# PACS-2 integrates interorganellar communication pathways of cellular homeostasis and apoptosis

By

Joseph Elliott Aslan

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CERTIFICATE OF APPROVAL

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This is to certify that the Ph.D. dissertation of

**Joseph Elliott Aslan**

has been approved

| | |
|---|---|
| Gary Thomas | Mentor |
| Michael Forte | Committee Member |
| Bruce Magun | Committee Member |
| William Skach | Committee Member |
| Philip Stork | Committee Member |
| Peter Gillespie | Committee Member |

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| | |
|---|---|
| αSNAP | soluble NSF attachment protein α |
| 53BP1 | p53 binding protein 1 |
| Ab | antibody |
| ActD | actinomycin D |
| Ad | adenovirus |
| Admut | adaptor protein binding mutation |
| AIDS | Acquired Immune Deficiency Syndrome |
| AIF | apoptosis inducing factor |
| Ala | alanine |
| AP | Adaptor Protein |
| Apaf-1 | Apoptotic protease activating factor 1 |
| APC | adenomatous polyposis coli protein |
| Apo2L | Apoptosis-inducing Ligand 2 |
| ARF | ADP Ribosylation Factor |
| ARR | Atrophin-1 Related Region |
| A-SMase | acidic sphingomyelinase |
| ATM | Ataxia Telangiectasia, mutated |
| ATP | adenosine triphosphosphate |
| Bad | BCL2 Antagonist of Cell Death |
| BAP31 | B-Cell receptor associated protein of 31 kD |
| Bcl-2 | B-Cell Lymphoma 2 |
| BDL | bile duct ligation |
| BH | Bcl-2 Homology |
| Bid | BH3 Interacting Domain death agonist |
| Bim | Bcl-2-interacting mediator |
| BiP | immunoglobulin heavy-chain-binding protein |
| C19ORF5 | chromosome 19, open reading frame 5 |
| Caspase | Cysteine-Aspartate protease |
| CD | cluster of differentiation |
| cdc | cell division cycle |
| CD-IC | cytoplasmic dynein intermediate chain |
| CDK | cell division kinase |
| CD-MPR | Cation Dependent Mannose 6 Phosphate Receptor |
| ced | Caenorhabditis Elegans Death Genes |
| Chk | checkpoint kinase |
| CHX | cycloheximide |
| CI-MPR | Cation Independent Mannose 6 Phosphate Receptor |
| CK1 | protein kinase CK1 (formerly called Casein Kinase 1) |
| CK2 | protein kinase CK2 (formerly called Casein Kinase 2) |
| CLC | voltage-dependent chloride channel |
| CML/CMML | chronic myelomonocytic leukemia |
| CMV | cytomegalovirus |
| CNG | cyclic nucleotide gated |

| | |
|---|---|
| COPI | coatomer protein complex I |
| COPII | coatomer protein complex II |
| cr | cleavage resistant |
| CTR | C-terminal Region |
| CTSD | cathepsin D |
| DIABLO | direct IAP-binding protein with low PI |
| DISC | death induced signaling complex |
| Dlp1 | dynamin like protein 1 (same as Drp1) |
| DNA | deoxyribonucleic acid |
| DR4 | Death receptor 4 |
| DR5 | Death receptor 5 |
| Drp1 | dynamin related protein 1 |
| EGFR | Epidermal Growth Factor Receptor |
| EMBO | European Molecular Biology Organization |
| EndoG | endonuclease G |
| ER | Endoplasmic Reticulum |
| ERGIC | ER-Golgi Intermediate Compartment |
| EST | expressed sequence tag |
| eYFP | enhanced yellow fluorescent protein |
| FACL-4 | long chain fatty acid-CoA ligase 4 |
| FADD | Fas associated death domain |
| FasL | Fas ligand |
| FBR | Furin (or cargo) Binding Region |
| FLIP | flice inhibitory protein |
| fmk | fluoromethyl ketone |
| FOXO | Forkhead Homeobox type O |
| FP | fluorescence polarization |
| FRET | fluorescence resonance energy transfer |
| GEF | Guanine nucleotide exchange factor |
| GFP | green fluorescent protein |
| GGA | Golgi associated, gamma adaptin ear containing |
| GRASP | Golgi reassembly stacking protein |
| GTP | guanosine triphosphosphate |
| HA | Hemagglutinin antigen |
| HAUSP | Herpesvirus-Associated Ubiquitin-Specific Protease |
| HCMV | human cytomegalovirus |
| His | histidine |
| HIV-1 | Human Immunodeficiency Virus |
| HT-1 | hereditary tyrosinemia 1 |
| ICD | inclusion cell disease |
| IP | Immunoprecipitation |
| IP3 | Inositol 1,4,5-trisphosphate |
| IRB | Institutional Review Board |
| Jnk | c-Jun kinase |
| Jo2 | anti-Fas (mouse specific) |
| kD | kilodalton |

