Novel Interactors of X-linked Inhibitor of Apoptosis Protein

Expression and Effects on Tumor Cell Death

HÅKAN STEEN
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Abstract

Programmed cell death, or apoptosis, has during the last decade received a lot of attention due to its involvement in a large number of pathological conditions. Since death is always irreversible, it is important for cells to fully control the initiation and execution of this process. One of many apoptosis-regulatory proteins is XIAP, which blocks the action of caspases, a family of proteases that are important during apoptosis. However, apoptosis inhibitors have to be tightly controlled since too little cell death can lead to the development of tumors and other diseases. This thesis is the result of an aspiration to fully understand the function and regulation of XIAP.

By using the yeast-2-hybrid system, we identified two novel binding partners of XIAP. The first, GPS2, was found to bind XIAP and inhibit its ability to block caspase-activity. In addition, GPS2 induced caspase-mediated cell death in two different tumour cell lines and XIAP inhibited this effect.

The second binding partner, Nulp1, preferentially bound XIAP in the presence of the apoptosis-inducer staurosporine. Nulp1 induced or sensitized cell lines to cell death when overexpressed, but this was not blocked by caspase-inhibitors or XIAP, suggesting a different reason for binding than apoptosis regulation. With the aim to understand the Nulp1-XIAP interaction, we continued to study Nulp1 in vivo and in vitro. We studied three different splice variants of Nulp1 and found that they were regulated by poly-ubiquitination and nuclear shuttling. Also, Nulp1 was expressed in embryonic mice, especially in the cortical plate, hippocampal neurons and cerebellar granular neurons. Expression of Nulp1 decreased with age but was still present in cerebellar deep nuclei and Purkinje cells of adult mice.

To summarize, we have identified GPS2 as an apoptosis-inducing factor and an inhibitor of XIAP in vitro, and Nulp1 as a XIAP-interacting protein during staurosporine-induced apoptosis.

Keywords: XIAP, tumor cell death, apoptosis, caspases, basic helix-loop-helix protein

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‘If you try the best you can, the best you can is good enough’

‘Optimistic’ by Radiohead
List of papers

This thesis is based on the following papers, referred to in the text by their roman numerals:


III  **Steen H**, and Lindholm D. Nulp1/Tcf25 isoforms are differentially regulated through ubiquitination and subcellular localization in neuroblastoma cells. (Manuscript)

IV  **Steen H**, Korhonen L, Hansson I, and Lindholm D. Nulp1 is expressed in developing mouse brain and in neuronal progenitor cells. (Manuscript)

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Abbreviations

AIF: Apoptosis Inducing Factor
Apaf-1: Apoptotic protease-activating factor 1
ATP: Adenosine Tri-Phosphate
BIR: Baculoviral IAP Repeat
BMP: Bone Morphogenic Protein
BRUCE: BIR Repeat containing Ubiquitin-Conjugating Enzyme
CARD: CAaspase Recruitment Domain
cIAP: Cellular IAP
DIABLO: Direct IAp-Binding protein with LOw pi
DISC: Death Inducing Signal Complex
EGFP: Enhanced Green Fluorescent Protein
EGL: External Granular Layer
ERK: Extracellular signal-Regulated Kinase
GPS2: G-protein Pathway Suppressor 2
GST: Glutathione S-Transferase
HAT: Histone Acetyltransferase
HDAC: Histone Deacetylase
IAP: Inhibitor of Apoptosis Protein
ID: Inhibitor of DNA-binding
IGL: Internal Granular Layer
JNK: c-Jun N-terminal Kinase
LMB: Leptomycin B
NF-κB: Nuclear Factor-κB
NGF: Neurotrophic Growth Factor
NKT: Natural Killer T-lymphocyte
Nulp1: Nuclear localized protein-1
PARP: Poly(ADP-ribose)polymerase
PCD: Programmed Cell Death
PFA: Paraformaldehyde
PKB: Protein Kinase B
RING: Really Interesting New Gene
Smac: Second mitochondria-derived activator of caspases
SRE: Serum Response Element
SRF: Serum Response Factor
STS: Staurosporine
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</tr>
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<td>TGF-beta activated kinase 1</td>
</tr>
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<td>Tcf25</td>
<td>Transcription factor 25</td>
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<td>TGF</td>
<td>Transforming Growth Factor</td>
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<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<td>TRAF</td>
<td>TNF-Receptor Associated Factor</td>
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Introduction

During embryogenesis, the number of neuronal and glial cells is about twice the amount found later in the fully developed organism. Due to environmental signals, such as the limited amount of neurotrophic factors secreted by target tissues, a large number of the neurons commit suicide, also called apoptosis or programmed cell death (PCD) (Pettmann and Henderson, 1998). The observation of naturally occurring cell death in the nervous system in the late 1940’s by Hamburger and Levi-Montalcini led to the realization that cell death is an important process in the developing embryo and also during later stages in life (Bennet et al., 2002). Since then, a large amount of work has been done to fully understand the role and mechanisms of apoptosis, with the insight that apoptosis is involved in most physiological processes and also in a large number of pathological conditions. As with many other cellular processes, the execution of apoptosis is tightly regulated and controlled by more than a hundred genes. Many of these genes have functional homologues in more primitive organisms, which suggest that programmed cell death is an evolutionary conserved process.

1. An overview of cell death

Cell death is usually divided into two types, apoptosis and necrosis. It was first thought that these two types where morphologically and functionally distinct but the view has changed in the last decade to one considering apoptosis and necrosis to be the two extremes on a scale with a number of intermediate cell death types in between, with features of both apoptosis and necrosis.

1.1 Apoptosis

The term ‘apoptosis’ was proposed by John Kerr and colleagues in 1972 and originates from the Greek words ‘apo’ and ‘ptos’, which translates to ‘separation’ and ‘falling off’, respectively (Duque-Parra, 2005; Kerr, 2002). Together, the two words have been used to describe the leaves falling off trees in the autumn or petals falling from flowers. The reason for using the word to label programmed cell death is because during the end stage of apoptosis
the cell is split into small membrane-bound vesicles, called apoptotic bodies, which resembles the falling of leaves from trees.

During apoptosis, a number of characteristic morphological features are usually seen (Fig. 1). The plasma membrane starts to roughen, the cytoskeleton gets disassembled, organelles start to shrink, the nucleus becomes condensed and its chromatin is cleaved into about 180 base-pair large fragments (Saraste and Pulkki, 2000). All of these features are the result of an increase in proteolytical activity in the cell, especially activity of the caspase protease family. For example, cleavage of nuclear lamins results in nuclear collapse and condensation (Gruenbaum et al., 2000), whereas activation of gelsolin and cleavage of p21-activated kinase-2 results in membrane blebbing and forming of apoptotic bodies (Fink and Cookson, 2005). One of the most important events in the apoptotic process is the translocation of phosphatidylserine from the inner to the outer side of the membrane (Schlegel and Williamson, 2001). This translocation is a signal for neighbouring cells to start phagocytize the apoptotic cell, ultimately leading to the removal of the cell.

1.2 Necrosis

Necrosis is, in contrast to apoptosis, a passive process and not genetically controlled (Fink and Cookson, 2005). Necrosis is often caused by damaging agents such as heat stress, toxic agents, radiation or mechanical injury. These agents are also able to trigger apoptosis in cells, but the determining factor is the intensity of the stressor and the energetic status of the cell. Cells with a low concentration of ATP or impaired ATP production are more prone to turn necrotic, since apoptosis is an energy-dependent process (ATP is required for the forming of the apoptosome, for kinase-signalling and for the maintenance of ion-homeostasis) (Skulachev, 2006). Although the morphological and biochemical features of necrosis may vary depending on cell type, stressor, environment etc., necrosis is typically characterized by an early increase in membrane permeability, organelle swelling and late disruption of the nucleus (Fig. 1) (Golstein and Kroemer, 2007). The end-point is the total collapse and disruption of the cell. Due to the increased permeability of the plasma membrane, pro-inflammatory substances leak into the surrounding tissue triggering a harmful inflammatory response. A classic example of necrosis and its toxic effects on neighbouring cells is in response to a traumatic brain injury, e.g. a blow to the head (Bramlett and Dietrich, 2004). The area closest to the point of impact becomes necrotic very rapidly, spills toxic and pro-inflammatory substances, which in turn affects cells not directly involved by the initial injury. This surrounding area is called penumbra and the major type of cell death present there is not necrosis but apoptosis. In this neuronal setting, one of the most toxic substances released
is the neurotransmitter glutamate, which in high concentration acts toxic on neurons by increasing intracellular calcium.

Figure 1. A simplified schematic of the morphological cellular changes during apoptosis and necrosis. N, nucleus.

2. A closer look at apoptosis

2.1 Caspases

The main players in the apoptotic process are the caspases, a family of cysteine proteases with a preference to cleave its substrate after aspartic acid residues (Degterev et al., 2003). Caspases are initially translated as inactive pro-caspases. Proteolytic cleavage of the pro-caspase is therefore required to form the active form of the caspase, which consists of two large and two small subunits from two cleaved pro-caspase proteins. Caspases and their homologues have been found in a number of diverse species, ranging from the nematode Caenorhabditis elegans, the dipteran Drosophila melanogaster and far up the evolutionary tree to mammals. At least 13 different caspases has been identified, although only 11 in human. Caspases are usually grouped into ‘initiator caspases’ or ‘effector caspases’ depending on which function they have in the apoptotic pathway. The initiator caspases include
caspase-2, -8, -9 and -10, and function as triggers of the caspase cascade by cleaving caspase-3, -6 and -7, the effector caspases. These effector caspases are the proteases responsible for cleaving important cellular substrates. A third group of caspases is called ‘inflammatory caspases’ and includes human caspase-1, -4, -5, and mouse caspase-11 and -12. Some of these are involved in both inflammatory and apoptotic processes whereas other have no known function during apoptosis.

2.2 Apoptotic pathways

There are two general pathways that lead to cleavage and activation of the effector caspases; the mitochondria-mediated and the receptor-mediated pathway (Fig. 2) (Danial and Korsmeyer, 2004). The mitochondria act as sensors of cellular stress and initiate an apoptotic response if the signals triggered by the stressors are strong enough. The proteins acting directly upstream of the mitochondria are the pro- and anti-apoptotic members of the Bcl-2 family (Cory and Adams, 2002). The pro-apoptotic members, such as Bax and Bak, are activated by apoptotic stimuli with the consequence of triggering permeabilisation of the outer mitochondrial membrane. This is done by Bax or Bak forming a pore in the outer membrane by oligomerisation. The anti-apoptotic members, such as Bcl-2 and Bcl-X_L, counteract this permeabilisation by binding and inhibiting Bax. As the outer mitochondrial membrane gets permeabilised, a number of pro-apoptotic proteins get released. The most important of these are cytochrome c, normally functioning in the mitochondrial electron-transport chain (Garrido et al., 2006). As cytochrome c enters the cytoplasm, it forms the apoptosome by binding Apoptotic protease-activating factor 1 (Apaf-1), procaspase-9 and ATP (Riedl and Salvesen, 2007). With the formation of the apoptosome, caspase-9 is activated which further triggers the activation of caspase-3, -6 and -7. These effector caspases will then take care of the final stages of the apoptotic process. Since activated caspases can cleave and activate other pro-caspases, the process is often called the caspase cascade.

