yield any significant differences in apoptosis levels between control- and cathepsin-transfected cells **(Fig. 3.2.6 C)**.

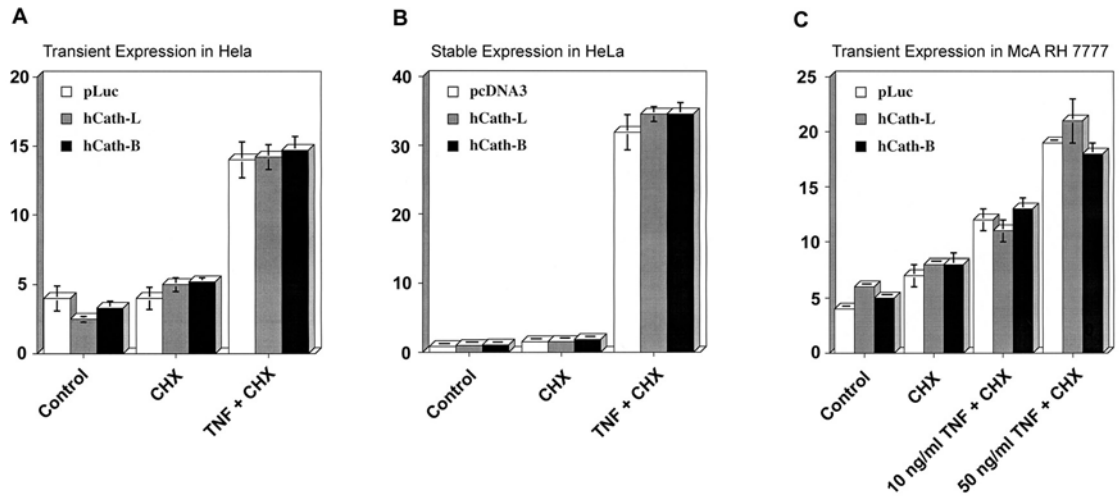


Fig. 3.2.6
Sensitivity to the TNF-mediated death signal is not enhanced by overexpression of hCath-L or hCath-B. *(A) Hela cells were transfected with pLuc, hCath-L, or hCath-B expression vectors, and 36h post transfection the cells were incubated in absence or presence of 5 µg/ml CHX alone or 10 ng/ml TNF + 5 µg/ml CHX. After 12h of treatment, cells were harvested for propidium iodide FACS analysis. Transfection efficiency in this experiment was 40% as determined by pEGFP cotransfection and EGFP FACS analysis, (B) HeLa cell pools selected for stable expression of pcDNA3, hCath-L, or hCath-B were incubated in absence or presence of 5 µg/ml CHX alone or 10 ng/ml TNF + 5 µg/ml CHX. After 12h of treatment, cells were harvested for propidium iodide FACS analysis, (C) McA RH 7777 rat hepatoma cells were transiently transfected with pLuc, hCath-L, or hCath-B expression vectors, and 36h post transfection were incubated in absence or presence of 1 µg/ml CHX alone, 10 ng/ml TNF + 1 µg/ml CHX, or 50 ng/ml TNF + 1 µg/ml CHX. After 12h of treatment cells were harvested for propidium iodide FACS analysis. Transfection efficiency in this experiment was 40% as determined by pEGFP cotransfection and EGFP FACS analysis. Data points were measured in triplicate and the data shown are representative for several independent experiments.*

3.2.6 Treatment of HeLa cells with TNF-α/CHX apparently triggers partial redistribution of lysosomal content into the cytosol

The mode of action of lysosomal proteases in apoptosis signalling is assumed to involve release of lysosomal content into the cytosol where lysosomal proteolytic activity is expected to trigger activation of proapoptotic factors such the Bcl-2 family member Bid with subsequent release of cytochrome c from the mitochondria and activation of caspases [Guicciardi, 2000; Turk, 2002]. Although the results presented thus far do not indicate any correlation between cathepsin expression levels and sensitivity of HeLa cells to TNF-α/CHX-mediated death, it was tested whether the integrity of lysosomes in TNF-α/CHX-treated HeLa cells is disturbed so that elevated cathepsin levels within the lysosomes can be expected to result in accordingly higher amounts of cathepsin in the cytosol in response to the TNF-α stimulus.

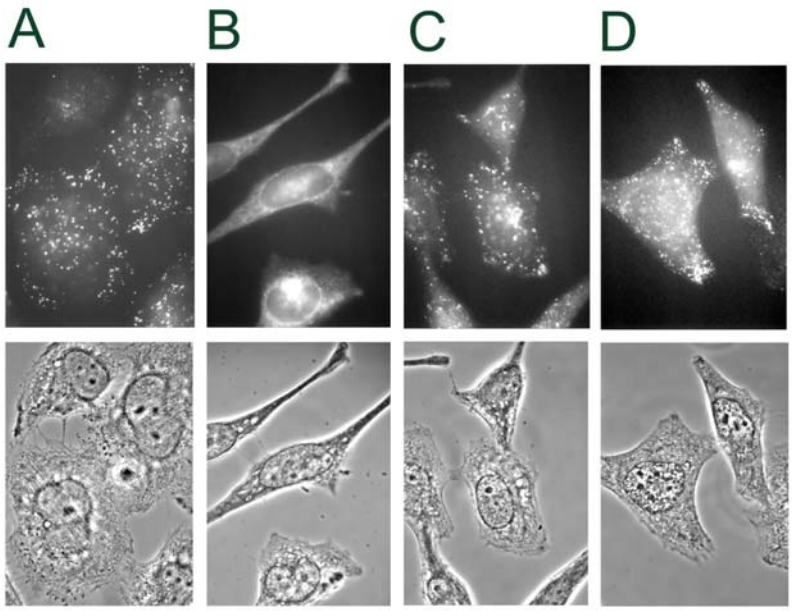


Fig. 3.2.7
Impact of TNF-α and NDI-HCl on lysosomal integrity. *HeLa cells were cultivated for 16h untreated (A) or in presence of 100 µM NDI-HCl (B), 2 µg/ml CHX (C), or 10 ng/ml TNF + 2 µg/ml CHX (D). Cells were then fixed and immunostained using an anti-Cath-L mAb and a secondary FITC-labeled anti-mouse mAb. Cells were microscopically inspected by fluorescence microscopy (upper panels) or by Differential Interference Contrast microscopy (lower panels), and images were taken using a digital imaging system. Magnification was 400-fold.*

As a positive control, HeLa cells were treated with the lysosomotropic detergent N-dodecyl-imidazole hydrochloride (NDI-HCl), which was reported to accumulate in the lysosomes and eventually to damage the lysosomal membrane with subsequent release of lysosomal content into the cytosol [Dubowchik, 1995]. Anti-Cath-L immunofluorescent staining of untreated control HeLa cells showed the expected spot-like lysosomal distribution pattern of endogenous Cath-L **(Fig. 3.2.7 A)**.

Treatment of HeLa cells with NDI-HCl resulted in an uniform staining of Cath-L within still viable cells, indicating effective lysosomal rupture and release of its content into the cytosol **(Fig. 3.2.7 B)**. Treatment of HeLa cells with TNF-α/CHX resulted in a considerable number of cells with a decrease in the staining intensity of discrete lysosomal spots and an apparent partial redistribution of Cath-L from those spot-like structures to a more evenly distributed pattern as shown in **(Fig. 3.2.7 D)**. Upon treatment with CHX alone such a redistribution effect could not be seen **(Fig. 3.2.7 C)**.

3.2.7 The lysosomotropic detergent NDI-HCl does not induce enhanced cell death in HeLa cells overexpressing hCath-L or hCath-B

As shown above, the lysosomotropic detergent NDI-HCl efficiently triggers redistribution of lysosomal content into the cytosol as indicated by the release of Cath-L **(Fig. 3.2.7 B)**. It was now examined, whether the cell death effect induced by NDI-HCl can be enhanced by overexpression of hCath-L or hCath-B.

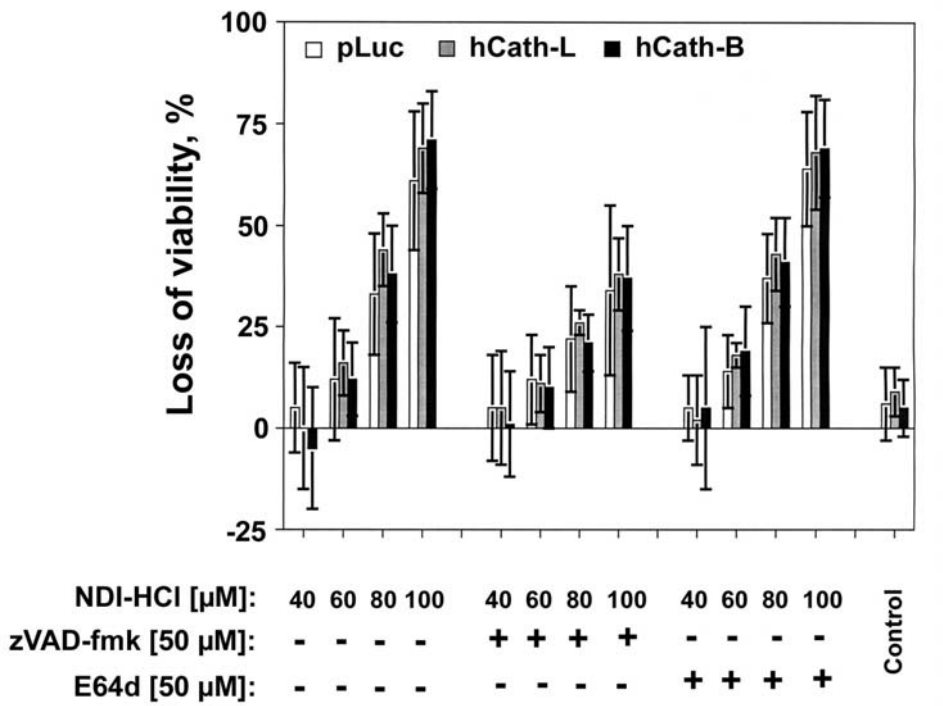


Fig. 3.2.8
Cell death induced by NDI-HCl is not enhanced by overexpression of hCath-L or hCath-B.
HeLa cells were transfected with pLuc, hCath-L, or hCath-B expression vectors, 24h later equal cell numbers were reseeded in wells of a 96 well plate and again 24h later were incubated in absence or presence of the indicated amounts of NDI-HCl in absence or presence of either 50 µM zVAD-fmk or 50 µM E64d. After 22h of treatment, cells were analysed by MTT assay as described in Materials and Methods. Transfection efficiency was determined by pEGFP cotransfection and was > 50%. The data shown are the means of 4 independent experiments in which each datapoint was measured in hexaplicate.

As shown in **Fig. 3.2.8**, expression levels of hCath-L or hCath-B did not have any significant effect on NDI-HCL-induced cell death in HeLa cells as determined by MTT assays. Cell death could be partially suppressed in presence of the pan-caspase inhibitor zVAD-fmk indicating the involvement of caspase-dependent mechanisms in NDI-HCL-mediated cell death. Interestingly, this type of cell death could not be inhibited at all in presence of the broad specificity cysteine protease inhibitor E64d **(Fig. 3.2.8)**.

3.2.8 RNAi as an approach to specifically downregulate cathepsin expression

As shown in paragraph 3.2.4, the commonly used cathepsin protease inhibitors cannot be regarded specific for a distinct type of cathepsin. CA074Me not only blocks Cath-B enzymatic activity but also Cath-L activity and maybe also that of other cysteine proteases. Specific downregulation of a distinct cathepsin acticity would be necessary in order to unambiguously identify those cathepsins or cathepsin-like proteases that contribute to TNF-mediated apoptosis signalling. Targeted downregulation of cathepsins should be possible by using the recently described RNAi approach which allows the knockdown of gene expression at the level of the mRNA message [Elbashir, 2001].