| | |
|---|---|
| KIAA | Kazusa DNA Research Institute (AA are reference characters) |
| KIF | Kinesin Family Member |
| KP | Kaiser Permanente |
| LC8 | dynein light chain 8 |
| mAb | monoclonal antibody |
| MAM | Mitochondria Associated Membrane |
| MAP1S | microtubule associated protein 1S |
| Mcl-1 | myeloid cell leukemia sequence 1 |
| Mdm2 | murine double minute 2 |
| MEF | mouse embryonic fibroblast |
| MHC-1 | major histocompatability complex 1 |
| MMP | Mitochondria Membrane Permeabilization |
| MR | Middle Region |
| MRE11 | meiotic recombination protein 11 |
| mtDNA | mitochondrial DNA |
| MTOC | microtubule organizing center |
| mTOR | mammalian target of rapamycin |
| Nef | HIV-1 Negative Factor |
| OHSU | Oregon Health & Science University |
| Op18 | oncoprotein 18 |
| p53 | tumor protein of 53 kD |
| PACS-1 | Phosphofurin Acidic Cluster Sorting Protein 1 |
| PACS-2 | Phosphofurin Acidic Cluster Sorting Protein 2 |
| PAK | p21 activated kinase |
| PARP | poly ADP-ribose polymerase |
| PDI | protein disulfide isomerase |
| PI | propidium iodide |
| $PIP_3$ | Phosphatidylinositol (3,4,5)-trisphosphate |
| PKA | protein kinase A |
| PKB | protein kinase B |
| PKC | protein kinase C |
| PKD-2 | polycystin-2, TRPP2 |
| PP2A | protein phosphatase 2A |
| PSS-1 | Phosphatidylserine synthase I |
| PUMA | p53 upregulated mediator of apoptosis |
| Ser | Serine |
| SERCA | sarco/endoplasmic-reticulum Ca2+ pump |
| SFK | Src family kinase |
| siRNA | small interfering RNA |
| Smac | second mitochondrial activator of caspases |
| SNARE | SNAP receptor |
| STS | staurosporine |
| TAg | SV40 large T antigen transformed |
| tBid | truncated Bid |
| TGN | *trans*-Golgi Network |

| | |
|---|---|
| Thr | threonine |
| TMRM | tetramethylrhodamine maleimide |
| TNFα | Tumor Necrosis Factor α |
| TNFR1 | TNF Receptor 1 |
| TRAIL | TNF Related Apoptosis Inducing Ligand |
| TRP | transient receptor potential |
| TTC | triphenyltetrazolium chloride |
| TUNEL | terminal deoxynucleotidyl transferase-mediated nick end labeling |
| UPR | unfolded protein response |
| UXT | ubiquitously expressed transcript |
| VAMP | Vesicle Associated Membrane Protein |
| VDAC-1 | voltage dependent anion channel 1 |
| VHL | von Hippel-Lindau factor |
| VMAT | Vesiclular Monoamine Transporter |
| VZV | Varicella-Zoster virus |
| WB | western blot |
| WT | wild type |
| ZAP-70 | Zeta-chain-associated protein kinase 70 |

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## Abstract

Eukaryotic cells are distinguished by an elaborate endomembrane system that regulates cellular and organismal homeostasis. While this system of membrane-bound organelles coordinates the proper functioning, proliferation and survival of cells, a mechanism of programmed self-destruction, apoptosis, emerges from the endomembrane system in times of damage and stress or during specific developmental stages to eliminate unneeded cells. Several details of how intracellular regulators manage either homeostasis or apoptosis are now understood. However, the manner in which such factors communicate with one another or share functions is unknown. In this dissertation I describe PACS-2, a sorting protein that regulates the trafficking of factors throughout the endomembrane system to medicate cellular homeostasis, interorganellar communication and apoptosis. PACS-2 coordinates communication between the endoplasmic reticulum and mitochondria to regulate calcium signaling, ER homeostasis and apoptosis. In response to apoptotic induction by chemotherapeutic agents such as Apo2L/TRAIL, PACS-2 is required to translocate Bid to mitochondria to initiate the activation of caspases to mediate cell death *in vitro* in cancer cell lines as well as *in vivo* in hepatocytes. PACS-2 is phosphorylated at $Ser_{437}$ by the pro-survival kinase Akt to establish a docking site for 14-3-3 proteins. Apoptotic induction triggers dephosphorylation of $Ser_{437}$, reprogramming PACS-2 to promote apoptosis rather than the trafficking of cargo. Together, these studies describe the phosphorylation state of PACS-2 $Ser_{437}$ as a molecular switch that regulates interorganellar communication, cellular homeostasis and apoptosis.