Receptor-mediated apoptosis is a more straightforward process. Binding of death-inducing ligands, such as TNF-α and FasLigand (FasL), to their cognate receptor induces the assembly of a death-inducing signal complex (DISC) which propagates the apoptotic signal by binding and activating an initiator caspase, such caspase-8 (Curtin and Cotter, 2003). The initiator caspase cleaves and activates effector caspases, such as caspase-3, and -7.
Figure 2. A schematic picture of the receptor-mediated and mitochondria-mediated apoptotic pathway.

The two apoptosis-inducing pathways do not only converge at the level of caspase-3. Caspase-8 has been shown to cleave Bid, a pro-apoptotic protein of the Bcl-2 family, into a truncated form called tBid. tBid is able to induce mitochondrial membrane permeabilisation by triggering a conformational change in Bax which in turn leads to Bax-oligomerization (Desagher et al., 1999). The activation of the effector caspases leads to cleavage of a large number of target proteins, including actin, lamins, inhibitor of caspase activated DNase (ICAD), Akt/Protein kinase B (PKB), Rb and Poly(ADP-ribose)polymerase (PARP) (Fischer et al., 2003).

The fate of a cell is determined by the balance of pro- and anti-apoptotic proteins. This balance is not only controlled at the post-translational level by proteolytic activity, but also by extra- and intracellular signals, and their effects on protein expression through transcriptional regulators.

2.3 Major regulators of apoptosis

Growth factors, cytokines and other receptor-binding ligands, as well as intracellular sensor proteins, all affect cells by inducing intracellular signal cascades resulting in alteration of the transcriptome, and subsequently the proteome. Four signal proteins that often orchestrate the cellular apoptotic response are Nuclear Factor-κB (NF-κB), c-jun N-terminal kinase (JNK), Akt/Protein kinase B (PKB) and p53.
NF-κB

NF-κB is a transcriptional regulator normally present in an inactive state in the cytosol and consists of a transcription factor dimer bound to the Inhibitor of κB (IκB), which is responsible for keeping the dimer inactive by cytoplasmic sequestration (Mattson and Meffert, 2006). Several different subunits of the dimer have been identified, including p50, p52, p65 (Rel-A), Rel-B and c-Rel, where p50/p65 is the most common NF-κB complex in neuronal cells. Different genes are regulated dependent on which subunits make up NF-κB. Activation of NF-κB is triggered by phosphorylation of IκB by IκB-kinase (IKK) leading to dissociation of the complex and translocation of NF-κB (i.e. p50/p65) to the nucleus for transcriptional regulation. Bcl-2 (Catz and Johnson, 2001), Bcl-XL (Lee et al., 1999), cIAP1 (You et al., 1997), XIAP (Stehlik et al., 1998), and Bax (Grimm et al., 2005) are just a few apoptosis protein whose genes are known to be regulated by NF-κB. NF-κB-activation has often been seen to protect cells against apoptosis but can also act pro-apoptotic depending on subunit composition, triggering stimuli and cell-type (Radhakrishnan and Kamalakaran, 2006). For example, NF-κB activation through TNF-treatment protects rat hippocampal neurons against metabolic and excitotoxic insults (Mattson and Meffert, 2006). An increase in NF-κB activity has also been seen in rat hippocampal neurons in response to transient global forebrain ischemia and after reperfusion-induced ischemia. However, NF-κB-activation in glial cells in response to damage can lead to the induction of pro-inflammatory cytokines, reactive oxygen and nitrogen species as well as excitotoxins, thereby inducing more cell death in the vicinity of the glial cells. Hence, the reigning hypothesis of the role of NF-κB in the nervous system is that it is somewhat of a double-edged sword; beneficial through its anti-apoptotic effect in neurons but detrimental due to the inflammatory and oxidative response in glial cells.

JNK

JNK is a member of the mitogen-activated protein kinase (MAPK) family and is activated by stressful stimuli including UV and λ-irradiation, toxins, ischemia, heat shock and inflammatory cytokines such as TNF-α (Nishina et al., 2004). JNK regulates in turn transcription factors including c-Jun, ATF-2, Elk-1, p53 and c-Myc, as well as non-transcription factors such as Bcl-2, Bel-XL, paxillin and MAP2. JNK can therefore control not only apoptotic processes but also proliferation, differentiation, migration and cytoskeletal structure. Three forms of JNK have been found, JNK1, 2 and 3, where JNK1 and JNK2 are ubiquitously expressed and JNK3 is only expressed in brain and heart (Kanda and Miura, 2004). Their exact function in apoptosis regulation is not known, since they have been found to be pro-apoptotic, anti-apoptotic and non-apoptotic. In concordance with the observed dual-nature of JNK signalling, knock-out studies have shown that E9.5 JNK1−/−/JNK2−/−
mice have decreased apoptosis in the hindbrain neuroepithelium and increased rate of cell-death in the forebrain indicating that JNK can play both an anti- and a pro-apoptotic role (Liu and Lin, 2005).

**Akt/PKB**

Growth factors usually push the cell towards survival and proliferation. One common factor in the intracellular signal cascade during growth factor-signalling is Akt/PKB. Akt/PKB is activated by phosphatidylinositol-3 kinase (PI3K) and phosphorylates pro-apoptotic factors such as Bad, caspase-9 and the Forkhead box transcription factors, class O (FOXO), which results in their inhibition and increased cellular resistance towards apoptosis (Brunet et al., 2001). The FOXO-proteins regulates genes involved in cell metabolism, cycle control, DNA repair, differentiation and cell death (Greer and Brunet, 2005). The cell death regulating genes that are positively controlled by FOXO includes the pro-apoptotic Bcl-2 proteins Bim (Dijkers et al., 2000) and NIP3 (Tran et al., 2002), and the death-inducing ligands FasL and TRAIL (Brunet et al., 1999; Modur et al., 2002).

**p53**

One of the most important transcription factors in regards to apoptosis and cell survival is p53. The p53 protein is normally kept at a low concentration by ubiquitin-mediated degradation in healthy cells (Brooks and Gu, 2006). DNA-damage, mitotic impairment, oncogene activation and oxidative stress triggers stabilization and activation of p53 through post-translational changes such as phosphorylation, acetylation and protein-protein interactions, which results in increased transcriptional activity. A low level of active p53, i.e. during mild stress, primarily activates genes such as p21 and p27, thereby inducing cell cycle arrest to give the cell time to repair the damage (Laptenko and Prives, 2006). If the damage persists or the damaging agent continues to compromise the genomic integrity, p53 accumulates further and starts to regulate apoptotic genes, including the pro-apoptotic Bcl-2 genes Bax, Noxa and Puma, resulting in cell death and subsequent removal. p53 has also been seen to directly affect the mitochondrial apoptotic pathway by binding Bcl-2 and Bcl-X\textsubscript{L}, thereby replacing Bax and Bak with the consequence of mitochondrial outer membrane permeabilisation (Fuster et al. 2007).

These four apoptosis regulators do not work in isolation or without any input from the other. For example, NF-\kappaB-activation in response to TNF-\alpha leads to a transcription-dependent inhibition of JNK-activity resulting in suppression of apoptosis (Tang et al., 2001), and Akt/PKB can phosphorylate MDM2/HDM2 which promotes ubiquitination and degradation of p53 (Mayo and Donner, 2001; Zhou et al., 2001a).
2.4 Translational regulation during cell stress

Cells also respond to stressors and damage by decreasing general transcriptional activity in an attempt to conserve ATP and resolve the damage (Graber and Holcik, 2007). This is done by inhibition the cap-dependent translation machinery that is responsible for the majority of all mRNA translation. However, some mRNA has an internal ribosomal entry site (IRES) that functions as a cap-independent translational initiation site, which facilitates synthesis of the protein despite the general synthesis repression. Interestingly, most of the IRES-containing mRNA codes for proteins involved in cell death-regulation and cell survival. Apaf-1, Bcl-2, cIAP1, p53 and XIAP are just a few proteins whose mRNA contains an IRES and that are still under translational control during cell stress.

3. The Inhibitor of Apoptosis Protein family

In theory, one active caspase can start a process that eventually leads to apoptosis and cell demise. Therefore, the cell has to have protection against accidentally activated caspases. It was long thought that as soon as the mitochondria released its pro-apoptotic content, the cells had passed its ‘point-of-no-return’ and were doomed to die, and that the decision to live or die was decided on the level of Bcl-2/Bax. However, with the discovery of a protein family of apoptosis-inhibitors, the Inhibitor of Apoptosis Proteins (IAPs), this view has been somewhat revised.

The IAP family consists of eight members and is characterized by the presence of the zinc-containing Baculoviral IAP Repeat (BIR) domain (Birnbaum et al., 1994). The BIR-domain is responsible for most protein interactions of the IAPs. The domain was first found in the genome of the Cydia pomonella granulosis virus, and is a part of its IAP protein (Crook et al., 1993). IAP was found to inhibit insect cell apoptosis induced by a p35-lacking (p35 is a viral anti-apoptotic protein unrelated to the IAPs) Autographa californica nuclear polyhedrosis virus. After that, mammalian homologues to IAP were quickly found with conserved anti-apoptotic properties (Ducette et al., 1996; Uren et al., 1996). The family consists of X-linked IAP (XIAP), cellular-IAP1 (cIAP1), cellular-IAP2 (cIAP2), survivin, neuronal apoptosis inhibitory protein (NAIP), BIR repeat containing ubiquitin-conjugating enzyme (BRUCE), Testis-specific IAP (Ts-IAP) and Melanoma IAP (ML-IAP). It was originally thought that there was large redundancy between the different IAPs, especially XIAP, cIAP1 and cIAP2, because of their ability to inhibit caspases in vitro (Deveraux et al., 1997; Roy et al., 1997), but further investigations have suggested that XIAP is the only inhibitor of the proteolytical activity of caspases (Eckelman and Salvesen, 2006). With time it has been revealed that each IAP covers its own niche, such as
survivin being important for cytokinesis and the cIAPs being involved in receptor-mediated apoptosis induced by TNF-α. An overview of the different IAPs and their known functions is shown in Table 1.