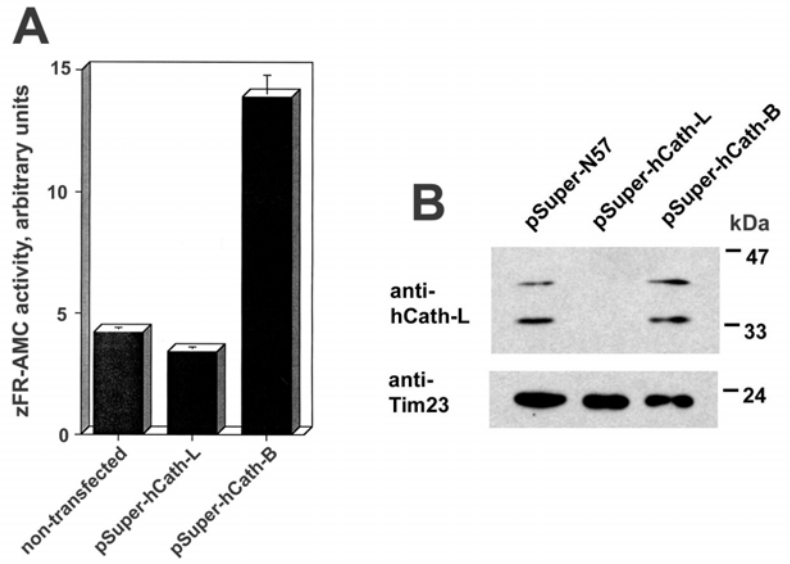


Fig. 3.2.9
Downregulation of hCath-L by RNA$_i$. *A. Test of the hCath-L RNAi construct in a cotransfection experiment: HeLa cells were left untreated or were cotransfected with the hCath-L expression vector together with the pSuper-hCath-L RNAi construct or the pSuper-hCath-B RNAi construct (the latter serving here as a negative control). 24h post transfection, the cells were harvested and the extracts were tested for zFR-AMC cathepsin enzymatic activity. B. Downregulation of endogeneous hCath-L: HeLa cells were transfected with the pSuper-hCath-L construct or as negative controls with pSuper-hCath-B or the arbitrary pSuper-N57 construct. 48h post transfection, endogeneous hCath-L levels were detected by anti-hCath-L immunoblot analysis (upper panel). Equal loading was verified by anti-Tim23 immunoblot analysis.*

RNA_i plasmid constructs against hCath-L and hCath-B were generated using the pSuper vector system (Oligoengine, for oligo sequences see chapter 2.1.6). In the scope of this thesis, thus far only a RNAi construct was identified that successfully allowed the downregulation of hCath-L. In a cotransfection experiment the hCath-L overexpression vector was cotransfected together with the pSuper-hCath-L RNAi construct. As shown in **Fig. 3.2.9 A**, pSuper-hCath-L but not pSuper-hCath-B could suppress the overexpression of hCath-L as measured by the cathepsin zFR-AMC enzymatic assay. Transfection of the pSuper-hCath-L construct also could downregulate endogeneous hCath-L levels as shown in **Fig. 3.2.9 B**. In contrast, the tested pSuper-hCath-B RNA_i construct for specifically downregulating hCath-B expression proved to be inactive in a hCath-B cotransfection experiment (data not shown). Therefore additional target sequences will have to be tested for successful downregulation of hCath-B.

4 DISCUSSION

Two projects are described within the scope of this thesis. Project 1 characterizes the ubiquitin-specific protease UBP41 as a dominant apoptosis-inducing protein whereas project 2 investigates the possible involvement of the cathepsins-B and –L in proapoptotic signalling. Interestingly, UBP41 and the cathepsins-B and –L have something in common: all of them are cysteine proteases that are components of one of the two major systems of protein degradation: UBP41 is a member of the large family of ubiquitin-specific proteases (USPs) that are an integral part of the ubiquitin/proteasome system whereas cathepsin-B and –L are major components of the hydrolytic content of the lysosomes. At the same time, the two projects discussed here represent examples for the impact of non-caspase cysteine proteases on apoptosis. The existence of caspase-independent mechanisms and the involvement of non-caspase proteases in the mediation and execution of programmed cell death has been discussed intensely during the last years. Initially, there was the common belief that apoptosis is inevitably linked to the activation of the caspases which are regarded to be the backbone of the apoptotic machinery (see paragraph 1.1.4.2). However, evidence accumulates that there exist caspase-independent apoptotic death pathways and that programmed cell death can occur in the complete absence of caspases (see paragraph 1.1.7). The existence of alternative, caspase-independent apoptosis mechanisms is actually not surprising when taking into account that it would be dangerous for a multicellular organism to exclusively depend on the functional integrity of a single protease family for such an essential biological process [Jaattela, 2003]. A number of factors have been proposed to mediate apoptotic events independently of caspases. Among those are proteins that are released from the mitochondria into the cytosol, such as the apoptosis-inducing factor (AIF), endonuclease G (endoG) and the serine protease Omi/HtrA2. Other factors that are suspected to contribute to caspase-independent cell death pathways are the cytosolic calpain proteases as well as the lysosomal cathepsin proteases. Thus, cathepsins have already been recognized previously as potential proapoptotic factors and, in this context, the isolation of a cathepsin-L cDNA in the genetic screen for apoptosis-inducing genes raised special interest and stimulated its further investigation (project 2), the results of which will be discussed in paragraph 4.2. In contrast, a direct link between ubiquitin-specific proteases and apoptosis did not exist at the time when the cDNA for UBP41 had been isolated from our screen for proapoptotic genes. Nevertheless, there were several reasons to believe that USPs, such as UBP41, might indeed play a role in the regulation of apoptosis. One reason was that a link between the ubiquitin/proteasome system and apoptosis had already been established because, e.g., many apoptosis signalling factors were known to be regulated by proteasome-mediated degradation (see paragraph 1.2.4). Since USPs were expected to play a major role in regulating ubiquitin/proteasome-dependent proteolysis by possibly targeting specific proteins for deubiquitination, it could be assumed that among

those putative USP targets there also might be proteins that are involved in apoptosis. Therefore, I decided to further characterize the UBP41-mediated apoptosis effect (project 1) which will be further discussed in paragraph 4.1.

4.1 UBP41 - a dominant apoptosis-inducing factor

The mouse orthologue of the ubiquitin-specific protease UBP41 was identified in the screen for dominant apoptosis-inducing genes **(Fig. 3.1.1**). It was demonstrated that also transfection of the human form of UBP41 (hUBP41) induces cell death in various human cell lines. The apoptotic mode of cell death induced by hUBP41 overexpression is supported by the detection of oligonucleosomal DNA fragmentation (**Fig. 3.1.3**), activation of caspase-3 (**Fig. 3.1.5**), and by the fact that cell death can be inhibited by the caspase inhibitor zVAD-fmk (**Fig. 3.1.10).**

4.1.1 Ubiquitin-specific proteases as possible regulators of apoptotic processes

The family of ubiquitin-specific proteases (USPs) represents an integral component of the ubiquitin-proteasome system in participating presumably in both, house-keeping and regulatory processes (see paragraph 1.2.3, and **Box 4.1**). As part of their house-keeping functions, USPs are able to remove ubiquitin-moieties from ubiquitylated proteins or peptides thereby influencing the available pool of monoubiquitin molecules as well as influencing the efficiency by which proteins are degraded at the level of the proteasome. Expecting an involvement of USPs in the regulation of the ubiquitin/proteasome system, it has been speculated that some USPs, via their deubiquitylating activity, might act to rescue distinct ubiquitylated protein substrates from degradation, whereas others may promote the degradation of distinct substrates by removing sterically inhibitory polyubiquitin chains and thereby enhancing the substrate's accessibility to the proteasome [D'Andrea, 1998].

Therefore, USPs are expected to have highly specific roles in regulating various biochemical and biological processes such as development, growth, and transcription, but also apoptosis. Indeed, there is accumulating evidence for an involvement of the ubiquitin-proteasome system in apoptosis by regulating the stability and activity of several factors including the Bcl-2 family of proteins, the IAPs, p53, IkB, as well as IKK [Jesenberger, 2002]. However, thus far there have been only few reports implicating USPs in the apoptotic process. Evidence for an impact of USPs on apoptosis could only be assumed, such as the fat facets gene product (Faf) in Drosophila or the related Fam protein in mouse which both appear to be crucial in development by contributing to the regulation of cell number [Fischer-Vize, 1992; Wood, 1997]. Intact Faf is necessary to maintain the correct number of 8

BOX 4.1 Selected members of the USP family

USP2: There is a number of isoforms belonging to the USP2 family: UBP41 was identified first from chicken [Baek, 1997]. Other chicken isoforms are UBP46, UBP52, and UBP66 [Baek, 1998]. All those *gallus gallus* USP2s share strong sequence similarity within the core enzymatic region and differ by N- or C-terminal extensions. Chicken USP2s were shown to hydrolyze various ubiquitylated test substrates. DNA sequences coding for human USP2 homologues were identified and were published in Genbank. UBP41 expression was reported to be stimulated in response to parathyroid hormone and thus might be involved in maintaining bone homeostasis [Miles, 2002].

USP4: USP4 is the synonym for Unp (ubiquitous nuclear protein) which initially was proposed to be an oncogene elevated in lung tumors [Gray, 1995]. The Unp locus is at 3p21.3 which is frequently deleted or rearranged in lung cancers. Two isoforms, UnpEL and UnpES, were identified which both are moderately expressed in various cell lines and appear to be localized mostly cytosolic, not nuclear [Frederick, 1998]. Results of this study contradict a role of Unp as an oncogene, rather supporting a possible function as a tumor suppressor. Interestingly, Unp contains a Rb binding motif, LxCxE, and indeed was shown to physically interact with the Rb protein *in vivo* [deSalle, 2001].

USP5: USP5 corresponds to the isopeptidase T (IsoT) protein of which two isoforms exist, IsoT-S and IsoT-L. IsoT can process linear and branched polyubiquitin chains. It has been proposed that IsoT physiologically acts at the terminal step of ubiquitin-dependent proteolysis, disassembling the polyubiquitin chain following the action of the poteasome on the target protein, presumably targeting free polyubiquitin chains after the chains are released from the target protein [Wilkinson, 1995].

USP6: USP6 corresponds to the *Tre2* oncogene which was identified in a transfection screen as a gene able to transform mouse NIH 3T3 cells, however, *Tre2* exists exclusively in the hominoid lineage of primates and only recently evolved during evolution (about 30 million years ago) from the chimeric fusion of two genes, USP32 and TBC1D3 [Paulding, 2003].

USP7: USP7 is the systematic name for the ubiquitin-specific protease HAUSP (herpesvirus-associated ubiquitin-specific protease) which originally was identified to associate with the herpes simplex virus activator of gene expression protein Vmw110. HAUSP was reported to be localized in the nucleus, in part within PML nuclear bodies [Everett, 1997]. Importantly, HAUSP was recently identified as a p53-interacting protein, mediating deubiquitination and therefore stabilization of p53 [Li, 2002]. HAUSP could rescue p53 from Mdm2-mediated ubiquitination and degradation and it also activated p53 for transactivation and apoptosis induction.

USP8: USP8 corresponds to UBPY, which was identified as a ubiquitin-specific protease that accumulates upon growth stimulation. Moreover, UBPY levels decreased in response to growth arrest, and downregulation of the UBPY transcript by an antisense approach caused G1 arrest. Therefore, UBPY appears to be a growth-regulated USP which itself has important impact on proliferation. Overexpression of wildtype UBPY resulted in an overall decrease of protein ubiquitination and in a severe inhibition of cell growth [Naviglio, 1998].

USP9: One form of USP9 is localized on the male Y chromosome and is therefore called USP9Y. *USP9Y* is suspected to be a male infertility gene since it is frequently deleted in infertile male mice with defective spermatogenesis [Lee, 2003]. Another variant of USP9 can be located on the X chromosome and is called *USP9x*. Interestingly, USP9y and USP9x appear to be the homologous proteins of the Drosophila fat facets (faf) gene and the mouse Fam gene.

USP16: USP16 was described as Ubp-M, being a protein which is phosphorylated during G2/M transition and is dephosphorylated during metaphase/anaphase transition. Interestingly, Ubp-M is able to deubiquitinate histone H2A and might be involved in the regulation of mitotic events, even though it localizes mainly to the cytoplasm. An active site mutant of Ubp-M accumulated with metaphase chromosomes and caused cell cycle arrest with subsequent apoptosis induction, possibly by binding to the ubiquitylated portion of histone H2A and by this interfering with normal structure and function of chromatin [Ying, 1999].

USP18: USP18 was originally identified in mice and was named UBP43 which was demonstrated to cleave ubiquitin from the test substrate Ub-ß-galactosidase and was proposed to play a role in hematopoiesis [Liu, 1999]. Human USP18 was later identified to be an IFN-ß inducible gene in human melanoma cells but also in various other cell lines and was proposed to contribute to growth control and differentiation [Kang, 2001]. Recently, it was reported that USP18 preferentially cleaves ISG15 conjugates rather than ubiquitin conjugates and might be regarded to be the first ISG15-specific protease [Malakhov, 2002].

USP21: USP21 was found to be able to cleave both, ubiquitin and Nedd8 conjugates and thus is a unique UBP with dual specificity for ubiquitin and Nedd8. Moreover, USP21 was able to deubiquitinate a broad range of ubiquitylated proteins, and overexpression of USP21 resulted in profound inhibition of cell growth [Gong, 2000].

DUB-1 and DUB-2: DUB-1 and DUB-2 are two USPs thus far only described in mouse, and yet no name has been assigned according to the unified USP nomenclature [Baker, 1999]. DUB-1 is an IL-3 inducible gene, whereas DUB-2 expression is induced by IL-2, and both might play an important role in cytokine-induced cell proliferation. Indeed, prolonged expression of DUB-1 inhibited cellular proliferation with little effect on cellular viability [Zhu, 1996].

CYLD: CYLD has been identified as a tumor-suppressor frequently mutated in cases of familial cylindromatosis. CYLD was shown to negatively regulate the NFkB survival signalling pathway by removing ubiquitin from the TRAF2 protein. Downregulation of the CYLD mRNA message results in enhanced activation of NFkB and in a reduction of cell death induced by TNF-α [Brummelkamp, 2003; Kovalenko, 2003; Trompouki, 2003].

photoreceptor cells per facet by specifically targeting the liquid facets protein (Lqf) for deubiquitination and stabilization. Lqf is involved in endocytosis and has been proposed to enable the communication between the photoreceptor cells and their neighbours, thereby preventing positive differentiation signals [Chen, 2002]. The *mus musculus* Fam protein is homologous to the *Drosophila* Faf protein and even can substitute for Faf function in the *Drosophila* eye [Chen, 2000b]. In mouse, Fam interacts with β-catenin and Af-6, two proteins involved in cell-cell junctions, and it is expressed in rapidly dividing cells, for example in the apoptotic region between the digits [Taya, 1999; Wood, 1997].

Of major interest are recently published data which demonstrate that the ubiquitin-specific protease HAUSP is able to directly deubiquitinate the tumor-suppressor protein p53 [Li, 2002]. HAUSP-mediated deubiquitination of p53 resulted in p53 stabilization and enhanced the proapoptotic activity of p53 (**Fig. 4.1.1**). In a colony-formation assay, HAUSP caused growth inhibition in cells with intact p53 status but not in p53 deficient cells. HAUSP presents the first example for a direct influence of USP-mediated deubiquitination on the stability and activity of an apoptosis-mediating factor (see also paragraph 1.2.3.2). It should be mentioned that, in contrast to my results found with hUBP41, overexpression of HAUSP alone did not result in significant cell death [Li, 2002].

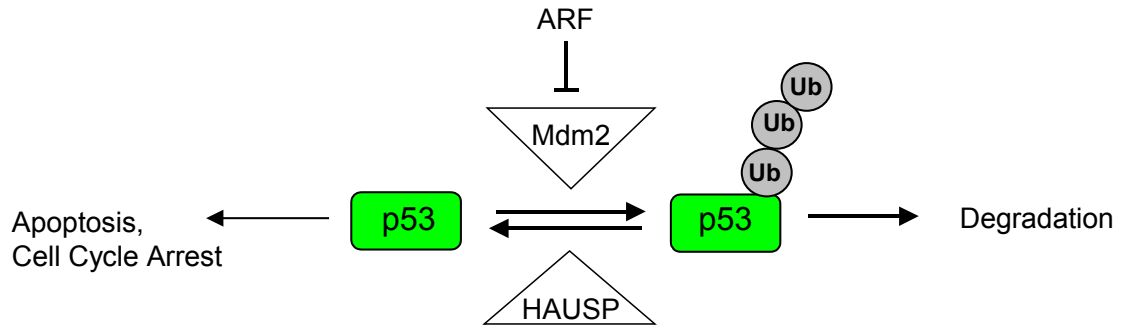


Fig. 4.1.1
p53 is a specific target for the deubiquitinating enzyme HAUSP. *Stability of p53 is regulated by Mdm2-mediated ubiquitination and subsequent degradation by the proteasome. HAUSP can deubiquitylate p53 and thus rescue it from degradation. Rescued and thus stabilized p53 can mediate apoptosis and/or cell cycle arrest. HAUSP was the first ubiquitin-specific protease that was shown to specifically target a proapoptotic factor for deubiquitination. According to Li et al. [Li, 2002].*

Another example for the direct involvement of a deubiquitinating enzyme in the regulation of survival and thus cell death pathways is the recently described *CYLD* gene product. As already mentioned in paragraph 1.2.3.2, CYLD is a tumor-suppressor which is frequently mutated in cases of familial cylindromatosis, an autosomal dominant predisposition to tumors of skin appendages. CYLD is a negative regulator of the pro-survival NF-κB signalling pathway by deubiquitylating the TNF receptor associated factor 2 (TRAF2) and thereby preventing the phosphorylation and activation of the IKK

complex by the IKK kinase complex (**Fig. 4.1.2**). Inactivation of CYLD by knockdown experiments leads to enhanced NF-κB activity and, in consequence, renders cells more resistant to apoptosis induction by TNF-α [Brummelkamp, 2003]. Thus, besides HAUSP, CYLD is the second known example for a deubiquitinating enzyme that targets a specific substrate with impact on cellular survival and apoptosis. CYLD is a paradigm for a deubiquitinating enzyme which, besides having impact on survival and cell death signalling, can possess tumor-suppressive properties whose impairment results in cancer.

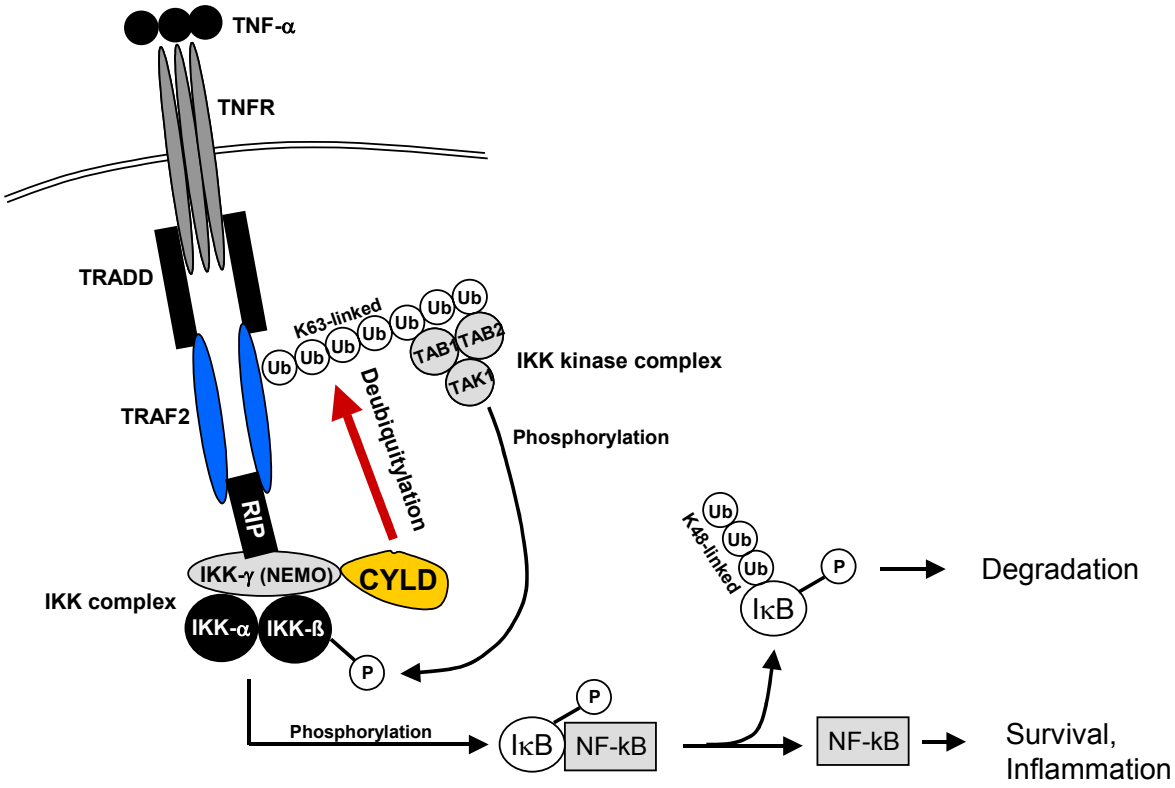


Fig. 4.1.2
The deubiquitinating enzyme CYLD specifically deubiquitylates the TRAF2 protein. *The NF-κB survival pathway can be activated in response to the ligation of the tumor necrosis factor receptor (TNFR) with TNF-α. The adaptor protein TRADD is recruited to the activated TNF receptor, which besides forwarding the signal to proapoptotic signalling components such as the DISC, also allows the binding of TRAF2, RIP, and the IKK complex. The IKK complex is phosphorylated and thereby activated by the IKK kinase complex and mediates phosphorylation and degradation of IκB with consequent activation of NF-κB. The IKK kinase complex is recruited to the TNFR-TRAF2-IKK complex when TRAF2 is modified by a multiubiquitin chain in which the ubiquitin units are linked with each other by their internal Lys63 residue. Lys63 linkage within multiubiquitin chains is unusual because in most polyubiquitins it is the Lys48 residue that is covalently attached to the C-terminus of another ubiquitin molecule. Lys48-linked multiubiquitin chains target their corresponding substrate proteins for recognition and degradation by the proteasome as is the case with e.g. IκB. In contrast, Lys63-linked multiubiquitin chains were discussed to preferentially play regulatory roles such as during DNA repair processes [Hoege, 2002]. CYLD physically interacts with the IKK-γ/NEMO subunit of the IKK complex and causes deubiquitination of TRAF2, thereby disrupting the interaction of TRAF2 with the IKK kinase complex which consequently is prevented from activating the IKK complex.*

4.1.2 The effect of UBP41 overexpression in comparison to overexpression of other members of the USP family

In this thesis, it was demonstrated that hUBP41 mediates the induction of apoptosis when overexpressed in various transformed human cell lines such as 293T, HeLa, and PC3 (see paragraph 3.1.2). hUBP41 is the first example for a ubiquitin-specific protease which upon overexpression displays proapoptotic activity, because there are no previous reports in the literature that describe apoptosis induction by the overexpression of USPs. In one case, cells were reported to undergo cell death when an active-site mutant of the ubiquitin-specific protease Ubp-M was overexpressed. The wildtype form of Ubp-M, however, did not have any apparent effect on transfected cells. Ubp-M has been discussed to selectively act on histones H2A and H2B, thereby contributing to the condensation of mitotic chromosomes. An explanation for the apoptosis-inducing effect of the active-site mutant form of Ubp-M might be its observed tight association with mitotic chromosomes during all stages of cell division, possibly triggerig cell cycle arrest and eventually apoptosis [Cai, 1999].