<table>
<thead>
<tr>
<th>Members</th>
<th>Function</th>
<th>Domains</th>
<th>Refs.</th>
</tr>
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<tbody>
<tr>
<td>XIAP</td>
<td>Suppresses apoptosis by binding and inhibiting caspase-3, -7 and -9. Ubiquitinates caspases, Smac/DIABLO, Murr1 and AIF. Co-factor in BMP/TGFβ-signalling. May modulate cell cycle.</td>
<td>3xBIR, RING</td>
<td>(Birkey Reffey et al., 2001; Burstein et al., 2004; Devraux et al., 1998; Levkau et al., 2001; MacFarlane et al., 2002; Suzuki et al., 2001b; Wilkinson et al., 2008; Yamaguchi et al., 1999)</td>
</tr>
<tr>
<td>cIAP1</td>
<td>Part of the TNFRI/II intracellular complex that activates NF-kB. Ubiquitinates XIAP, cIAP2, Mad1, RIP, Smac and TRAF2.</td>
<td>3xBIR, RING, CARD</td>
<td>(Conze et al., 2005; Hu and Yang, 2003; Li et al., 2002; Park et al., 2004; Rothe et al., 1995; Silke et al., 2005; Xu et al., 2007)</td>
</tr>
<tr>
<td>cIAP2</td>
<td>Part of the TNFRI/II intracellular complex that activates NF-kB. Ubiquitinates BCL10, Smac, caspase-3 and -7.</td>
<td>3xBIR, RING, CARD</td>
<td>(Hu et al., 2006; Hu and Yang, 2003; Huang et al., 2000; Rothe et al., 1995)</td>
</tr>
<tr>
<td>Survivin</td>
<td>Required for proper cytokinesis. Potentiates function of XIAP. Suppresses apoptosis through inhibition of caspase-9 and Smac.</td>
<td>1xBIR</td>
<td>(Ambrosini et al., 1997; Dohi et al., 2004; Li et al., 1998; Tamm et al., 1998)</td>
</tr>
<tr>
<td>NAIP</td>
<td>Inhibits caspase-3, -7 and -9 in vitro. Involved in neuronal protection against calcium-, amyloid-beta- and ischemia-induced apoptosis</td>
<td>3xBIR, NACHT</td>
<td>(Lesne et al., 2005; Liston et al., 1996; Mercer et al., 2000; Xu et al., 1997)</td>
</tr>
<tr>
<td>BRUCE</td>
<td>Inhibits caspase-3 and -7 in vitro. Ubiquitinates caspase-9 and Smac/DIABLO. May regulate p53.</td>
<td>1xBIR</td>
<td>(Bartke et al., 2004; Hao et al., 2004; Ren et al., 2005)</td>
</tr>
<tr>
<td>Ts-IAP</td>
<td>Suppresses apoptosis induced by BAX and caspase-9.</td>
<td>1xBIR, RING</td>
<td>(Richter et al., 2001)</td>
</tr>
<tr>
<td>ML-IAP</td>
<td>Inhibits caspase-3 and -9 in vitro. Suppresses apoptosis induced by etoposide and BAX overexpression. Promotes Smac/DIABLO degradation.</td>
<td>1xBIR, RING</td>
<td>(Kasof and Gomes, 2001; Lin et al., 2000; Ma et al., 2006)</td>
</tr>
</tbody>
</table>

**Table 1.** Summary of the Inhibitor of Apoptosis Protein family and their known functions. CARD, Caspase recruitment domain; NACHT, domain present in NAIP/CIITA/HET-E/TP1.
4. X-linked Inhibitor of Apoptosis Protein

X-linked Inhibitor of Apoptosis Protein (XIAP), as well as cIAP-1 and -2, consists of three consecutive N-terminal BIR-domains and a C-terminal RING-domain (Really Interesting New Gene) (Fig. 3). XIAPs anti-apoptotic properties have primarily been attributed to its caspase-inhibiting function (Deveraux et al., 1997). Even though XIAP has three BIR domains, only two seem to be involved in caspase inhibition. BIR2, with the addition of the linker region between BIR1 and BIR2, is able to inhibit active caspase-3 and -7 (Riedl et al., 2001; Scott et al., 2005; Takahashi et al., 1998), whereas BIR3 binds and inhibits the formation of the active caspase-9 tetramer (Deveraux et al., 1998; Shiozaki et al., 2003). The BIR1-domain is mainly involved in XIAP homodimerization and binding to TAB1 (Lin et al., 2007; Lu et al., 2007). XIAP inhibits caspases with a binding affinity in the nanomolar range, making it the most efficient caspase-inhibitor of the IAPs. However, it is also important for the cell to be able to regulate XIAP, both due to XIAPs potentially tumorigenic properties and for the need to tightly regulate the apoptotic process. This is primarily done by the inhibitory IAP-binding proteins.

Figure 3. This figure shows the structural architecture of XIAP and the regions involved in binding XIAP-interacting protein.

4.1 XIAP-inhibitors

Four proteins have been identified as XIAP-inhibitors, namely Smac/DIABLO, Omi/HtrA2, ARTS and XAF1. The first three inhibitors reside in the mitochondria and do not bind XIAP until they are released to the cytoplasm after mitochondrial membrane permeabilisation (Fig. 4) (Du et al., 2000; Gottfried et al., 2004; Suzuki et al., 2001a). Smac/DIABLO and Omi/HtrA2 bind BIR3 of XIAP in the same way as processed caspase-9, i.e. through their N-terminus consisting of an IAP-binding motif (IBM) (Srinivasala et al., 2001; Suzuki et al., 2001a). This motif consists of the tetrapeptide AVPI (Smac/DIABLO) or AVPS (Omi/HtrA2) and competes
for binding with caspase-9 (AVPY), which leads to an increase in free and active caspase-9. In addition to blocking caspase-9 binding, Smac and Omi also bind the BIR2-domain and prevent caspase-3 and -7 inhibition, though the exact mechanism behind this interaction is not fully known (Huang et al., 2003). Omi/HtrA2 can also cleave XIAP into smaller fragments because of its serine protease activity, thereby further decreasing XIAPs anti-apoptotic abilities (Srinivasula et al., 2003). ARTS and XAF1 are believed to bind XIAP and trigger the translocation of XIAP to the nucleus (Fig. 4) (Gottfried et al., 2004; Liston et al., 2001). How this affects the function of XIAP is not very clear, but in the case of XAF1 it decreases the anti-apoptotic ability of XIAP. In addition to translocation, an increased expression of ARTS decreased the amount of XIAP suggesting an effect of ARTS on XIAP stability.

4.2 XIAP as an ubiquitin ligase
XIAP has also the ability to target bound proteins for degradation through poly-ubiquitination. To make a protein poly-ubiquitinated, three kinds of enzymes are needed; an ubiquitin-activating enzyme (termed E1), an ubiquitin conjugating enzyme (E2) and finally a substrate recognizing ubiquitin ligating enzyme (E3) (Ciechanover, 2005). The C-terminal RING-domain of XIAP functions as an E3-ligase, and XIAP has been shown to ubiquitinate a number of proteins, including itself (Yang et al., 2000), caspase-3 (Suzuki et al., 2001b), caspase-9 (Morizane et al., 2005), and Smac/DIABLO (MacFarlane et al., 2002). These proteins are therefore thought to not only get inhibited, in the case of the caspases, but also get targeted for destruction. The importance of this for the anti-apoptotic function of XIAP has been studied, but due to conflicting results this issue therefore needs further investigation. In a recent paper by Wilkinson et al., XIAP was shown to bind and ubiquitinate AIF during apoptotic conditions (Wilkinson et al., 2008). However, this did not lead to proteasomal degradation. The exact function of ubiquitinated AIF was not found, but the authors suggested a pro-survival function of AIF since expression of both AIF and XIAP lead to reduced levels of reactive oxygen production.

The finding by Yang et al. (2000) that XIAP could ubiquitinate itself raised a number of questions such as if it happens continuously or if some signal can trigger auto-ubiquitination. In a number of studies, the level of XIAP decreased in response to different apoptosis inducers, such as cisplatin (Dan et al., 2004), etoposide and glucocorticoids (Yang et al., 2000), and by inhibiting the proteasome the decrease in XIAP was ablated. This indicates a requirement for a triggering signal or protein interaction for the auto-ubiquitination to be effective in decreasing the amount of cellular XIAP. Two studies have touched upon the possible mechanisms behind the regula-
tion of self-ubiquitination. Dan et al. (2004) showed that the anti-apoptotic kinase Akt/PKB could phosphorylate XIAP and thereby making it more resistant to ubiquitination, whereas Fu and co-workers (Fu et al., 2003) identified a splice-variant of Smac, named Smac3, which increased auto-ubiquitination of XIAP when binding to it during apoptosis. As with XIAP-mediated ubiquitination of associated protein, it is not known how important auto-ubiquitination is for the regulation or function of XIAP since most systems do not show an increased degradation of XIAP in response to apoptosis.

Figure 4. A schematic picture of XIAP and its interacting partners during mitochondria-mediated apoptosis. However, it is not known if all interactions take place at the same time or even in the same system. pUb, poly-ubiquitination.

4.3 XIAP in cell signalling

In 1999, a paper published by Yamaguchi and co-workers showed that XIAP was involved in bone morphogenetic protein (BMP)-signalling in *Xenopus*
embryonic development (Yamaguchi et al., 1999). XIAP bound activated BMP-receptor Ia which lead to the recruitment and activation of TGF-beta associated kinase-1 (TAK1) and its binding-partner TAK1-binding protein-1 (TAB1). Further studies by several groups revealed that XIAP could mediate BMP- and TGFβ1-signalling by recruiting TAB1/TAK1 to the intracellular part of the receptor (Birkey Reffey et al., 2001), which subsequently activated both NF-kB and JNK1 (Hofer-Warbinek et al., 2000; Sanna et al., 1998). Even though the roles of JNK1 and NF-kappaB in apoptosis are unclear by showing both pro- and anti-apoptotic properties in different systems, additional studies showed that XIAP could protect embryonic kidney cells and MCF7-cells against Fas, TNF-α and caspase-1-induced apoptosis (Sanna et al., 2002a; Sanna et al., 2002b). JNK1 and TAK1 were necessary for this inhibitory function of XIAP, indicating that XIAP can inhibit apoptosis in two different ways. However, it is still not clear how important XIAP-mediated JNK1/NF-kB-activation is for its anti-apoptotic function, or how important XIAP is for BMP/TGF-β-signalling, due to the lack of studies concerning these issues.

5. Physiological importance of XIAP

5.1 Knock-out studies

Even though a lot of evidence is suggesting XIAP to be a potent and important protector of cells, especially when overexpressed, it is still debated what role XIAP plays in the living organism. One of the first studies trying to highlight this question was made by Harlin and colleagues in 2001 by knocking out the Xiap gene in mouse (Harlin et al., 2001). Unfortunately, no phenotype was detected in the Xiap -/- mice and the only difference found between wild-type and Xiap -/- mice were an increased expression of cIAP-1 and cIAP-2. This was thought to be a compensatory mechanism and part of the reason why no phenotype was found. Recently it was reported that the RING-domains of cIAPs are able to target XIAP for degradation by ubiquitination (Silke et al., 2005), and a similar effect of XIAP-RING on cIAPs would then explain why the amount of cIAPs is increased in XIAP-deficient mice. Further studies on XIAP-deficient mice have actually led to the discovery of some functional and structural phenotypes. The first phenotype of XIAP-deficient mice was found in 2004 by Burstein and colleagues and surprisingly it was unrelated to apoptosis (Burstein et al., 2004). By doing a yeast two-hybrid screen they identified Murr1 as an interactor to XIAP. Murr1-expression leads to export of copper from the cell by an unknown mechanism, and XIAP was found to decrease Murr1 levels by poly-
ubiquitination and proteasomal degradation. Therefore, a loss of XIAP resulted in high levels of Murr1 and decreased levels of intracellular copper, which was also seen in the liver of XiapΔ/Δ-mice. However, the physiological consequence of decreased levels of intracellular copper caused by lack of XIAP has not been further studied. A year later, it was published that XIAP is developmentally regulated in mammary glands during mouse mammmopoi-
esis (Olayioye et al., 2005). Differentiation of the lobuloalveolar ducts in the mammary glands of XiapΔ/Δ-mice were reduced, which lead to lower density of alveoli and less dilated lumens. No change in proliferation or apoptosis in mammary gland cells was detected. It was found that XiapΔ/Δ-cells had elevated ERK-activity and delayed NF-kappaB activation, which was believed to be a consequence of XIAPs role in TGF-β and BMP-signalling.