Overexpression experiments have also been reported with the ubiquitin-specific proteases DUB-1 [Zhu, 1996], USP21 [Gong, 2000], and UBPY [Naviglio, 1998] but in all three cases no apoptosis has been observed. Instead, DUB-1 overexpression was reported to result in growth suppression by arresting cells in G_1, and overexpression of USP21 and of UBPY also mediated a profound growth inhibitory effect. Results of this thesis demonstrate that overexpression of hUSP21-HA but also hUSP18-HA do not not result in apoptosis induction in 293T cells (**Fig. 3.1.5**). It must be mentioned that it cannot totally be ruled out that this is explained by the fact that expression levels of hUSP18-HA and hUSP21-HA could not be obtained as high as was the case with hUBP41-HA overexpression (**Fig. 3.1.5 C**). Indeed, it appears that appropriately high expression levels of hUBP41-HA are necessary in order to obtain a reasonable degree of apoptosis induction. Therefore, if hUBP41-mediated sensitivation for apoptosis induction plays a role under physiological or pathological conditions, tissues with strong hUBP41 / hUSP2 expression levels such a kidney or skeletal muscle (**Fig. 3.1.16**) or testis [Miles, 2002] are most likely to be affected.

In this context, it might be worth noting that in the attempt to generate 293T clone pools stably expressing hUBP41-HA, hUSP18-HA, hUSP21-HA, as well as their corresponding enzymatic mutant forms, only in case of hUBP41-HA it was not able to recover any surviving cell clone, whereas from 293T cells transfected with the other constructs many clones could be rescued under selection conditions (not shown). In this sort of experiment, the transfection of a pool of cells is expected to result in cells with varying amounts of overexpression, from very low to very high levels of hUBP41. The fact that not one single hUBP41-transfected 293T cell clone (not even a clone with minor overexpression) could be recovered under selection conditions may indicate that not even a slight increase of hUBP41 levels is tolerable for a cell to survive over a long term. From this observation one

also may draw the conclusion that endogenous hUBP41 activity is in balance with other cellular events and that a disturbance of this balance by overexpression of hUBP41 will result in cell death.

4.1.3 Possible explanations for the apoptotic effect of UBP41 overexpression

Overexpression of hUBP41-HA resulted in dramatic deubiquitination of a wide range of substrates (**Fig. 3.1.12**), and a severe interference with the ubiquitin-proteasome system should be expected. The apoptotic effect of UBP41 overexpression might be explained by various different possible scenarios, which will be discussed below and are schematically summarized in Fig. **4.1.3**.

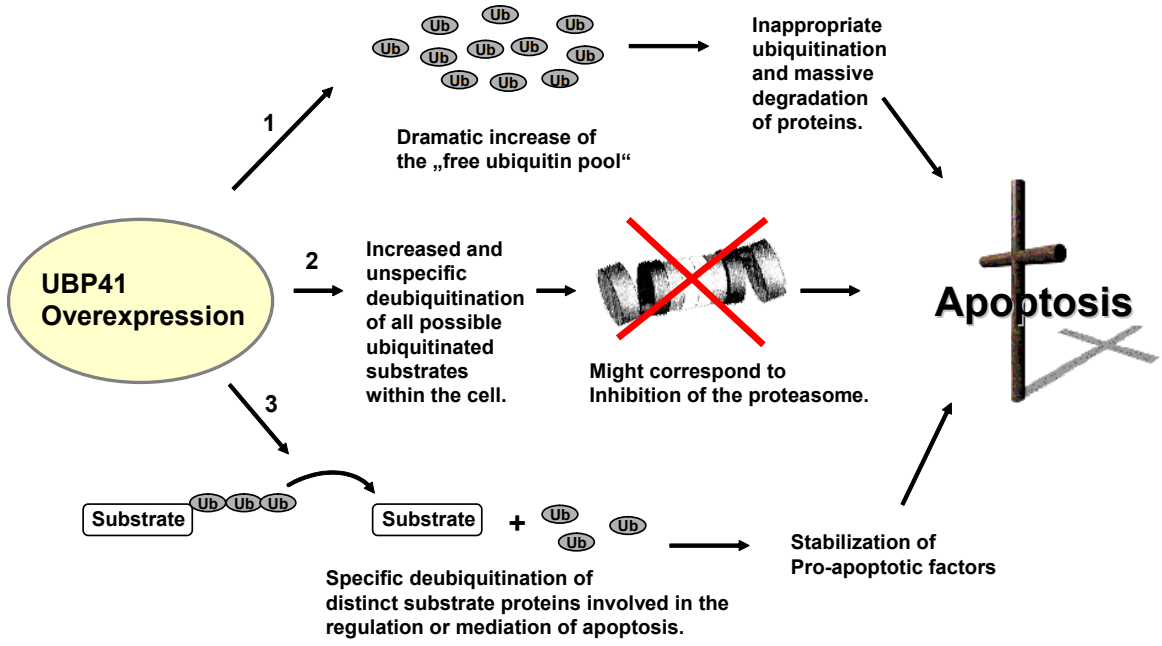


Fig. 4.1.3
Possible explanations for the apoptosis-inducing effect of UBP41 overexpression. *UBP41 overexpression might cause a massive increase in the free mono-ubiquitin pool and thus might result in cell death either by stimulating inappropriate protein degradation by the proteasome or by mediating a chaotropic effect in partially unfolding proteins **(1)**. Another possibility would be the broad range deubiquitination and the consequent stabilization of a vast number of proteins, possibly being comparable to an inhibition of the proteasome. Inhibition of the protasome is known to result in apoptosis induction **(2)**. In case that only specific proteins are stabilized as a consequence of UBP41 overexpression, it would be the accumulation of proapoptotic factors that might be responsible for apoptosis induction **(3)**.*

4.1.3.1 Apoptosis induction by the accumulation of monoubiquitin

Increased deubiquitinating activity within the cell, such as caused by UBP41 overexpression, might possibly lead to an inappropriate level of free monomeric ubiquitin that might interfere with cellular processes, e.g. by its putative chaotropic action towards other proteins. This chaotropic effect of ubiquitin is characterized by a partial unfolding of proteins due to the interference of ubiquitin with intramolecular non-covalent binding forces of proteins [Wenzel, 1993]. But this scenario appears not to be likely since when wildtype ubiquitin was overexpressed in HeLa cells and 293T cells, no effect concerning cell death induction could be observed (data not shown) and, moreover, anti-ubiquitin immunoblots already demonstrated very high basal levels of monoubiquitin in untreated 293T and HeLa cells which did not recognizably change in hUBP41-HA transfected cells (see the prominent protein band at < 10 kDa in **Fig. 3.1.12**).

4.1.3.2 Apoptosis by a general disturbance of the cellular ubiquitination status

Overexpression of UBP41 resulted in the deubiquitination of a broad range of proteins (**Fig. 3.1.12**), suggesting that the ubiquitination status of the cell is severely disturbed. Interestingly, the capacity to cause broad range deubiquitination upon overexpression has been previously observed with USP21 [Gong, 2000] and UBPY [Naviglio, 1998], but in both cases not apoptosis but a strong growth inhibitory effect was observed. It certainly cannot be excluded that the growth inhibition observed in those studies is a consequence of slow and gradual cell death induction that had not been recognized by the corresponding investigators. Furthermore, a similar decrease of overall ubiquitination levels and concurrent apoptosis induction has been reported for cells rendered deficient in the ubiquitin-activating enzyme E1 [Monney, 1998]. E1 deficiency resulted in broad range protein deubiquitination and apoptosis, but in contrast to hUBP41-mediated apoptosis, this type of cell death was caspase-independent. The broad range deubiquitylating effect of E1 deficiency was accompanied by the accumulation of p21, p27, cyclin D1 and p53, but not Bax. The authors proposed that as yet unidentified short-lived proteins might be stabilized, accumulate and act as death effectors in the induction of apoptosis.
Therefore, broad range deubiquitination has previously been observed in other cell systems either in response to USP overexpression or by impairment of the ubiquitination system in E1-deficient cells. In the latter case, the broad range deubiquitination effect eventually resulted in apoptosis, but also in this study it remained elusive whether apoptosis induction was caused by the accumulation of distinct proapoptotic protein factors or by the general defect in the ubiquitination system.

A general disturbance of the cellular ubiquitination status by UBP41 overexpression may correspond to an interference with the proteasomal degradation system as a whole, i.e. might be analogous to the

inhibition of the proteasome. Indeed, treatment of cells with proteasome inhibitors has been recognized to induce apoptosis under certain conditions [Shah, 2001; Adams, 2002]. For this reason, the response of HeLa cells to the proteasome inhibitor MG132 was examined and compared to the overexpression of hUBP41-HA in the same cell system. It became apparent that MG132 primarily leads to a cell cycle arrest in G2/M and presumably only subsequently results in moderate apoptosis induction (**Fig. 3.1.15**) which is in line with previous reports [Chen, 2000; Shah, 2001]. In contrast, overexpression of hUBP41-HA did not arrest cells in the cell cycle but apparently was driving cells directly into apoptosis (**Fig. 3.1.15**). Thus, even though a strong interference with the ubiquitin-proteasome system must be assumed upon hUBP41-HA overexpression, the resulting cellular changes are clearly distinct from a general inhibition of proteasome activity by the common drug MG132. Proteasome inhibitors have recently been introduced into clinical studies due to their potential capability to enhance the proapoptotic effect of chemotherapeutical drugs towards otherwise resistant tumor cells [Adams, 1999]. Since overexpression of Ubp41 induces apoptosis with distinct differences to a common proteasome inhibitor (MG132), it might be rewarding to identify and characterize drugs that act on the level of protein ubiquitination in addition to the development of drugs that directly target the proteasome for the therapeutic induction of cell death in tumor cells. Interfering with the ubiquitination status might present an additional approach to target cancer cells for destruction and might be of advantage under certain conditions in certain cell or tissue systems.

In the attempt to interfere with the ubiquitination status in an alternative way, the ubiquitin double mutant Ub K48R, G76A was expressed in 293T. Overexpression of Ub K48R, G76A did not result in apoptosis induction (**Fig. 3.1.13**). The ubiquitin double mutant is certainly not expected to confer deubiquitination comparable to that observed for hUBP41 HA **(Fig. 3.1.12**) but rather to influence the dynamics of polyubiquitin chain formation with a bias toward shorter chain length [Finley, 1994].

4.1.3.3 Apoptosis induction as a cause of specific protein stabilization

Since hUBP41 overexpression apparently causes deubiquitination of a majority of cellular proteins (**Fig. 3.1.12**), it consequently can be expected that many proteins, which normally are regulated by the ubiquitin-proteasome system, will be rescued from degradation and therefore will accumulate within the cell. The arbitrary accumulation of a broad range of cellular proteins would correspond to a general disturbance of the ubiquitin/proteasome system as discussed in paragraph 4.1.3.2. However, at least within the scope of this thesis work, it could not be demonstrated that broad range deubiquitination directly corresponds to broad range stabilization of protein substrates. The cell cycle proteins p21, p27 and cyclin B1 have been well described to be ubiquitylated and to be degraded by the proteasome. Consistently, protein levels of p21, p27, and cyclin B1 accumulated in the presence of the drug inhibitor of the proteasome, MG132. In contrast, overexpression of hUBP41-HA did not

stabilize those proteins (**Fig. 3.1.14 B**). Moreover, 2-dimensional gel electrophoresis of protein extracts from hUBP41- or control-transfected cells did not reveal any striking differences in the overall protein expression pattern (data not shown).

While no endogenous proteins could be demonstrated to be stabilized by hUBP41-HA overexpression, the artifical test substrate Ub-G76V-GFP [Dantuma, 2000] was stabilized when co-expressed with hUBP41-HA. (**Fig 3.1.14 A**). This result at least leaves the possibility that despite the apparent broad range deubiquitination effect of hUBP41-HA, only distinct, usually short-lived substrates are actually stabilized, leading to the observed cell death effect. It should be noted, that the significance of the results obtained with the test substrate Ub-G76V-GFP is somewhat questionable since not only co-expression of hUBP41-HA but also of the active site mutant hUBP41-C24A-HA resulted in stabilization of the GFP test substrate, almost as efficient as with hUBP41-HA itself (**Fig 3.1.14 A**). Possibly, the mutant enzyme hUBP41-C24A-HA can still bind to Ub-G76V-GFP, however it is not able to deubiquitinate this substrate. Therefore, it might be speculated that hUBP41-C24A-HA keeps Ub-G76V-GFP bound in a complex in which the ubiquitin moiety is shielded from recognition by the proteasome and in this way Ub-G76V-GFP is rescued from degradation (**Fig. 4.1.4**).

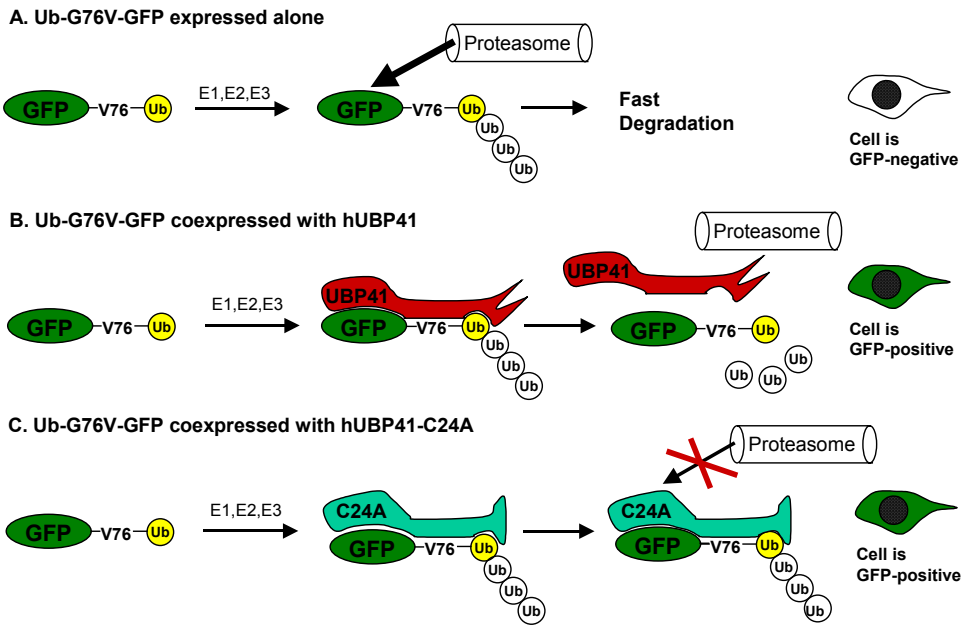


Fig. 4.1.4
Effect of hUBP41 and hUBP41-C24A on the test substrate Ub-G76V-GFP. *Ub-G76V-GFP is a GFP protein artificially fused at its N-terminus to the C-terminus of a ubiquitin-monomer. The C-terminus of the ubiquitin was changed from Gly to Val in order to make the fusion more stable towards deubiquitination. A. When Ub-G76V-GFP is overexpressed alone, it is multiubiquitylated and efficiently degraded by the proteasome: the cells are GFP-negative. B. When coexpressed with UBP41, Ub-G76V-GFP still can be ubiquitylated, but this is reversed by the action of UBP41. The monoubiquitylated Ub-G76V-GFP cannot be targeted by the proteasome and therefore is not degraded: the cells are GFP-positive. C.When coexpressed with UBP41-C24A, this enzymatic mutant form of UBP41 may still bind to, but cannot deubiquititinate Ub-G76V-GFP. UBP41-C24A therefore possibly remains bound to Ub-G76V-GFP in a complex and shields it from the proteasome: the cells are GFP-positive.*

In support of this possibility, when EYFP fusions of hUBP41 and its active-site mutant were observed under the fluorescence microscope (**Fig. 3.1.9**) it could be seen that in contrast to wildtype hUBP41, the mutant enzyme obviously forms prominent aggregates within the cell, i.e. it might associate with certain targets within the cell in an inappropritately tight manner. If this is the case, it may explain the significant cell-death effect of the active-site mutant hUBP41-C24A-HA in the HeLa cell system (**Fig.3.1.7** and **Fig. 3.1.8**). In this context, it should be noted that the already mentioned enzymatically inactive mutant of Ubp-M was described to tightly associate with mitotic chromosomes, thereby possibly mediating its apoptotic effect upon overexpression [Cai, 1999].

4.1.4 The possible role of UBP41 as a modulator of apoptosis under physiological and pathological conditions

The data presented in this study mainly describe the response of human cancer cell lines to high level overexpression of genes, in this case of UBP41. It is questionable whether the protein levels obtained in transient transfection experiments will ever be reached under physiological conditions. Nevertheless, it cannot be ruled out that UBP41 expression might be strongly deregulated under pathological conditions, thereby causing or at least contributing to inappropriate or premature cell death induction in certain settings.

Under physiological conditions, UBP41 possibly should not predominantly be regarded as a dominant apoptosis-inducing factor as it is under conditions of high level overexpression but rather as a factor that modulates the sensitivity of cells to death stimuli. While UBP41 is obviously capable of influencing the ubiquitination status of a broad range of proteins (**Fig 3.1.12**), it realistically must be assumed that not only proapoptotic factors are among the proteins stabilized but antiapoptotic proteins as well. Instead of specifically stabilizing distinct proapoptotic proteins, it therefore might be more likely that hUBP41 overexpression interferes with the well balanced ratio between pro- and antiapoptotic mediators. Whereas in a normal, viable cell proapoptotic signalling is counteracted by the presence and action of an equivalent or more than equivalent number of antiapoptotic factors, the situation might be inversed after hUBP41 overexpression so that in total the proapoptotic signalling machine can overpower the antiapoptotic mechanisms (**Fig. 4.1.5**).

Thus, the status of UBP41 expression might influence the balance between pro- and antiapoptotic factors. Consequently, a minor change in the UBP41 level not necessarily will lead to apoptosis induction by itself but would contribute to the cellular status that determines whether a cell is more or less sensitive to the induction of apoptosis in the context of survival or death signals from within or outside the cell (**Fig. 4.1.5**).

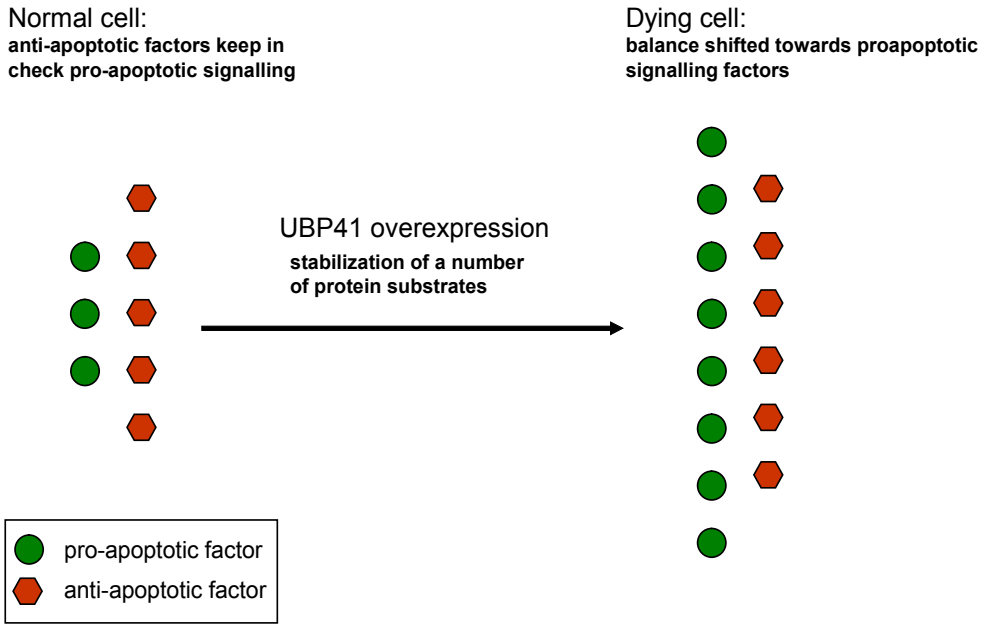


Fig. 4.1.5
A hypothetical mode of action of hUBP41 overexpression. *hUBP41 overexpression may interfere with the well balanced ratio between pro- and antiapoptotic mediators. Whereas in a normal, viable cell proapoptotic signalling is counteracted by the presence and action of an equivalent or more than equivalent number of antiapoptotic factors, the situation might be inversed after hUBP41 overexpression so that in total the proapoptotic signalling machine can overpower the antiapoptotic mechanisms.*