The purpose of the previous studies was to understand the physiological function and role of XIAP in mouse, with the intent of confirming interesting findings in human systems. Even though animal models are needed, the difference between rodents and humans are most often too big to draw any direct conclusions about physiological processes. Interestingly, Rigaud and colleagues came across a group of patient with an inherited disease diagnosed as X-linked lymphoproliferative syndrome (XLP) (Rigaud et al., 2006). These patients had low numbers of Natural Killer T-lymphocytes (NKT-cells), which lead to lymphohistiocytosis, hypogammaglobulinaemia and lymphoma caused by the Epstein-Barr virus (NKT-cells are hypothe-
sized to be important in the innate defence against Epstein-Barr virus). By performing a genotype analysis, it was found that all affected individuals either had a premature stop-codon due to a mutation or deletions in BIRC4, the gene coding for XIAP, resulting in truncated versions of XIAP contain-
ing only BIR1. Lymphocytes from the affected patients were more sensitive to Fas Ligand (FasL)- and TNF-related apoptosis-inducing ligand (TRAIL)-
induced cell death. Reconstitution of functional XIAP in the lymphocytes reversed the susceptibility to FasL and TRAIL, indicating that XIAP was important in protecting the cells against receptor-mediated apoptosis. It was therefore concluded that the loss of NKT-cells were due to those cells being the most sensitive of the lymphocytes. Mouse deficient in XIAP did not have a reduced number of NKT-cells, highlighting the actual differences between mouse models and humans, and the difficulties in using data from animal models for clinical trials.

5.2 XIAP in differentiated cells

A number of studies made by Deshmukh and colleagues have focused on apoptosis in neurotrophic growth factor (NGF)-dependent sympathetic neu-
rons. These neurons, when maintained with NGF, are resistant to microinjec-
tion of cytochrome c, which is a potent apoptosis-inducer in most cells
(Deshmukh and Johnson, 1998). This resistance is reported to be caused by XIAP, since both Smac/DIABLO-injected and XIAP-deficient sympathetic neurons undergo rapid apoptosis when injected with cytochrome c (Deshmukh et al., 2002; Potts et al., 2003). Also, when NGF is withdrawn the neurons die by mitochondrial release of cytochrome c and decreased XIAP-levels. So why is XIAP potent in this system and not in other? One regulatory mechanism seems to be involving Apaf-1 of the apoptosome. During differentiation of PC12-cells (a pheochromocytoma cell line), sympathetic neurons and cardiomyocytes, the amount of Apaf-1 decreases thereby limiting the activation-rate of caspase-9 during induction of apoptosis (Potts et al., 2005; Wright et al., 2004). As a consequence, the ratio of active caspase-9 and XIAP becomes more in the favour of XIAP, thus making these post-mitotic cells more resistant towards cytochrome c-induced apoptosis.

6. Using XIAP as a therapeutic tool

6.1 XIAP in tumors

Since apoptosis is important in the innate defence against tumor formation, it would be beneficial for the tumor to increase its expression of IAPs, thereby blocking apoptosis. A number of studies have been made to investigate this possibility and the IAPs correlating best with tumor survival and chemoresistance were survivin and XIAP. For example, increased level of survivin mRNA has been reported to be a prognostic marker for poor outcome in gastric (Miyachi et al., 2003), urothelial (Schultz et al., 2003) and hepatocellular carcinoma (Schultz et al., 2003). Regarding XIAP, studies made on a number of different tumor types, including acute myeloid leukaemia and clear-cell renal carcinoma, showed that an increased level of XIAP correlated with decreased patient survival (Ramp et al., 2004; Tamm et al., 2004a; Tamm et al., 2004b). Interestingly, increased expression of XIAP has also been seen to be a good prognostic factor in radically resected non-small-cell lung carcinoma indicating a somewhat more complex relationship between the expression level of XIAP and tumor progression (Ferreira et al., 2001). A more direct approach to assess the importance of XIAP in tumor survival has been to decrease the level of XIAP. Decreasing XIAP, by either short inhibitory RNA (siRNA) or antisense expression, has shown to sensitize a variety of human tumor types, including breast cancer (Zhang et al., 2005), hepatocellular carcinoma (Yamaguchi et al., 2005), glioma (Hatano et al., 2004), melanoma (Chawla-Sarkar et al., 2004) and ovarian carcinoma cells (Sasaki et al., 2000), to cytotoxic drugs. Although most studies have been done using cell lines, studies made by Cao et al. (2004) and Hu et al. (2003) showed a
sensitizing role of antisense XIAP to chemotherapeutics in xenografts. A second-generation mixed-backbone antisense oligonucleotide targeting XIAP, called AEG35156/GEM®640, is currently in clinical trials (LaCasse et al., 2006).

6.2 XIAP in neuroprotection
XIAP has also been used as a protective agent in a number of neurological damage models. By introducing increased expression, either by adenoviral infection or fusing XIAP to protein transduction domains, XIAP has conferred protection against cerebral ischemia (Guegan et al., 2005; Xu et al., 1999), hippocampal ischemia (Xu et al., 1999), retinal ischemia (Renwick et al., 2006) and ganglion cell axotomy (Kugler et al., 2000). Transgenic mice overexpressing XIAP in neurons are also more resistant to brain ischemia (Trapp et al., 2003; Wang et al., 2004) and neurodegenerative diseases, including amyotrophic lateral sclerosis (Inoue et al., 2003; Wootz et al., 2006) and the MPTP-model of Parkinson’s disease (Crocker et al., 2003).

Despite the current difficulties in inducing or increasing expression of specific proteins as a part of therapeutic treatment, XIAP is still a promising target for the treatment of certain tumor-types and other proliferative disorders. If gene-therapy will become a useful therapeutic approach in the future, XIAP will most probably be an interesting candidate in the treatment of neurodegenerative diseases. In the meantime, it is important to continue the study of XIAP in apoptosis and other cellular processes.

7. Novel interactors of XIAP
G-protein pathway suppressor 2 (GPS2) and Nuclear localized protein-1 (Nulp1) are two proteins this thesis is focusing on together with XIAP. In Paper I and II we show that XIAP is able to bind both GPS2 and Nulp1 in mammalian cells, and in Paper III and IV we study the function, expression and regulation of Nulp1. GPS2 and Nulp1 are both involved in transcriptional regulation, although in somewhat different ways.

7.1 GPS2
GPS2 is a protein originally identified by its ability to inhibit a lethal G-protein-mediated pheromone signal pathway in yeast (Spain et al., 1996). Since the pheromone signal is dependent on kinases similar to the mammalian mitogen-activated protein kinases (MAPK), the effect of GPS2 on these pathways in mammalian cells was subsequently studied. Indeed, GPS2 inhibited JNK1 activity in mouse 3T3-fibroblasts. Further studies showed that
this inhibitory function was the result of increased histone deacetylase (HDAC)-dependent repression due to GPS2 being an integral subunit of the Nuclear Co-repressor complex (N-CoR) (Zhang et al., 2002). Histone acetylation and deacetylation are post-translational modifications that alters the structure of chromatin, where acetylation opens up the structure for the RNA-polymerase complex and deacetylation decreases the accessibility (Shahbazian and Grunstein, 2007). Although the exact function of GPS2 in the N-CoR-complex is not known, it does seem to be important for the interaction between the DNA-binding transcription factor and the rest of the N-CoR complex. However, GPS2 can also bind the histone acetylase-transferase p300 instead of N-CoR and thereby facilitate transcriptional activation (Peng et al., 2000), but the mechanism behind the change in binding preference is still not known. Transcription factors that GPS2 helps bind p300 include p53 (Peng et al., 2001), RFX4_v3, HNF4α and the Farnesoid X Receptor (FXR) (Sanyal et al., 2007), whereas GPS2 recruits N-CoR to LRH-1 and FXR (Sanyal et al., 2007). Interestingly, GPS2 facilitates both transcriptional activation and repression for FXR depending on which promoter FXR binds to. In addition, GPS2 helps activate reporter genes containing serum response elements (SRE) and tissue plasminogen activation response elements (Degenhardt and Silverstein, 2001).

7.2 Nulp1

Nulp1, also referred to as Transcription factor 25 (Tcf25) or KIAA1049, is a protein that was first studied by Olsson and colleagues because of its high expression during mouse embryonic kidney development (Olsson et al., 2002). Analysis of the peptide sequence suggested that Nulp1 had, from the N-terminal to the C-terminal, an acidic transactivation domain, a basic helix-loop-helix (bHLH) domain, a nuclear localization signal (NLS) and a Domain of Unknown Function-654 (DUF654). Transcription factors of the bHLH-family all share the bHLH-domain (Murre et al., 1989a). The basic region of this domain is responsible for DNA-binding, whereas the helix-loop-helix part is required for homo- or heterodimerization with other bHLH proteins (Fairman et al., 1993; Murre et al., 1989b). In addition, bHLH proteins have different C-terminal domains, such as PAS- (Per-Arnt-Sim), Orange- and leucine zipper-domains, that determines what additional regulators are recruited to the bound promoter-site (Jones, 2004). Nulp1 mRNA was seen to be ubiquitously expressed in mouse embryo but the expression declined post-natally in most tissues except brain and lungs. Although no function was found at that time, Cai et al. later investigated its role during heart development and found that human Nulp1 (hNulp1) had transcriptional repressive properties, especially towards serum response factor-mediated transcription (Cai et al., 2006). The repressive action of Nulp1 was partially
inhibited by the HDAC inhibitor Trichostatin A, suggesting that Nulp1 could recruit HDACs to the SRE of the luciferase reporter plasmid.

A number of studies using cDNA arrays have picked up Nulp1 mRNA to be regulated in their experimental systems. The most interesting findings include an increase of Nulp1 mRNA in the midbrain of adult mice in response to acute ethanol treatment (Rulten et al., 2006), an increase in the heart of mice treated with melatonin (Anisimov et al., 2002), a decrease in Nulp1 mRNA during the differentiation of hematopoietic progenitor cells in vitro (Choong et al., 2004), and a decrease in T47D-cells in response to estrogen treatment (Buterin et al., 2006). Two studies have also touched upon Nulp1 in tumors. Alsøe and colleagues identified Nulp1 as a potential tumor marker for prostate cancer, due to the identification of Nulp1-antibodies in the serum of a prostate cancer patient (Alsoe et al., 2008). Also, Su et al. detected high levels of Nulp1 mRNA in prostate, kidney, pancreas and lung squamous tumors compared to other forms examined (Su et al., 2001). This is not necessarily a consequence of up-regulation of Nulp1 in tumors, but could instead be due to inherently high expression in those tissues.

Apart from these studies, nothing is known about the function of Nulp1 or its role in embryonic development.
Aims

General aim:
To further study the cellular role of XIAP by finding new interaction partners by the yeast two-hybrid method. This was done to more precisely predict possible cellular and physiological consequences of using XIAP as a pharmacological target in the treatment of proliferative disorders and neurodegenerative diseases.