4.1.5 Genomic sequence analysis of the USP2 gene locus

In this study, a cDNA clone named UI-64 was isolated in the screen for apoptosis-inducing genes. By sequence analysis using BLAST [Altschul, 1997] this clone was identified as the mouse orthologue of the ubiquitin-specific protease UBP41 which originally was isolated and characterized in chick skeletal muscle [Baek, 1997]. The sequence alignment in **Fig. 3.1.2** reveals that the coding sequence of the original cDNA clone (UI64) from the screen lacks the first five aminoterminal amino acids when compared to the published mouse UBP41 sequence (mUBP41, NCBI gi:7949157). At that time, when sequence analysis was done for the clone UI64, the mUBP41 sequence was the only mouse sequence hit in the database. In the meantime, several isoforms of UBP41 were discovered and according to a unifying nomenclature [Baker, 1999] are now known as USP2 isoforms. Recently (Oct 2003), the sequences of three murine USP2 (mUSP2) isoforms were published in Genbank. The mUSP2 transcript variant 1 (Accession NM_016808, gi:7949157) corresponds to the former mUBP41 entry, whereas mUSP2 transcript variant 2 (NM_198091, gi:37674278) and mUSP2 transcript variant 3 (NM_198092 , gi:37674280) possess N-terminal extensions when compared to variant 1 (**Fig. 4.1.6**).

```
mUSP2_var3    (1) MSQLSSTLKRYTESSRYTDAPYAKPGYGTYTPSSYGANLAASFLEKEKLG
     _var3   (51) FKPVSPTSFLPRPRTYGPSSILDCDRGRPLLRSDIIGSSKRSESQTRGNE
     _var3  (101) RPSGSGLNGGSGFSYGVSSNSLSYLPMNARDQGVTLSQKKSNSQSDLARD
     _var3  (151) FSSLRTSDGYRTSEGFRIDPGNLGRSPMLARTRKELCALQGLYQAASRSE

mUSP2_var1    (1) --------------------------------------------------
mUSP2-var2    (1) ------------------MRTSYTVTLPEEPPAAHFPALAKELRPRSPLS
mUSP2_var3  (201) YLTDYLENYGRKGSAPQVLTQAPPPS-RVPEVLSPTYRPSGRYTLWEKSK
UI64              --------------------------------------------------

mUSP2_var1    (1) -----------MLNKAKNSKSAQGLAGLRNLGNTCFMNSILQCLSNTREL
mUSP2-var2   (33) PSLLLSTFVGLLLNKAKNSKSAQGLAGLRNLGNTCFMNSILQCLSNTREL
mUSP2_var3  (250) GQASGPSRSSSPGRDTMNSKSAQGLAGLRNLGNTCFMNSILQCLSNTREL
UI64          (1) ----------------MNSKSAQGLAGLRNLGNTCFMNSILQCLSNTREL

mUSP2_var1   (40) RDYCLQRLYMRDLGHTSSAHTALMEEFAKLIQTIWTSSPNDVVSPSEFKT
mUSP2-var2   (83) RDYCLQRLYMRDLGHTSSAHTALMEEFAKLIQTIWTSSPNDVVSPSEFKT
mUSP2_var3  (300) RDYCLQRLYMRDLGHTSSAHTALMEEFAKLIQTIWTSSPNDVVSPSEFKT
UI64         (35) RDYCLQRLYMRDLGHTSSAHTALMEEFAKLIQTIWTSSPNDVVSPSEFKT

mUSP2_var1   (90) QIQRYAPRFMGYNQQDAQEFLRFLLDGLHNEVNRVAARPKASPETLDHLP
mUSP2-var2  (133) QIQRYAPRFMGYNQQDAQEFLRFLLDGLHNEVNRVAARPKASPETLDHLP
mUSP2_var3  (350) QIQRYAPRFMGYNQQDAQEFLRFLLDGLHNEVNRVAARPKASPETLDHLP
UI64         (85) QIQRYAPRFMGYNQQDAQEFLRFLLDGLHNEVNRVAARPKASPETLDHLP

mUSP2_var1  (140) DEEKGRQMWRKYLEREDSRIGDLFVGQLKSSLTCTDCGYCSTVFDPFWDL
mUSP2-var2  (183) DEEKGRQMWRKYLEREDSRIGDLFVGQLKSSLTCTDCGYCSTVFDPFWDL
mUSP2_var3  (400) DEEKGRQMWRKYLEREDSRIGDLFVGQLKSSLTCTDCGYCSTVFDPFWDL
UI64        (135) DEEKGRQMWRKYLEREDSRIGDLFVGQLKSSLTCTDCGYCSTVFDPFWDX

mUSP2_var1  (190) SLPIAKRGYPEVTLMDCMRLFTKEDILDGDEKPTCCRCRARKRCIKKFSV
mUSP2-var2  (233) SLPIAKRGYPEVTLMDCMRLFTKEDILDGDEKPTCCRCRARKRCIKKFSV
mUSP2_var3  (450) SLPIAKRGYPEVTLMDCMRLFTKEDILDGDEKPTCCRCRARKRCIKKFSV
UI64        (185) SLPIAKRGYPEVTLMDCMRLFTKXDILDGDEKPTCCRCRARKRCIKKFSV

mUSP2_var1  (240) QRFPKILVLHLKRFSESRIRTSKLTTFVNFPLRDLDLREFASENTNHAVY
mUSP2-var2  (283) QRFPKILVLHLKRFSESRIRTSKLTTFVNFPLRDLDLREFASENTNHAVY
mUSP2_var3  (500) QRFPKILVLHLKRFSESRIRTSKLTTFVNFPLRDLDLREFASENTNHAVY
UI64        (235) QRFPKILVLHLKRFSESRIRTSKLXTFVNFXLRDLDLREFASENXNHAVY

mUSP2_var1  (290) NLYAVSNHSGTTMGGHYTAYCRSPVTGEWHTFNDSSVTPMSSSQVRTSDA
mUSP2-var2  (333) NLYAVSNHSGTTMGGHYTAYCRSPVTGEWHTFNDSSVTPMSSSQVRTSDA
mUSP2_var3  (550) NLYAVSNHSGTTMGGHYTAYCRSPVTGEWHTFNDSSVTPMSSSQVRTSDA
UI64        (285) NLYAVSNHSGTXMGGXYTAYCRSPVTGEWHTFNDSSVTPMSSSQVRTSDA

mUSP2_var1  (340) YLLFYELASPPSRM
mUSP2-var2  (383) YLLFYELASPPSRM
mUSP2_var3  (600) YLLFYELASPPSRM
UI64        (335) YLLFYELASPPSRM
```

Fig. 4.1.6
Amino acid alignment of the three mouse USP2 isoforms together with the sequence derived from the cDNA clone UI64 from the screen. *Recently, the sequences of three murine USP2 sequences were released in Genbank, here designated mUSP2_var1-3. The three isoforms are identical in their last 340 amino acids with mUSP2 transcript variants 2 and 3 possessing N-terminal extensions. By length, the translated UI64 cDNA clone sequence is most closely related to mUSP2 variant 1. However, the N-terminus of UI64 corresponds to an internal methionine of the mUSP2 variant 3. This explains why UI64 lacks the first five aminoterminal amino acid residues when compared to the transcript variant 1. The X residues in the UI64 sequence are due to inaccuracies during sequencing.*

The sequence of the cDNA clone UI64 starts with a methionine that presumably corresponds to an internal methionine of the mUSP2 transcript variant 3 (see **Fig. 4.1.6**). Therefore, taking into account this recent sequence information, the cDNA clone UI64 is most likely a fragment of the mUSP2 transcript variant 3. This explains why UI64 lacks the first five aminoterminal amino acid residues when compared to the transcript variant 1.

At the time when the UBP41 project was started (in the year 2000), the NCBI Genbank contained one single entry for a human form of UBP41 (NM_004205.1, gi:4759291). According to this sequence, hUBP41 was isolated by RT PCR and was used for all further studies. In the meantime, NCBI databank entries were revised several times for hUBP41 as can be seen in the revision history for the NCBI Genbank accession number NM_004205. As is the case with mouse UBP41, more sequence information about hUBP41 has been released recently. The protein that was originally named human UBP41 (consisting of 353 amino acid residues) is now designated human USP2 (hUSP2) of which two isoforms are described: the long transcript variant 1 (Accession NM_004205, gi:21361711) and the short transcript variant 2 (Accession NM_171997, gi:28565284). The originally published human UBP41 sequence is a perfect match of the last 352 C-terminal amino acids of the shorter hUSP2 variant 2 which consists of 396 amino acids and thus possesses a N-terminal 43 amino acid extension when compared to hUBP41 (**Fig. 4.1.7**).

It remained elusive whether hUBP41 is just a fragment of hUSP2 variant 2 or exists independently as a third hUSP2 variant. Recent information provided by the Human Genome Sequence server at NCBI (second release of the finished human reference genome from Oct 7, 2003) for the first time allowed the identification of the complete hUSP2 coding sequence with its corresponding genomic counterparts (exon sequences) on chromosome 11q23.3 (**Fig 4.1.8**). Previous genomic sequence releases did not contain matching sequences for the extreme N-terminal part of hUBP41, hUSP2 variant 1, and hUSP2 variant 2.

Genomic sequence analysis, using this revised sequence release, suggests that hUBP41 might be a fragment of the human USP2 transcript variant 2 (**Fig. 4.1.8**) because hUBP41 is identical in sequence as well as in exon/ intron organisation when compared to hUSP2 variant 2 but it is missing the first 129 bp. Importantly, at the putative start position of the UBP41 coding sequence the expected ATG codon is missing: instead, a CTG codon is found. This raises doubts whether the hUBP41 sequence investigated within the scope of this thesis does exist on its own or indeed is only a fragment of the USP2 variant 2. Notably, the same situation is found when the *mus musculus* mUBP41 sequence is analysed: as indicated above, three mUSP2 isoforms have been published in Genbank of which the mUSP2 transcript variant 1 corresponds exactly to the previously published mUBP41 sequence and therefore would be the mouse orthologue of the putative human UBP41.

The mouse USP2 locus is found on chromosome 9, and sequence analysis indicates that also for mUBP41 (alias mUSP2 transcript variant 1) no start ATG but instead a CTG codon is present. Consequently, with respect to those data, also the existence of the mUBP41 transcript would appear questionable and mUBP41 could be suspected to represent only a fragment of the longer mUSP2 transcript variant 2. However, the mUBP41 transcript has been identified in several independent cDNA sequencing projects and has been annotated as a putative full-length cDNA [Strausberg, 2002; Kawai, 2001]. This leaves open the possibility, that mUBP41 and also hUBP41 exist as USP2 isoforms on their own, and that translation can be initiated at a putative alternative CTG start

codon. At least there exist examples for the capacity of CTG codons as a start for translation [Arnaud, 1999].

Fig. 4.1.7
Alignment of hUBP41 with the two published hUSP2 variants. *The hUBP41 sequence (essentially as published in Genbank gi:4759291) was aligned with the two hUSP2 variants: hUSP2 variant 1, here designated hUSP-L (gi:21361711), and hUSP2 variant 2, here designated hUSP-S (gi:28565284). The C-terminal part of hUSP variant 2 is identical to the complete hUBP41 sequence whereas there are a few differences to variant 1, possibly due to polymorphisms.*

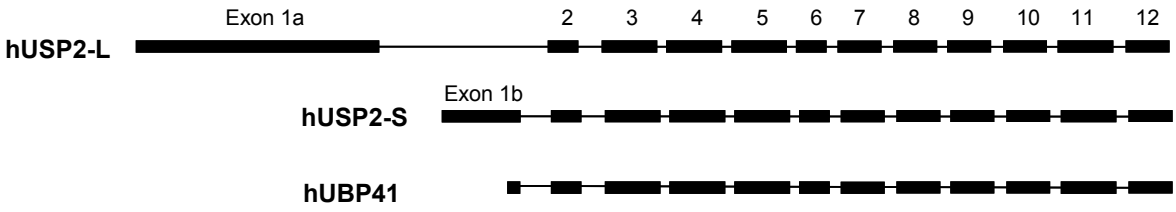


Fig. 4.1.8
Genomic organization of the USP2 locus on chromosome 11q23.3. *This sequence analysis is based on the second release of the finished human reference genome from Oct 7, 2003. The human USP2 variant 1 (here designated hUSP2-L) consists of 12 exons distributed over a total length of 16646 bp. Exon 1a (773 bp) is unique to hUSP2-L whereas exons 2 to 12 (as well as the corresponding intron sequences) are identical to the human USP2 variant 2 (here designated hUSP2-S). The coding sequence of hUSP2-S starts with the unique exon 1b which is located in a region between exon 1a and exon 2. Therefore, hUSP2-L and hUSP2-S are very likely to be splice isoforms. The difference in two amino acid positions as shown in Fig. 4.1.6 are presumably due to single nucleotide polymorphisms. The hUBP41 sequence is identical to that of USP2-S but the first exon of hUBP41 possesses only the last 15 bp of exon 1b. At the putative start position of hUBP41, the genomic sequence does not contain a start ATG but instead displays a CTG codon, thus the start adenosine is missing.This strongly suggests that the formerly published sequence of human hUBP41 (Genbank gi: 4759291) is only a fragment of the USP2 transcript variant 2 with the first 43 N-terminal amino acids missing. However, it cannot be excluded that CTG serves as an alternative start codon.*

As a conclusion, sequence analysis of the human USP2 locus raises doubts about the existence of hUBP41 as a transcript on its own. Strongest doubts arise from the fact, that the putative hUBP41 coding sequence does not possess a start ATG but instead displays a CTG. From this one should assume that hUBP41 is only a fragment of the longer hUSP2 transcript variant 2. On the other hand, the cDNA of mouse USP2 transcript variant 1 – which is almost identical to human UBP41 - has been identified in several independent EST sequencing programs, even though according to analysis of the mouse genome also the mUBP41 coding sequence is not expected to possess a start ATG but also the CTG codon. Therefore, it remains elusive whether or not UBP41 exists as a USP2 isoform on its own.