Specific aims:

Paper I:
To study the interaction between XIAP and GPS2, and its functional implications during apoptosis.

Paper II:
To study the interaction between XIAP and Nuclear localized protein-1 (Nulp1), the functional implications of this interaction and possible novel effects of Nulp1 on apoptosis.

Paper III:
To further study the function and regulation of Nulp1 by characterizing three splice forms of the Nulp1/Tcf25-gene.

Paper IV:
To study the expression of Nulp1 in developing mouse brain and in differentiating neuronal progenitor cells.
Materials and methods

Yeast-2-hybrid screen
cDNA coding for BIR 1-3 of human XIAP, to minimizing the risk of finding binding partners only involved in the ubiquitination-process, was cloned into expression vector pGBK7 and further transformed into yeast strain Y190. This strain was mated with yeast strain AH109, containing a human foetal cDNA library inserted into pGADT7, and cultured on Trp-/His-/Leu-/Met-synthetic dropout-plates containing aminotriazole and X-a-gal. Library cDNA from pGADT7 of growing blue colonies where then isolated and sequenced. Further control experiments were performed to confirm true interactors of XIAP BIR1-3.

β-galactosidase assay
To determine which part of XIAP binds GPS2, we employed a quantitative liquid β-galactosidase assay using deletion constructs of XIAP in the pGBK7 vector and GPS2 in the pACT2 vector. Yeast lysates were prepared and the reaction was carried out at 30°C in the presence of 4 mg/ml o-nitrophenyl-β-D-galactoside. The absorbance was measured at 420 nm and correlated to the strength of binding between GPS2 and each XIAP construct.

Cell culture
Mouse N2a neuroblastoma cells, human Saos2 osteosarcoma cells, human HeLa cervical carcinoma cells and rat C6 glioma cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-Glutamine and 10% foetal calf serum. The cells were kept at 37°C in a humidified atmosphere containing 5% CO₂ and were subcultured three times a week. The cells were seeded 24h before transfection with Transfectin (Bio-Rad), which was done according to manufacturer’s protocol.

To obtain neuronal progenitor cells, striatum from E17 Wistar rats were dissociated using a Hank's buffered saline solution containing 0.54% glu-
cose, 0.13% trypsin, 0.02% kynurenic acid, 0.07% hyaluronidase and 40 μg/ml Dnase. The enzymes were inactivated by Earle’s balanced salt solution supplemented with 20 mM HEPES and 4% BSA. The cells were further purified, and incubated at 37°C in 5% CO2 atmosphere in culture flasks. The medium consisted of 15 mM HEPES (pH 7.5), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, B27 supplement in DMEM-F12 and 20 ng/ml epithelial growth factor (EGF). To induce cell differentiation, the NPCs were plated onto culture dishes coated with 50 μg/ml poly-DL-ornithine and cultured for five days.

Hippocampal neurons were prepared from E17 old rats and cultured for 7 days on poly-DL-ornithine-coated dishes in Neurobasal medium with B27 supplement.

Cloning and reverse transcriptase PCR
cDNA from N2a, Saos2, HeLa and C6 cells was synthesized using Thermo Script RT PCR System (Invitrogen). PCR was performed using primers targeting conserved regions in mouse, rat and human Nulp1. Human, mouse and rat β-actin were used as controls. Nulp1 isoform C and D was cloned by PCR from the N2a cDNA library by using primers complementary to the start and end of the Nulp1-C coding sequence based on GenBank entry NM_001037878. Sequences of all primers are shown in Table 2.

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Table 2. Primers used to detect expression of Nulp1 in different cell lines (Nulp1, human/mouse/rat β-actin) and to clone Nulp1-C/D from N2a-cells.

GST-preparation and GST pull-down
Human XIAP and GPS2 was cloned into pGEX4T3 and transformed into E. Coli-strain BL21. Positive clones were screened for high expression of GST, GST-GPS2 and GST-XIAP. GST-, GST-GPS2- and GST-XIAP-expression were induced by 1 mM isopropyl-β-D-thiogalactopyranoside, and the cells were lysed in PBS + 1 mg/ml lysozyme followed by freeze-thawing and sonication. The GST-proteins were purified from the bacterial lysate by adding glutathione-conjugated sepharose beads, which were then subsequently washed to remove unspecifically bound proteins. GST and GST-XIAP were
then added to N2a-lysate to pull down interacting proteins. The beads were washed and bound proteins were analyzed by immunoblotting.

Immunoprecipitation

N2a-cells or HeLa-cells were lysed with lysis buffer (see Paper I, II and III for more details) supplemented with 1 mM sodium orthovanadate, 10 mM NaF and 1x protease inhibitor cocktail. Protein concentration was measured with Bio-Rad DC Protein Assay and equal amounts were transferred to each sample. The samples were pre-cleared with Protein A (Invitrogen) or G Sepharose beads (GE Healthcare) for 20 minutes, followed by incubation with antibodies for three hours at 4°C. Protein A or G Sepharose beads were added to each sample and incubated overnight at 4°C. The beads were washed three times with lysis buffer and incubated overnight at 4°C. The beads were washed three times with lysis buffer and analyzed by immunoblotting.

Immunoblotting

Samples were boiled for 5 minutes in SDS-sample buffer before being applied to and separated on a SDS-page gel. Afterwards, the proteins were transferred to a nitrocellulose membrane by electrophoretic blotting. The membranes were blocked with TBS-T blocking buffer (TBS + 0.5% Tween-20 + 5% milk) for 1h. Primary antibodies, diluted in TBS blocking buffer, were added to the membrane and incubated overnight at 4°C. Antibodies used are summarized in Table 3. After washing with TBS-T, horse-radish-conjugated secondary antibodies in TBS-T blocking buffer were added to the membrane and incubated for 1h in room temperature (RT). The antibody staining was visualised with Super Signal West Pico solution (Pierce).

Immunocytochemistry

Cells were seeded on poly-L-lysine-coated coverslips and cultured overnight before treatment was carried out. The cells were fixed in 4% paraformaldehyde (PFA), permeabilised with PBS + 0.1% Triton-X and blocked with 4% goat serum for 1h at RT. Primary antibodies, diluted in 4% goat serum, was then added and incubated overnight at 4°C. Antibodies used for immunocytochemistry are summarized in Table 3. After washing with PBS + 0.1% Triton-X, Cy2 or Cy3-conjugated secondary antibodies (1:1000 in PBS + 4% goat serum) were added for 1h at RT. The cells were further washed, and the cell-nuclei were stained with 200 ng/ml DAPI before being mounted on glass slides using Gel Mount (Sigma-Aldrich). The cells were analyzed using a Zeiss LSM confocal microscope.
Immunohistochemistry
C57BL/6-mice of different ages were anesthetized using Avertin and perfused with 4% PFA. Brain samples were dissected, dehydrated, embedded in paraffin, cut in 5 μm thick sections and deparaffinized. The sections were further dehydrated in ethanol and boiled in citrate buffer for 10min. 4% goat serum was used to block unspecific binding. Antibodies and concentrations used are summarized in Table 3. Secondary Cy2- or Cy3-cinjugated antibodies were used to visualize the immunoreactivity of the primary antibody. The sections were mounted on glass slides using Kaiser’s glycerol-gelatin mounting medium and analyzed using a Zeiss LSM confocal microscope.

MTT-assay
N2a-cells were transfected for 48h before MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well at a concentration of 500 μg/ml for 2h at 37°C. The medium was removed and the formazan-crystals were dissolved in isopropanol/HCl (500:1). The absorbance, correlating to the amount of viable cells in each well, was measured at 570 nm and the background at 690 nm.

Flow cytometry
Measuring DNA-content of GFP-expressing cells by flow cytometry was based on the methodological study made by Chu et al. (Chu et al., 1999). The cells were washed once in PBS before being fixed in 1% PFA + 0.1% NaN₃ for 1h at 4°C. The cells were then washed twice in PBS and further permeabilised in 70% ethanol / 30% PBS overnight at 4°C. Afterwards the cells were washed once in PBS and resuspended in PBS supplemented with 40 μg/ml propidium iodide and 100 μg/ml RNase A. The samples were incubated for 30min at 37°C and kept on ice until measuring on a FACScalibur flow cytometer (Becton Dickinson). The data was analysed using CELLQUEST® software (Becton Dickinson).

Caspase assays
To measure caspase-3-like activity, BioMol’s Caspase-3 cellular activity assay kit PLUS was used. In short, the caspase-3 substrate Ac-DEVD-pNa was added to cell lysate and further incubated in 37°C. The increase in absorbance at 405 nm, which correlates to the caspase-3-like activity in the sample, was measured spectrophotometrically every 15 minute for 3 hours. Worth noting is that caspase-7 is also able to cleave the substrate, which is why we use the term ‘caspase-3-like activity’ (Talanian et al., 1997).
The cell-free caspase-3 assay was done with the same reagents as above, with the addition of recombinant active caspase-3 (Biomol) and affinity-purified GST, GST-XIAP and GST-GPS2. Detection and analysis of cleaved Ac-DEVD-pNa were done as above.

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<td>Ubiquitin</td>
<td>ms</td>
<td>1:4000</td>
<td></td>
<td></td>
<td>Biomol</td>
</tr>
<tr>
<td>XIAP</td>
<td>ms</td>
<td>1:5000</td>
<td>1:200</td>
<td></td>
<td>BD Transd. Labs</td>
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<tr>
<td>XIAP</td>
<td>rb</td>
<td></td>
<td></td>
<td>1:500</td>
<td>C.m. by Biogenes</td>
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**Table 3.** Antibodies used in Paper I-IV. rb, rabbit; ms, mouse; gt, goat; IB, Immunoblotting; IC, Immunocytochemistry; IH, Immunohistochemistry. C.m., Custom made.
Results and Discussion

Paper I

To further understand the physiological importance and function of XIAP, we performed a yeast two-hybrid screen with XIAP BIR1-3 as bait to identify new binding partners of XIAP. We identified G-protein pathway suppressor 2, GPS2, as an interactor of XIAP. To determine which part of XIAP GPS2 binds to, we performed a β-galactosidase-assay using different parts of XIAP. Both BIR1-3 and BIR1-2 bound GPS2 but not the BIR3-RING domains of XIAP, indicating interaction between some part of BIR1-2 and GPS2.

To confirm the interaction in a mammalian system, we performed a GST-pulldown assay using GST or GST-XIAP as bait. The mouse neuroblastoma cell line N2a was used to express GFP or GPS2-GFP, with the result of GST-XIAP pulling down GPS2-GFP but not GFP. Also, we performed co-immunoprecipitation using GFP or GPS2-GFP-transfected HeLa-cells and antibodies against GFP, with the result that endogenous XIAP was detected as GPS2-GFP was precipitated. Finally, we used antibodies against endogenous XIAP, with the result of GPS2-GFP being co-immunoprecipitated. These results confirm that XIAP and GPS2 are able to bind each other in mammalian cells.

Even though interaction may occur in lysate, the two proteins have to be localized to the same cellular compartment for the interaction to take place. Therefore we studied the whereabouts of XIAP and GPS2 by expressing GFP-XIAP and GPS2-Red fusion proteins in HeLa cells. GFP-XIAP and GPS2-Red were co-localized in aggregates in the cytosol, indicating physiological relevant interaction or abnormal aggregation of the two exogenous proteins. To exclude interaction due to effects of overexpression, we studied endogenous GPS2 and XIAP by immunocytochemistry. Both proteins were present in the cytosol of N2a-cells and were overlapping indicating possible interaction. Due to the punctuate distribution of GPS2, we stained GPS2-GFP-transfected N2a-cells with mitotracker, a mitochondria-specific dye, but no overlap of GPS2-GFP with mitochondria was seen. GPS2 was also detected as a 40kDa band in N2a- and Saos2-cells by immunoblotting. An additional band at 50kDa was detected in N2a-cells and may represent a modified GPS2 that remains to be studied in more detail.
Previously, GPS2 has primarily been seen in the nucleus of cells (Breiding et al., 1997), because of its function in transcriptional regulation, but the observation of cytosolic GPS2 by us and other groups suggests that it also plays a role outside of the nucleus (Degenhardt and Silverstein, 2001; Zhang et al., 2008).

Since XIAPs primary function is the inhibition of apoptosis, we expressed GPS2-GFP in N2a-cells to study if GPS2 had any effect on apoptosis. The fraction of cells with condensed or fragmented nuclei was 24% in GPS2-GFP-transfected cells compared to 3% in control cells, and with the addition of 20 nM staurosporine (STS) for 16 hours the fraction was approximately 53% and 10%, respectively. In addition, immunoblotting analysis showed that transfection of N2a-cells with GPS2-GFP induced caspase-3 activation as well as cleavage of the caspase substrate PARP. This clearly indicates that an increased expression of GPS2 triggers apoptosis in N2a-cells and increases the susceptibility to apoptosis induced by STS. By co-expressing XIAP-c-myc, GPS2-GFP-induced cell death was abolished and GPS2-GFP+staurosporine-induced cell death was decreased although not to the levels of GFP-transfected cells. This suggests that GPS2 acts at the same level or upstream of XIAP, and that an increased level of XIAP can block the effect of GPS2.

As previously mentioned, GPS2 augments p53-mediated transcription in U2OS osteosarcoma cells (Peng et al., 2001). In GPS2-overexpressing U2OS-cells, treatment with etoposide, a toposiomerase II-inhibitor triggering DNA-cleavage, delayed apoptosis compared to control cells. However, GPS2 sensitized the cells to apoptosis induced by UV-radiation. These two conflicting results might seem contradictory at first, but considering that p53 can induce both cell cycle arrest and apoptosis, the results were most likely a consequence of GPS2-mediated p53-activity.

To determine if the observed increase in apoptotic cells where a consequence of p53 activity, we used the p53-defect osteosarcoma cell line Saos-2 (Masuda et al., 1987). When expressing GPS2-GFP in Saos-2-cells, about 28% of the cells had fragmented DNA after 48 hours of transfection compared to 4.5% in the control. This indicates that p53 is not essential for GPS2-induced cell death in Saos-2 cells. Treating the GPS2-GFP-expressing cells with 20 nM STS for 24 hours further increased the amount of apoptotic cells to 45%, indicating a synergistic effect of GPS2 and STS, since STS-treated GFP-expressing cells only had 8% cells with fragmented DNA.

Caspase-3 and -7 are two important proteases during the execution of apoptosis and are inhibited by the BIR2-domain, together with the linker between BIR1 and 2, of XIAP. Therefore, we measured the amount of active caspase-3 and -7 in N2a-cells expressing GPS2-GFP. GPS2-GFP increased the amount of cleaved caspase-substrate compared to GFP-expressing cells. This effect was inhibited by co-expression of myc-XIAP but not when cells
were treated with both GPS2-GFP and 20 nM staurosporine. Although the implication of GPS2 and XIAP interaction on caspase-3/7 activity was not apparent in this cellular system, an \textit{in vitro} caspase-3 assay showed that GPS2 suppressed the ability of XIAP to inhibit recombinant caspase-3 activity. Increasing the amount of GPS2 in the assay further decreased XIAPs ability to inhibit active caspase-3. These results suggest that GPS2 acts pro-apoptotic by binding BIR1-2 of XIAP and replacing the caspases thereby increasing their activity.

Exactly how GPS2 binds to XIAP remains to be studied, but the lack of an obvious IBM indicates a different way of binding compared to Smac/DIABLO and Omi/HtrA2. The observation that GPS2 inhibits XIAP gives us an explanation why GPS2 sensitizes both N2a-cells and Saos-2 cells to STS-induced apoptosis. However, GPS2 also induced caspase-3 cleavage in N2a-cells by itself, which should not happen if GPS2 only inhibits XIAP and there is no active caspase-3 present in the cells. The observed activation of caspase-3 could either be the consequence of an effect of GPS2 upstream of XIAP, such as mediating p53-transactivation, or the presence of very low amounts of XIAP-blocked caspase-3 that would trigger the caspase cascade when released. This is not unlikely considering we are studying tumor cells that may have a basal level of active caspases. There is also the possibility that GPS2 affects apoptosis in N2a-cells through the N-CoR-complex and/or JNK-inhibition. These possibilities are needed to be studied in more detail to determine how important XIAP-inhibition is for the pro-apoptotic function of GPS2.

Finally, we studied the expression of GPS2 in N2a-cells during STS-induced apoptosis and in human colon carcinoma. The level of GPS2 was decreased as N2a-cells were treated with increasing amount of STS for 24 hours. This decrease in GPS2 was counteracted by co-treatment with either Boc-D-FMK, a pan-caspase inhibitor, or DEVD-fmk, a caspase-3 and -7 inhibitor. These results suggest that GPS2 are negatively regulated by caspases during STS-induced apoptosis. GPS2-levels, as well as XIAP-levels, were seen to be higher in human colon carcinoma tissue as compared to the surrounding tissue. This suggests a regulatory mechanism in tumor cells of matching the expression of XIAP with that of GPS2, to shift the balance towards tumor survival.

The observation that increased expression of GPS2 induced caspase-mediated, p53-independent apoptosis does raise some question of the function of GPS2. When considering all published data about GPS2, it is clear that there are two sides to the story. First, as an integral subunit of the N-CoR complex, GPS2 is involved in HDAC-dependent inhibition of the JNK-mediated stress pathway. This was most likely the reason why GPS2 was able to inhibit cell arrest and ensuing apoptosis in yeast with a constitutive active pheromone-response pathway (Spain et al., 1996). Second, GPS2 may
act as a transcriptional activator by binding p53 or the bovine papilloma virus E2-protein, with subsequent recruitment of the histone acetylase p300 (at least in the case of E2). Our results suggest a third function for GPS2, as a cytoplasmic protein that can act pro-apoptotic through binding and blocking XIAP-mediated caspase-inhibition. It remains to be studied in what context this interaction has physiological relevance.

Paper II

The second interactor of XIAP we decided to study closer was the putative transcriptional regulator Nuclear localized protein-1 (Nulp1), also identified by using XIAP BIR1-3 as bait in a yeast-2-hybrid screen. Since very little is known about the expression and function of Nulp1, we started to study the presence of Nulp1 in a number of different cell lines. By using reverse transcriptase-PCR, we detected Nulp1 mRNA in human Saos2-cells, human HeLa-cells, mouse N2a-cells and rat C6-cells. Also, by using a novel antibody against amino acid residues 109-122 of Nulp1, we detected endogenous Nulp1 in all four cell lines. This indicates that Nulp1 plays a role in proliferating tumor cells and not only during specific differentiation programmes, which has been seen with other bHLH-proteins such as MyoD, NeuroD and the neurogenins (Kageyama et al., 2005). The presence of a possible NLS in Nulp1 suggested that it was a nuclear protein. By using our antibody, we detected endogenous Nulp1 primarily in the nucleus of N2a and Saos2-cells hence confirming the predicted localization. Nulp was also seen to a lesser extent in the cytoplasm in small dot-like aggregates suggesting a possible cytoplasmic function. Also, by doing nuclear fractionation of N2a-cells, we detected the majority of Nulp1 in the nucleus as well as a small amount in the cytoplasm.

Our finding that Nulp1 is able to bind XIAP in yeast points towards a function of Nulp1 in the regulation of apoptosis. In fact, Olsson and colleagues observed that HEK293-cells did not recover after overexpression of GFP-Nulp1 through electroporation, suggesting involvement of Nulp1 in cell death regulation (Olsson et al., 2002). This observation was not studied in-depth, but incited us to investigate a possible effect of Nulp1 on cell viability. We subcloned mouse Nulp1 isoform b into the GFP-vector and expressed Nulp1 as an GFP-fusion protein in Saos2-cells. After 48 hours of transfection, GFP-Nulp1 increased DNA-fragmentation from 4.5% in control-cells to 37% in GFP-Nulp1-expressing cells. XIAP did not decrease the cell death induced by GFP-Nulp1 neither did treatment with the caspase inhibitor Boc-D-FMK, suggesting that caspases are not required for Nulp1-induced cell death.
However, transfection of N2a-cells did not result in increased cell death but did lead a reduction in the number of cells after 48 hours of transfection as seen with the MTT-assay, which is used to measure the amount of viable cells in a culture. Further analysis was done using KI-67 staining and flow cytometry. KI-67 is a nuclear protein only expressed in proliferating cells, i.e. cells in the G1-, S-, G2- or M-phase but no in the G0-phase (Scholzen and Gerdes, 2000). Ki67-staining and flow cytometry showed that GFP-Nulp1 increased the fraction of cells in the G0 and G0/G1-phase of the cell cycle, respectively. This could be either due to direct effect on the cell cycle machinery, or due to a more indirect stressful effect. Hence, we stimulated GFP- and GFP-Nulp1-expressing N2a-cells with different amounts of staurosporine (STS) for 24 hours and measured the amount of cells with fragmented DNA. When treating the cells with 100 or 300 nM STS, more GFP-Nulp1-expressing cells had fragmented DNA, indicative of apoptosis. Taken together, our data suggests that Nulp1 shifts the cell towards a pro-death response and thus, depending on cell type, either sensitizes the cell to further pro-apoptotic insults or induces cell death directly. The finding that GFP-Nulp1 induces cell death in Saos2-cells even during caspase-inhibition points towards an effect of Nulp1 on a parallel pathway to caspase-3/7-activation. Both apoptosis-inducing factor (AIF) and endonuclease G are released during mitochondrial permeabilization, with the consequence of translocating to the nucleus and propagating DNA fragmentation (Li et al., 2001; Susin et al., 1999). Omi/HtrA2 (Suzuki et al., 2001a), calpains (Camins et al., 2006) and cathepsins (Chwieralski et al., 2006) also confer proteolytical activity in some cell death systems, making it plausible that Nulp1 could facilitate cell death independently of caspases. However, more studies are needed to rule out caspases in Nulp1-induced cell death.

When transfecting N2a-cells and Saos2-cells, we observed that GFP-Nulp1 was primarily localized to the cytoplasm. The distribution was diffuse but also punctuate in some cells with high expression of GFP-Nulp1. The discrepancy in localization between exogenous GFP-Nulp1 and endogenous Nulp1 was not due to the presence of GFP, since both Flag-Nulp1 and C-terminally bound GFP had the same distribution as GFP-Nulp1. One explanation could be that we are using Nulp1 isoform b (625 amino acids) and that the larger isoform c (676 aa), that has an alternate 52 amino acid C-terminus, might have a different subcellular localization. Cai and colleagues observation that hNulp1 (676 aa) localized to the nucleus supports this possibility (Cai et al., 2006).