4.1.6 Characterization of the rabbit anti-hUSP2 polyclonal antibody

To characterize the endogenous hUSP2 expression and as a means to investigate the existence of USP2 isoforms on the protein level, an anti-hUBP41 polyclonal antibody was raised in rabbit and the obtained serum was affinity purified (see paragraph 3.1.4). The purified antibodies recognized the immunopeptide GST-IP-B that was used for immunization and overexpressed hUBP41 was recognized as well (**Fig. 3.1.16**). However, the antibody is also expected to be immunogenic against hUSP2 transcript variants 1 and -2 because the immunopeptide taken for immunization corresponds to

amino acids 119-150 of hUBP41 which are identical to both hUSP2 variants. Therefore the anti-hUBP41 polyclonal antibody is actually an anti-hUSP2 polyclonal antibody.

When these antibodies were used for detection of endogenous hUSP2, a pattern of five bands was detected (**Fig. 3.1.17**). Three bands corresponded to proteins larger than hUBP41, two smaller bands also appeared. Most likely would be that the band somewhat below the 47 kDa marker band would be USP2 variant 2 (which has a predicted molecular mass of 45 kDa) and the band higher than the 47 kDa band possibly USP2 variant 1 (predicted molecular mass 68 kDa). Unfortunately, no band was detected that was of the size expected for hUBP41, indicating that no hUSP2 variant of this kind is expressed at a high enough level in the cell lines tested.

4.1.7 Remaining questions and perspectives for future research

With respect to the previous two paragraphs (4.1.5 and 4.1.6), it remains to be elucidated what is the identity of the proteins that are recognized by the polyclonal anti-hUSP2 antibody (**Fig. 3.1.17**). It should be assumed that the two hUSP2 transcript variants 1 and –2 are among the proteins detected. It also is an open question whether hUBP41 is expressed in endogenous form or if it is merely a fragment of hUSP2 transcript variant 2. It remains to be tested whether overexpression of the hUSP2 transcript variant 2 also triggers apoptosis in human cell lines as efficiently as does hUBP41 overexpression.

In case that hUBP41 or its hUSP2 variants were detectable by the polyclonal antibody on the protein level, it might be rewarding to optimize RNAi conditions (as described in paragraph 3.1.4) to successfully downregulate the endogenous UBP41 / USP2 proteins. Then the sensitivity to apoptosis-inducing reagents could be compared between wildtype cells and cells with downregulated hUBP41 / hUSP2. If hUBP41 or its related hUSP2 variants play a role in determining the cellular balance between pro- and antiapoptotic factors, thereby influencing the sensitivity of tumor cells to apoptosis (as hypothesized in **Fig. 4.1.3**), then one might expect that cells with downregulated UBP41 / USP2 are possibly less sensitive to cell death induction.

Also depending on the successful detection and identification of the hUSP proteins by the anti-hUSP2 antibody, it could be investigated on the protein level whether USP2 levels are decreased in tumors of the kidney compared to normal kidney tissue as was observed on the mRNA level using the Tumor/Normal Profiling Array (**Figs. 3.1.20** and **3.1.21**). If the data from this Tumor/Normal Profiling Array can be varified, UBP41 itself or its USP2 variants might be candidate tumor suppressors which, when downregulated, allow neoplastic cells to be less sensitive to apoptosis. This hypothesis is certainly highly speculative but it should be noted that the deubiquitinating enzyme CYLD has been presented as an example for a USP whose downregulation results in increased resistance of cells to apoptosis and which has been identified as a tumor-suppressor that is frequently inactivated in tumor disease (see paragraph 4.1.1). In addition it shall be mentioned, that the hUSP2 gene is localized to the

chromosomal region 11q23.3 which has been reported to be lost in various cases of cancer and for which the existence of tumor-suppressors has been predicted [O'Sullivan, 2001; Monni, 1999, Herbst, 1999].

4.2 Cathepsins and their possible involvement in proapoptotic signalling

Caspases are thought to build the central proteolytic network involved in the execution of apoptotic signalling pathways induced by virtually all kinds of death stimuli [Earnshaw, 1999; Nagata, 1999; Robertson, 2000]. In recent years it has been recognized that besides caspases also other proteases such as cathepsins, calpains, and the proteasome may play a role as cofactors in mediating several cell death pathways, e.g. Cath-B in TNF-α-induced apoptosis [Leist, 2001a; Leist, 2001b]. Cath-B has been reported to be released from the lysosomes to the cytosol in response to TNF-α [Foghsgaard, 2001; Guicciardi, 2000] and to contribute to apoptotic downstream events such as cytochrome c release and the activation of executioner caspases [Guicciardi, 2000; Stoka, 2001]. Those observations suggest a possible role for cathepsins as positive effectors of cell death pathways (see paragraph 1.3), and the fact that tumors frequently contain high levels of cysteine proteases led to the hypothesis that Cath-B-like proteases may prove useful in selectively targeting tumor cells for apoptosis induction (**Fig. 4.2.1**).

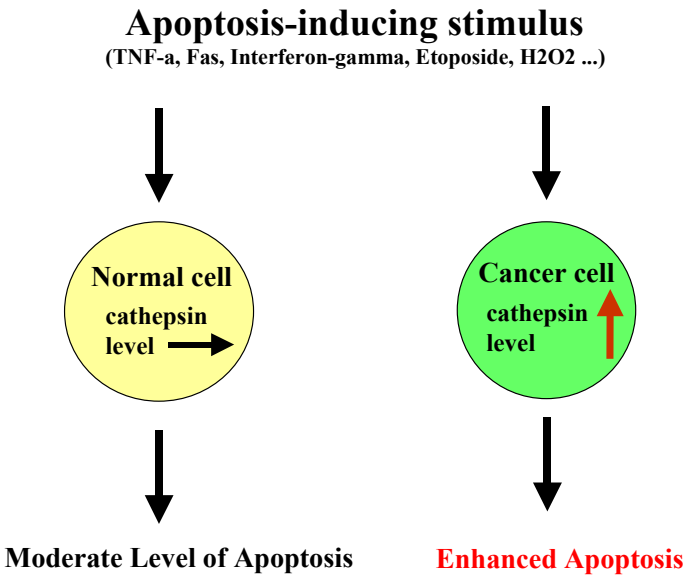


Fig. 4.2.1
Hypothesis: do elevated cathepsin expression levels sensitize cancer cells to apoptosis induction? *Whereas elevated cathepsin expression is frequently observed in tumors and is commonly regarded as a tumor marker correlated with poor prognosis, cathepsins on the other hand have recently been proposed to be involved in proapoptotic signalling (see also Fig. 1.12). If this were true, cells with elevated cathepsin expression levels would be expected to be more sensitive to certain death-inducing stimuli. It therefore has been hypothesized that this property might be exploited to selectively target tumor cells with high cathepsin expression levels for enhanced cell death induction in response to cytotoxic agents [Foghsgaard, 2001].*

4.2.1 Inhibition of cathepsin-B and -L appears to influence TNF-α-mediated apoptosis

The central objective of this study was to test the hypothesis, whether increased expression levels of Cath-L or Cath-B would enhance the sensitivity of HeLa cells to TNF-mediated apoptosis. The results obtained do not support this hypothesis, although at the same time inhibitor studies provided evidence for an influence of cathepsins in the TNF signalling pathway: apoptotic DNA fragmentation induced by cotreatment of HeLa cells with TNF-α and CHX could be significantly suppressed by various cathepsin inhibitors in a dose dependent manner (**Figs. 3.2.3 A,B).** This effect could be confirmed in PC3 cells (**Fig. 3.2.4**) and is also in line with observations in other cell systems shown in previous reports [Foghsgaard, 2001; Guicciardi, 2000]. Interestingly, apoptosis induced by irradiating the cells with UV light could not be diminished in presence of E64d (**Fig. 3.2.3 C**). Thus, in HeLa cells, cathepsins appear to contribute to the extrinsic receptor-mediated TNF cell death pathway, but not to the intrinsic apoptosis signalling pathways induced by UV.

4.2.2 Commonly used cathepsin inhibitors lack specificity

The inhibitor CA074Me is usually regarded to be highly selective for Cath-B, and therefore the results shown in **Fig. 3.2.3** and **Fig. 3.2.4** would suggest that Cath-B is a non-caspase cysteine protease contributing to TNF-mediated apoptosis in the HeLa cell system. In the attempt to verify the specific inhibitory activity of CA074Me, it turned out that CA074Me not only completely blocked hCath-B enzymatic activity but also hCath-L activity to almost the same extent (**Fig. 3.2.5**). This observation was confirmed by a recent publication, also demonstrating that CA074Me is not a selective inhibitor of Cath-B [Montaser, 2002]. Therefore, it cannot be ruled out that, besides Cath-B, CA074Me also inhibits other cysteine proteases such as Cath-L and by this exerts its inhibitory effect. The possibility that E64d might even be able to unspecifically inhibit caspases was excluded by the observation that E64d is not able to significantly inhibit caspase activity in a cell-free system (data not shown). From the inhibitor studies (see paragraph 3.2.3) it therefore can be concluded, that Cath-B itself or Cath-B-like proteases, such as Cath-L, contribute to TNF-mediated DNA fragmentation.

4.2.3 No influence of elevated cathepsin expression levels on sensitivity of HeLa cells to TNF-mediated apoptosis

There are several reports about an involvement of lysosomal cysteine proteases such as Cath-L and Cath-B in cancer progression and metastasis either by direct degradation of the extracellular matrix or by activation of other proteases such as urokinase-type plasminogen activator [Turk, 2000]. In many cases, strongly increased expression levels of Cath-L and Cath-B were detected in tumor cells and tumor tissues when compared to normal counterparts, and frequently the increase in cathepsin expression and activity correlated with malignant progression and metastasis. Consequently, cathepsins are regarded to be tumor markers with increased expression levels correlating with poor prognosis [Chauhan, 1991; Duffy, 1996; Kirschke, 1997; Lah, 1998; Sivaparvathi, 1996a; Sivaparvathi, 1996b; Sivaparvathi, 1996c; Sloane, 1990; Yan, 1998]. The tumor-promoting effect of cathepsins is obviously in contrast with the observation that cathepsins such as Cath-B can mediate proapoptotic signals triggered by TNF-α [Foghsgaard, 2001; Guicciardi, 2001; Mathiasen, 2001], but also bile-salts [Roberts, 1999], sphingosine [Kagedal, 2001b], B cell antigen receptor [Katz, 2001], and L-2,5-dihydrophenylalanine [Kiso, 2001]. But exactly this contrast might prove useful in developing a cancer therapy selectively targeting tumor cells for cell death with high level expression of cathepsins [Foghsgaard, 2001]. Even though this hypothesis appears attractive, up to now, no studies have been presented investigating a possible correlation between elevated cellular cathepsin levels and sensitivity to apoptotic stimuli such as TNF-α.