Although human Nulp1 and XIAP interacted in yeast, it is still necessary to confirm the interaction in mammalian cells due to differences in the cellular environment. The confirmation of the Nulp1-XIAP interaction in mammalian cells was not as straight forward as in the case of GPS2 and XIAP. GST-pulldown using GST-XIAP and GFP-Nulp1-transfected N2a-cells did
show that Nulp1 and XIAP interacted but the binding between the two proteins were only slightly more pronounced than the unspecific binding between GFP and the GST-XIAP-sepharose beads. Since caspases and the inhibitors of XIAP, i.e. Smac/DIABLO, Omi/HtrA2 and ARTS, only bind XIAP during apoptosis, we stimulated the GFP or GFP-Nulp1-transfected N2a-cells with 50 nM STS for 24 hours before performing co-immunoprecipitation with anti-GFP. As suspected, STS-treatment increased the amount of co-immunoprecipitated XIAP, and interaction between endogenous XIAP and Nulp1 was also seen when precipitating Nulp1 from STS-treated N2a-cells. These results indicate the requirement for an apoptosis-dependent modification of one or both proteins, and/or the need for a third protein to stabilize the interaction. XIAP has previously been shown to be both mono- and poly-ubiquitinated in response to apoptotic stimuli (Lotocki et al., 2003; Yang et al., 2000). However, conjugation of ubiquitin-moieties, which are about 76 amino acids long, to XIAP or Nulp1 would be detected due to its large size. One other possibility is phosphorylation of XIAP by Akt/PKB. XIAP was shown to be phosphorylated by Akt/PKB in A2780S ovary carcinoma cells, with the consequence of decreasing auto-ubiquitination and stabilizing XIAP (Dan et al., 2004). Treating the cells with the apoptosis-inducer cisplatin decreased Akt-activity and increased degradation of XIAP. It is feasible that dephosphorylation of XIAP during apoptosis might trigger interaction with other protein, such as Nulp1. This could also explain why XIAP and Nulp1 interact in yeast but barely in untreated N2a-cells.

Nulp1 and XIAP have to be present in the same subcellular compartment for binding to occur. Thus, we expressed GFP-Nulp1 in N2a-cells and stained with anti-XIAP to detect endogenous XIAP. GFP-Nulp1 was only detected in the cytoplasm of N2a-cells, as reported above, while XIAP was primarily present in the perinuclear area. The appearance of GFP-Nulp1 was mostly diffuse but in some cells GFP-Nulp was present in small dot-like aggregates, which co-localized with XIAP.

Immunoprecipitation of GFP-Nulp1 after treatment with the proteasome inhibitor MG132 revealed that Nulp1 was poly-ubiquitinated. This indicates that Nulp1 is regulated through ubiquitin-mediated proteasomal degradation.

This paper shows that the bHLH-protein Nulp1 can influence cell death regulation in both mouse and human tumor cells. The death-inducing or sensitizing effect of Nulp1 could be of value in designing treatment strategies against resistant tumor types. The presence of endogenous Nulp1 in the cytosol and the exclusive cytoplasmic localization of GFP-Nulp1 indicate that the death-inducing effect of Nulp1 originates from the cytosol, and not through transcriptional regulation in the nucleus. This raises the question if the different known isoforms also have different properties regarding subcel-
cular localization and effect on cell death. Answers to these questions could also lead to the finding of why XIAP and Nulp1 interact.

**Paper III**

In paper II we studied Nulp1 isoform b (Nulp1-B), the 625 amino acid form that was the first Nulp1 isoform to be cloned and characterized (Olsson et al., 2002). The existence of Nulp1 isoform c, a longer 676 amino acid splice variant, might indicate that Nulp1-B has dominant negative properties due to its truncated DUF654-domain, which spans amino acid residues 239-641 of Nulp1-C. Also, as a predicted bHLH protein, it was surprising to see Nulp1-B exclusively located to the cytoplasm of studied cells. For that reason, we decided to clone Nulp1-C and compare the function and subcellular localization of isoform b and c. Interestingly, when cloning Nulp1-C from N2a cDNA we also identified an isoform lacking exon 3 (Nulp1-D). Exon 3 is a 75 base-pair exon that encodes amino acids 119-143 that includes the basic motif as well as the NLS. Since this region is believed to be of importance for both Nulp1’s transcriptional activity and nuclear localization, we included this form in the study.

When expressed as GFP fusion proteins in N2a-cells, the Nulp1 isoforms were all cytoplasmic as previously seen with Nulp1-B in Paper II. However, a number of bHLH proteins are regulated through nucleocytoplasmic shuttling, which requires both an NLS and a nuclear export signal (NES). For example, OLIG2 is a bHLH protein important in oligodendrocyte differentiation, as well as in inhibiting astrocyte development (Fukuda et al., 2004; Zhou et al., 2001b). When neural stem cells are induced to differentiate into astrocytes, OLIG2 is exported from the nucleus thereby abolishing its antagonistic actions on astrocyte differentiation (Setoguchi and Kondo, 2004). To see if Nulp1 possibly contain an NES we used the NES prediction tool NetNES 1.1, resulting in a predicted NES between amino acid residues 383-391 of Nulp1-B/C (corresponding to 358-366 of Nulp1-D). NES-dependent export of proteins is mediated by Crm1, an exportin that binds the NES and is actively transported out of the nucleus along with its cargo (Pemberton and Paschal, 2005). To determine if Nulp1 is regulated by nuclear export, we expressed the different Nulp1 isoforms while inhibiting Crm1 using Leptomycin B (LMB). Nulp1-C and –D, but not Nulp1-B, was detected in the nucleus of N2a-cells in the presence of LMB. These results indicate that the predicted NLS in the basic region of Nulp1 is not required for nuclear translocation. The inability of Nulp1-B to enter the nucleus is also surprising, suggesting that the C-terminal part of Nulp1 is the determinant factor for subcellular localization. This could either be due to the presence of an unidentified NLS in the Nulp1-C-specific C-terminal or that the C-terminal
facilitates interaction with an NLS-containing partner. A number of bHLH proteins, such as NPAS1 and CLOCK, are dependent on their dimerization partner for proper nuclear translocation (Kondratov et al., 2003; Teh et al., 2006). This would, in the case of Nulp1, indicate that the isoforms differed in their binding preferences.

When overexpressed in N2a cells, we noticed that the levels of each Nulp1 isoforms differed considerably. Immunoblot analysis showed that both GFP-Nulp1-C and –D were expressed at a higher concentration in the cells than GFP-Nulp1-B. By inhibiting protein synthesis using cycloheximide, we measured the half-life of each isoform. GFP-Nulp1-B had a half-life of 16h, whereas the half-life of GFP-Nulp1-C and –D was 55h and 76h, respectively. Since we observed that GFP-Nulp1 was polyubiquitinated in Paper II, we compared the extent of ubiquitination of each isoform. As suspected, GFP-Nulp1-B had the highest levels of polyubiquitination, followed by GFP-Nulp1-C and GFP-Nulp1-D.

Ubiquitin-mediated proteasomal degradation is one common way of regulating the activity of bHLH proteins. BHLH factors such as MyoD (Abu Hatoum et al., 1998), E47 (Van der Put et al., 2004), Stra13 (Ivanova et al., 2001) and ID1 (Trausch-Azar et al., 2004) all have short half-lives due to poly-ubiquitination. Exogenously expressed MyoD has a half-life of ~1h, but co-expression with its binding partners E12 or E47 increased the half-life to ~4h (Lingbeck et al., 2005). A similar effect was seen on the stability of E12 and E47 when MyoD was co-expressed. This suggests that the monomeric form is more susceptible to ubiquitination than the dimerized form and that this mechanism could be a way for the cell to alter the protein levels of both monomers just by regulating one of the bHLH proteins.

The existence of an isoform lacking the DNA-binding basic motif suggests that its dimerization partner is negatively regulated by binding this smaller form. So far, no dimerization partner to Nulp1 has been identified. However, a number of bHLH factors also function through homodimerization, which lead us to investigate if Nulp1-D may homodimerize with Nulp1 and function as a dominant negative isoform. By performing an immunoprecipitation-assay using FLAG-Nulp1-D- and GFP-Nulp1-B/C/D-expressing N2a-cells we detected interaction between FLAG-Nulp1-D and Nulp1-C/D, indicating that Nulp1 is able to homodimerize in vitro and that Nulp1 lacking exon 3 may function as an inhibitor to Nulp1. The finding that Nulp1-B did not bind very well to Nulp1-CΔ3 could indicate that Nulp1-B has a lower affinity to Nulp1-C/CΔ3 thereby making it more accessible to ubiquitin-mediated degradation. However, more studies are needed to determine the tertiary status of Nulp1-B and the importance of dimerization for its regulation and function.

Finally we wanted to study if the observed lethal effect of Nulp1 in Saos2-cells (Paper II) was isoform-specific and transcription-dependent.
Expression of all three Nulp1 isoforms induced cell death compared to cells expressing the empty vector, but no difference between the different isoforms was observed. Similar results was seen in N2a-cells treated with 100 nM STS for 24 hours. These results show that the death-inducing effect of Nulp1 is not isoform b specific or dependent on direct transcriptional regulation by Nulp1.

Our results from this paper points towards a picture of Nulp1 being a protein under strict control by 1) the expression of a dominant negative isoform, 2) ubiquitin-mediated degradation, and 3) nucleocytoplasmic shuttling. A number of issues are still needed to be clarified before determining the importance of each regulatory mechanism in Nulp1 function, such as if and when Nulp1-D is expressed, what triggers nuclear shuttling and retention, and if ubiquitination is a continuous process or regulated by upstream signalling. The connection between Nulp1 and XIAP has not been touched upon here but so far the common denominator between the two proteins is their involvement in the ubiquitin-proteasome system; XIAP as an E3-ligase and Nulp1 as an ubiquitinated protein. In a recently published study by Xu and colleagues it was shown that cIAP1 could enter the nucleus and polyubiquitinate Mad1, a bHLH protein known to inhibit Myc by sequestering its dimerization partner Max (Xu et al., 2007). This was the first time an IAP had been seen to regulate a transcription factor of the bHLH family.

Paper IV

By studying the expression of a protein in an organism, it is possible to get a sense of in which processes the protein is involved in. Since bHLH proteins are often seen to regulate developmental programmes, such as neuronal differentiation, we wanted to investigate when and where Nulp1 was expressed during mouse brain development. Previous studies on Nulp1 have shown that its mRNA is expressed in a number of tissues during mouse embryonic development, including the CNS (Olsson et al., 2002). Since the development and differentiation of the brain is dependent on bHLH proteins, we wanted to look at the expression of Nulp1 in more detail by using a novel antibody specific for Nulp1 (also used in Paper II).

Cortex, hippocampus, striatum and brain stem from embryonic (E17.5), newborn (P0) and adult (3 months old) mice were analysed with immunoblotting, as well as cerebellum from P0 and adult mice. The expression of Nulp1 decreased with age, especially between P0 and adult, in all tissues analysed. In addition, three proteins with different molecular weights were identified with the Nulp1-antibody, which could correspond to any of the four known Nulp1 splice-variants (Nulp1 A-D) or to post-translationally
modified Nulp1. The form with the highest molecular weight was primarily expressed in embryonic brain and decreased rapidly after birth, whilst the two smaller forms had their highest expression in newborn mice. Cerebellum had the highest expression of Nulp1 in adult mice compared to cortex, hippocampus, striatum and brain stem. Only the smallest form of Nulp1 was detected in adult brain.

The presence of different forms of Nulp1 in the brain, regardless if its due to alternative splicing or PTMs, indicates that there is a change or modulation of Nulp1 function with development. Considering the possible existence of a dominant negative form of Nulp1 (e.g. Nulp1-D, Paper III) it is plausible that the function in adult is actually the opposite of embryonic Nulp1. This is worth having in mind when studying the role of Nulp1 in mouse embryonic and post-natal development.

With the apparent high expression in cerebellum, we focused our attention at the pattern of Nulp1 expression in the developing cerebellum. At E19, most cells in the cerebellum had distinct expression of Nulp1. Between P0 and P6, Nulp1 was primarily expressed in the two cerebellar granular layers, the external granular cell layer (EGL) and internal granular cell layer (IGL), and in cells of the deep nuclei. In P10 cerebellum, Nulp1 was still highly expressed in cells of deep nuclei, and had also appeared in Purkinje cells lining the outer part of the IGL. At this stage, most cells of the EGL had already migrated to the IGL, and the expression in the IGL was not as pronounced as in earlier stages. Between P10 and 2.5 months old cerebellum, the only major change in Nulp1-expression was the appearance of Nulp1-positive cells in the molecular layer. To see if Nulp1 was specifically expressed in dividing cerebellar progenitor cells, we co-labelled cerebellum from newborn mice (P0) with anti-Nulp1 and anti-Ki67. Nulp1 was present in the Ki67-positive proliferating cells of the outer EGL but the expression was higher in the cells of the inner EGL that had already exited the cell cycle for later migration into the IGL. Co-staining with the neuronal progenitor cell (NPC) specific marker Nestin also showed that Nulp1 was present in cerebellar progenitor cells. These results show that Nulp1 is expressed in NPCs and is further up-regulated in post-mitotic cerebellar granule neurons.

The increased expression of Nulp1 in post-mitotic and migrating granule neurons suggests a possible role of Nulp1 in differentiation and/or migration. Furthermore, the results also suggest that Nulp1 is expressed in two phases during cerebellar development. The first phase is characterized by high expression in post-mitotic granular cells of the EGL and IGL, whereas the second phase is characterized by expression in Purkinje cells, the deep nuclei and in the molecular layer. It would be interesting to study if the expression or presence of the largest Nulp1 form is specific to the first phase, and if the two smaller forms are restricted to the cells of the second phase.
Expression of Nulp1 in mouse brain was primarily detected in the neocortex of embryonic day 13 (E13) mice, as seen with immunohistochemistry. At E15 the cortex had increased in thickness, making it possible to differentiate the cortical layers more easily. Nulp1 was expressed in the outermost layers, most likely corresponding to the marginal zone, cortical plate and the subplate. Co-staining with anti-Doublecortin, specifically expressed in migrating neuronal cells, showed an overlap specifically in the marginal zone. A few Nulp1-positive cells were dispersed along the ventricular surface of the ventricular zone. At E19, Nulp1 was present at a relatively high level in the ventricular zone of the lateral ventricle, indicating an increase in expression between E15 and E19. Co-staining with anti-Nestin showed that a number of Nulp1-positive cells were also positive for Nestin.

Comparing the expression of Nulp1 with transcription factors involved in cortical development, the closest match in regard to spatial expression are Math2 and Tbr1 (Hevner, 2006). Expression of these two factors starts in post-mitotic neurons as they migrate from the intermediate zone into the cortical plate. Although no direct connection between Nulp1 and Math2/Tbr1 has been seen here, it could be speculated that Nulp1-expression is initiated by the same upstream events that trigger Math2/Tbr1-expression. The primary cells in the ventricular zone are the radial glial cells and the neuroepithelial cells. Both have the ability to function as neuronal progenitor cells and produce glial cells or neurons destined to become projection neurons in the cortical plate. The presence of Nulp1 in Nestin-positive cells at the ventricle surface suggests that it may have an important function in neuronal progenitor cells.

To further study the expression of Nulp1 in proliferating and differentiating neuronal cells, we prepared cultures of neuronal progenitor cells (NPC) from the striatum of E17 Wistar rats. After keeping the cells proliferating by using EGF, we labelled the cells with antibodies against Nestin, Nulp1 and Ki67. About 90% of all nestin-positive cells, indicative of stem cell characteristics, were Nulp1-positive, and the same was true of Nulp1-positive, i.e. >90% of all Nulp1-positive had expression of nestin. However, only about 50% of all Ki67-positive cells were Nulp1-positive meaning that Nulp1 was both present in dividing cells and in post-mitotic cells. We also differentiated the NPCs by plating them on poly-DL-ornithine. After five days of differentiation, the resulting neuroblasts, astrocytes and oligodendrocytes were positive for Nulp1. These results show that Nulp1-expression overlaps with the stem-cell marker Nestin in a majority of NPCs, but also continues to be expressed during and after the transition to specialized brain cells.

Finally, we studied the expression of Nulp1 during hippocampal development in more detail. Immunostaining showed that many cells in the hippocampus expressed Nulp1 during early postnatal development. The expression pattern became more neuronal like with development and in adult hip-
pocampus Nulp1 expression was confined to the neuronal layers of CA1-4 and to the dentate gyrus.

When considering all results from this paper, it appears that Nulp1 is important both during differentiation and afterwards in the brain. Nulp1 was primarily expressed in the cortical plate, the two granular layers of the cerebellum as well as in hippocampal CA1-3. All of these areas are rich in newly formed post-mitotic neurons in the process of finding their synaptic targets. The decrease in Nulp1 protein levels after birth hints at a reduced need for Nulp1 in the adult brain. Also, the shift in Nulp1 forms might indicate that the function of Nulp1 changes even earlier. Interestingly, Northern blotting against Nulp1 mRNA has previously shown that Nulp1 transcripts levels remain unchanged in brain throughout mouse development and adulthood. A number of regulatory mechanisms exist at the translational level, such as microRNA, 5’- and 3’-UTR-binding factors, and at the post-translational level (e.g. poly-ubiquitination) that could explain the discrepancies between our results and previous Northern blot-studies. The continuous steady-state levels of Nulp1 mRNA in adult mouse brain might indicate that the protein is inducible in response to some external cue, such as inflammation, cell stress, or during the process of neuronal regeneration. Previously mentioned cDNA array studies support this possibility.
Conclusions

These are the conclusions and take-home messages from each paper:

Paper I: GPS2 binds BIR1-2 and blocks XIAPs caspase-inhibitory function in vitro. GPS2 induces apoptosis through caspase-3 activation.

Paper II: Nulp1 induces cell death in human Saos-2 cells and sensitizes N2a-cells to staurosporine-induced apoptosis. In addition, Nulp1 binds XIAP in staurosporine-treated N2a-cells.

Paper III: N2a-cells express a novel Nulp1 isoform lacking the basic region of the bHLH-domain. Nulp isoform C and D are regulated by nucleocytoplasmic shuttling and all three isoforms are poly-ubiquitinated and have different stability. The cell death-inducing action of Nulp1 is not isoform-specific or dependent on transcriptional regulation by Nulp1.

Paper IV: Nulp1 is expressed in cortex, cerebellum, hippocampus, striatum and brain stem of developing mouse, and expression decreases with age. Nulp1 is highly expressed in the granular layers of developing mouse cerebellum and in the cortical plate of embryonic mouse cortex. Both mouse neuroepithelial NPCs and cultured rat striatal progenitor cells express Nulp1. Rat neuroblasts, astrocytes and oligodendrocytes retain expression of Nulp1 after differentiation in vitro.
GPS2 and Nulp1 are two proteins that, at least on the surface, are involved in transcriptional regulation. GPS2 both recruits the histone acetylase p300 to bound transcriptional activators and is a part of the N-CoR complex that represses transcriptional activation by its histone deacetylase subunit (HDAC3). Not much is known about Nulp1, but it has been shown that it is able to repress SRF-mediated transcription through histone deacetylase activity. Even more interesting is the fact that GPS2 is able to activate reporter plasmids controlled by serum response elements (Degenhardt and Silverstein, 2001). Whether this is pure coincidence or if it points towards a functional connection between GPS2 and Nulp1, and therefore also XIAP, in transcriptional regulation remains to be seen. The fact that XIAP has been seen to translocate to the nucleus in some systems without any known consequence (Keane et al., 2001; Nowak et al., 2004) could be a hint that XIAP affects HDAC-associated proteins, such as GPS2 and Nulp1, on their home turf. The recent finding that XIAP’s family relative cIAP1 is able to alter the stability of the bHLH member Mad1 does not contradict this possibility.

As discussed previously, the findings that GPS2 induces apoptosis by itself in both Saos-2- and N2a-cells might indicate that GPS2 also acts upstream of XIAP. More studies in Saos-2 cells would be useful to find out if GPS2 acts at other steps in the apoptotic pathway, without having to be concerned about p53. The previously reported JNK-inhibitory function of GPS2 has not been studied here and due to the ambiguous role of the JNK pathway in cell death it is possible that JNK-inhibition might lead to apoptosis in our systems. So far, most studies concerning the role of JNK in Saos-2 and N2a cells report that JNK-activation mediates pro-apoptotic stimuli, such as cisplatin, doxorubicin, oridinin and amyloid-beta (Jin et al., 2007; Longpre et al., 2006; Mikami et al., 2006; Sang et al., 2002; Zhang et al., 2003). Still, since there are three different forms of JNK and a total of 10 isoforms with somewhat non-redundant functions, it is possible that GPS2-induced JNK-inhibition could induce PCD.

The extent and importance of XIAPs involvement in BMP/TGF-β- and JNK1-signalling is still not fully understood, but it is possible that GPS2 also affects XIAP in its function as an adapter between the receptor and TAB1. Interestingly, Birkey-Reffey et al. showed that BIR1-3 of XIAP was necessary for XIAP-induced JNK1-activation. Therefore it would be interesting to
see if GPS2 inhibits this function in our system, which then would be an additional mode of JNK-inhibition by GPS2.

Regarding Nulp1 and its interaction with XIAP, many more experiments are needed to fully understand why the two proteins bind each other during apoptosis. To begin with, the precise function of Nulp1 is not yet known. Until we can measure some non-apoptotic transcriptional-dependent ‘activity’ of Nulp1 in our systems, it will be difficult to observe if XIAP can modulate the predicted transcriptional function of Nulp1. There is, of course, still the possibility that Nulp1 can influence any of the numerous functions of XIAP, and the first issue would be to thoroughly study if Nulp1-ubiquitination is dependent on XIAP.
I must admit that I’m not too fond of these kinds of ‘lists’, because it always feels like you are forgetting someone and that’s the last thing I want to do. Anyhow, here we comes the list and please be forgiving if I have forgotten anyone.

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