Since the inhibitor studies presented in this study indicate a contribution of Cath-B-like cysteine proteases in TNF-α/CHX-induced apoptosis in HeLa cells (**Fig. 3.2.3** and **Fig. 3.2.4**), it was further investigated whether sensitivity of HeLa cells to the TNF-α/CHX signal is enhanced by increased hCath-L or hCath-B levels. High level overexpression of hCath-L or hCath-B by itself did not result in apoptosis induction in HeLa cells (**Fig. 3.2.2**) whereas overexpression of hCath-D resulted in moderate but significant apoptosis as could be expected from a previous report [Deiss, 1996]. It also was not possible to detect a significant increase in sensitivity to TNF-α/CHX-induced apoptosis in HeLa cells transiently transfected with hCath-L or hCath-B (**Fig. 3.2.6 A**). In those transient transfection experiments transfection efficiencies between 40% and 60% were usually obtained as judged be GFP cotransfection, and overall cathepsin activities were increased by at least 6- to 10-fold for hCath-L and 10- to 20-fold in case of hCath-B what presumably is comparable to what is frequently detected in tumor tissues [Chauhan, 1991; Lah, 1998; Yan, 1998]. Under those experimental conditions it should be possible to detect any significant increase in sensitivity that might have been correlated with cathepsin overexpression. Additionally, HeLa cell pools were generated that stably overexpress hCath-L or hCath-B, respectively. In this case it was assumed that the majority of stable transfected cells overexpress the corresponding cathepsin, although at at a lower level than in transient expression

experiments. Also those stable transfected pool clones did not show any increased sensitivity to the TNF-α death signal (**Fig. 3.2.6 B**).

Additionally, the rat hepatoma cell line McA RH 7777 was used for another transient expression experiment, which also did not indicate any change in sensitivity to TNF-α/CHX upon cathepsin overexpression (**Fig. 3.2.6 C**). This additional cell line was chosen because hepatocyte apoptosis has been discussed in particular to be influenced by Cath-B proapoptotic activity [Guicciardi, 2000; Guicciardi, 2001; Roberts, 1999].

4.2.4 Inhibition but not overexpression of cathepsins influences TNF-mediated apoptosis – possible explanations

As shown in **Fig. 3.2.7**, lysosomal integrity is apparently disturbed when HeLa cells are treated with TNF-α/CHX as indicated by the partial redistribution of Cath-L from a lysosomal spot-like pattern to a more diffuse staining. Thus, increased expression levels of cathepsins are expected to result in correspondingly higher amounts of cathepsin activity in the cytoplasm in response to TNF-α/CHX. How then can be explained that TNF-α/CHX-induced apoptosis is inhibited in presence of the cathepsin inhibitors E64d and CA074Me (**Fig. 3.2.3** and **Fig. 3.2.4**) but that cathepsin overexpression does not enhance TNF/CHX-induced cell death (**Fig.3.2.6**)?

Possibly, endogenous basal levels of lysosomal Cath-L or Cath-B activity are sufficient to provide the lysosomal stimulus contributing to the proapoptotic signalling cascade for cell death execution. In this case, other factors than Cath-L or Cath-B levels would be rate limiting in the execution process of apoptosis, and an increase in Cath-L or Cath-B levels would not further accelerate or enhance the signal coming from the TNF receptor. It also cannot be excluded that other cathepsin-like proteases than Cath-L or Cath-B are mainly responsible for the contribution to TNF-α apoptotic pathways, since the inhibitors E64d and apparently also CA074Me are not specific inhibitors of distinct cathepsins (Fig.2 and [Montaser, 2002]).

Both explanations would be in line with the observation, that overexpression of hCath-L or hCath-B not even shows any death-enhancing effect on NDI-HCl-induced cell death (**Fig. 3.2.8**), even though in this case it can be assumed that upon treatment with NDI-HCl the overexpressed cathepsins are almost completely released from the lysosomal compartments to the cytosol (**Fig. 3.2.7**). It should be pointed out, that NDI-HCl-induced death behaves different than TNF-α/CHX-mediated death because NDI-HCl results in cell death that cannot be suppressed by E64d at all, and zVAD-fmk can only partially block this cell death (**Fig. 3.2.8**), presumably due to the extreme extent of lysosomal rupture

caused by this lysosomotropic detergent (**Fig. 3.2.7**) leading to both, a caspase-dependent apoptotic mode and caspase-independent mode of cell death.

4.2.5 Conclusions and perspectives for future research

In conclusion, there is evidence that Cath-B-like cysteine proteases contribute to certain aspects of apoptotic signalling pathways. Whereas the possibility appears attractive to exploit the proapoptotic features of cathepsins in specifically targeting tumor cells with elevated cathepsin expression levels for cell death induction, overexpression experiments presented in this study do not support such an hypothesis. Since this is the first report directly investigating a possible correlation between cathepsin expression levels and sensitivity to an apoptotic stimulus, further studies are certainly necessary to confirm and extend the here presented data for other cell systems and additional death stimuli besides TNF-α. Moreover, downregulation of cathepsin expression levels via the RNAi methodology (instead of using cathepsin inhibitors with limited specificity) should prove extremely useful in further determining the possible role of cathepsins in the mediation of cell death signals such as those triggered at the TNF receptor complex.

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Patent participation

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Curriculum Vitae

Stefan Andreas Clemens Gewies

A. PERSONAL DATA

Birthplace and Date: Berlin, Germany, May 7, 1971

Citizenship Status: German

B. EDUCATION

Time Period	Occupation
since Jan. 2000	Ph.D. Student at the Max-Planck-Institute for Biochemistry in Martinsried in the Independent Junior Research Group for Apoptosis Research, Group of PD Dr. Stefan Grimm
March 1998 - Dec. 1999	Research Assistant at the Department of Pathology, University of Iowa, Iowa City (USA) Research Group Prof. Michael B. Cohen M.D.; Studies in Receptor-mediated Apoptosis in Prostatic Carcinoma Cell Lines.
July 1997 - Febr. 1998	Research Assistant at the Charité, Medical School Berlin, Institute of Medical Genetics, Director Prof. Regine Witkowski, Department of Molecular Genetics, Group PD Dr. Peter Nürnberg; .
May 1996 - June 1997	Community Service at the Charité Medical School Berlin, Institute of Medical Genetics, Department of Molecular Genetics.
May 1995 - Spring 1996	Diplomarbeit at the Technical University Berlin, Institute for Biochemistry and Molecular Biology, research group Dr. Ullrich Keller. Title: "Expression and directed inactivation of immunophilin genes in *Streptomyces*."
January - May 1995	Final examinations for Diploma degree at the Technical University Berlin, department of chemistry.
July - December 1994	Internship in the pharmaceutical company "Gödecke AG Berlin": validation of microbiological quality control procedures.
Oct. 1990 - July 1994	Study of Chemistry at the Technical University Berlin.
Aug. 1983 - June 1990	Catholic Gymnasium Salvator, Berlin: Abitur (graduation).

Abbreviations

AICD	Activation-induced cell death
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis-inducing factor
AMC	Aminomethylcoumarine
ANT-1	Adenine nucleotide translocator 1
Apaf-1	Apoptotic protease activating factor 1
APC	Anaphase-promoting complex
ARF	Alternative reading frame within the Ink4a/ARF gene locus
ATP	adenosine triphosphate
Bad	Bcl-2 agonist of cell death
Bak	Bcl-2 agonist/killer
Bax	Bcl-2 associated X-factor
Bcl-2	B cell lymphoma gene 2
Bcl-XL	Bcl-2 related gene x, large splice isoform
BH domain	Bcl-2 homology domain
Bid	BH3-interacting DD agonist
Bim	Bcl-2 interacting mediator of cell death
Bmf	Bcl-2-modifying factor
BIR	*Baculovirus* inhibitor repeat
BLAST	Basic local alignment search tool
CA074Me	Inhibitor of cathepsin-B-like cysteine proteases
CAD	Caspase-activated DNase
CARD	Caspase recruitment domain
Caspase	Cysteine-dependent aspartate-specific protease
Cath-B	cathepsin B
Cath-D	cathepsin D
Cath-L	cathepsin L
cDNA	complementary DNA
CDK	Cyclin-dependent kinase
C. elegans	*Caenorhabditis elegans* – a nematode worm
ced-3	*Caenorhabditis elegans* death gene 3 – homologue of mammalian caspase-1
ced-4	*Caenorhabditis elegans* death gene 4 – homologue of mammalian Apaf-1
ced-9	*Caenorhabditis elegans* death gene 9 – homologue of mammalian Bcl-2
c-FLIP	cellular FLICE inhibitory protein
CHX	*Cycloheximide*
CIDE-A	*Cell death-inducing DFF45-like effector A*
DAPI	Diamidino-2-phenylindole
DR5	Death receptor 5
$\Delta\Psi$	inner mitochondrial transmembrane potential
dATP	deoxyadenosine triphosphate
DD	Death domain
DED	Death effector domain
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
DUB	Deubiquitinating enzyme
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme

E3	ubiquitin ligase
E4	accessory factor for multiubiquitylation
E64d	broad range cathepsin inhibitor
E6-AP	E6-associated protein
EGFP	Enhanced green fluorescent protein
Egl-1	Egg-laying defective gene 1 in *C. elegans*
endoG	Endonuclease G
EPO	Erythropoietin
ER	Endoplasmic Reticulum
EYFP	Enhanced yellow fluorescent protein
faf	Fat facets gene in *Drosophila*
FACS	Fluorescence aided cell sorting, flow cytometric analysis
Fam	Fat facets in mouse
FLICE	former name for caspase-8
GSH	Glutathione
GST	Glutathione S-transferase
HA	Haemagglutinin-derived peptide tag sequence (YPYDVPDYA)
HAUSP	*Herpes* virus associated USP
hCath-B	human cathepsin B
hCath-D	human cathepsin D
hCath-L	human cathepsin L
HECT	Homologous to E6-AP carboxyl terminus, ubiquitin ligase motif
HIF-α	Heat shock inducible factor α
HIV	Human Immunodeficiency Virus
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of CAD
ICE	Interleukin-1β-converting enzyme
IκB	Inhibitor of NF-κB
IL-3	Interleukin-3
lqf	Liquid facets gene in *Drosophila*
Mdm2	Murine double minute 2 gene
MG132	Cbz-leu-leu-leucinal, aldehyde inhibitor of the proteasome
NADH	Nicotinamide adenine dinucleoti de, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NCBI	National Center for Biotechnology Information
NDI-HCl	
NF-κB	Nuclear factor κB
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PI FACS	Flow cytometric analysis using PI for determining DNA content
PKB	Protein kinase B
pro-IL-1β	pro-Interleukin-1β
pro-IL-18	pro-Interleukin-18
PT	Permeability transition
PTP	Permeability transition pore
Rb	Retinoblastoma protein

RING domain	Really interesting new gene domain with ubiquitin ligase activity
RNA_i	RNA interference
ROS	Reactive oxygen species
RT PCR	Reverse transcription PCR
SAIP	Small apoptosis-inducing protein
SCF	Skp1/Cullin/F-Box complex
siRNA	small inhibitory RNA
Smac	Second mitochondrial activator of apoptosis
Spike	Small protein with inherent killing effect
SUMO	Small ubiquitin-like modifier
tBid	truncated Bid
TBS	Tris buffered saline
TNF	sometimes used as a short form of TNF-α
TNF-α	Tumor necrosis factor α
TNFR	Tumor necrosis factor receptor
TRADD	Tumor necrosis factor receptor associated death domain protein
TRAF2	TNF receptor associated factor 2
TRAIL	Tumor necrosis factor related apoptosis-inducing ligand
Ub	Ubiquitin
UBP	Ubiquitin-processing protease
UCH	Ubiquitin carboxy terminal hydrolase
USP	Ubiquitin-specific protease
UV	Ultraviolet
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau, a tumor syndrome
WT	wild type
XIAP	X-chromosome linked IAP
zDEVD-AMC	Benzoxy-Asp-Glu-Val-Asp-aminomethylcoumarine
zFR-AMC	Benzoxy-Phe-Arg-aminomethylcoumarine
zVAD-fmk	Benzoxy-Val-Ala-Asp-fluoromethylketone