# Chapter 1. Introduction

## 1.1 Overview

The central hypothesis of this dissertation is that the phosphofurin acidic cluster sorting protein 2 (PACS-2) controls a key step in the intracellular signaling and trafficking pathways that carry out apoptotic cell death programs. Apoptosis is a fundamental cellular response crucial to the development and homeostasis of eukaryotic organisms that results in the elimination of unneeded or damaged cells. On a biochemical level, apoptotic programmed cell death is ultimately carried out through the cleavage of specific substrates by proteases of the caspase family, leading to the dissolution and collapse of membranous and organellar structures and the efficient destruction of genetic material. Apoptosis is tightly controlled at an organellar level by a number of regulatory factors, most notably proteins of the Bcl-2 family. The biochemical mechanisms of Bcl-2 protein and caspase action are well understood, however, the manner in which these proteins target organellar systems to direct apoptotic programs remains enigmatic. The experiments in this dissertation establish PACS-2 as a multifunctional sorting protein that links communication between the endoplasmic reticulum (ER) and mitochondria with apoptosis. These experiments identify PACS-2 as a regulator of cell death pathways mediated by the pro-apoptotic Bcl-2 protein family member Bid. Investigation of the regulation of the apoptotic activation of PACS-2 reveals that PACS-2 is a target of the pro-survival kinase Akt which phosphorylates PACS-2 at Serine 437 to establish a binding site for anti-apoptotic 14-3-3 proteins. These mechanistic studies have implications for diseases of inflammation, proliferation and cancer and establish PACS-2

as a pro-apoptotic protein with essential functions in organellar communication and apoptotic pathways that rely on organellar pathways that act through the mitochondria to coordinate cell death induction.

This chapter serves as an introduction to the PACS family of proteins and their roles in intracellular trafficking pathways and organelle dynamics. This primer of PACS function leads into a review of intracellular trafficking pathways of apoptosis in Chapter 2, which describes the roles of Bcl-2 proteins and other factors in modulating organelle-regulated apoptotic pathways. Such processes include mitochondrial permeabilization and cytochrome c release, the activation and cytosolic release of lysosomal cathepsins, and a fragmentation of the Golgi apparatus. The review presented in Chapter 2 provides a summary of classic and current studies of apoptosis and provides a novel viewpoint describing how apoptotic interorganellar communication takes place through the trafficking of cargo between organelles or through direct membranous contacts between organelles. Such organellar contacts include ER-mitochondrial contacts at mitochondria associated membranes, or "MAMs" as well as lysosomal/mitochondria contacts and a "scrambling" of Golgi and mitochondrial membranes at the induction of apoptosis. Roles of PACS-2 as an intracellular trafficking protein that regulates Bid activation, organellar mobility and ER-mitochondrial communication are discussed throughout the chapter.

Studies in Chapter 3 establish PACS-2 as a regulator of ER-mitochondrial communication as well as mitochondrial apoptotic pathways mediated by the Bcl-2 family member Bid. This work was published in *The EMBO Journal* in 2005 and

presented in a poster session at the 2005 Keystone Symposium on Cellular Senescence and Cell Death in Keystone, Colorado. Together with other members of the Thomas lab, I establish that PACS-2 is a pro-apoptotic protein that regulates the translocation of Bid to mitochondria. Through siRNA depletion studies of PACS-2 in tumor cell lines, we demonstrated that PACS-2 is required for the mitochondrial pathway of apoptosis. An examination of key markers of the pathway to apoptosis shows that PACS-2 is required to cleave and translocate the Bcl-2 family member Bid to the mitochondria, a requisite step in mitochondrial membrane permeabilization. Accordingly, cells depleted of PACS-2 fail to release cytochrome c in response to apoptotic induction and do not activate executioner caspase-3. This work also describes the intracellular location of PACS-2 and its role as a mediator of ER-mitochondrial communication as PACS-2 depletion effects ER homeostasis, mitochondrial morphology, ER-mitochondrial communication and apoptosis at a specific step after initiator caspase activation but before translocation of the BH3-only domain protein Bid to the mitochondria.

Chapter 4 demonstrates that PACS-2 is required for death-receptor mediated apoptosis *in vitro* and *in vivo* and describes a mechanism by which the apoptotic activity of PACS-2 is regulated by the pro-survival kinase Akt. This work has been submitted for publication and was presented in part in June 2006 at the 6$^{th}$ International Symposium of the International Cell Death Society in Angra dos Reis, Brazil. Notably, we show PACS-2 is required in vivo in mouse liver in to induce apoptosis in response to the TNFα related apoptosis inducing ligand, or TRAIL, a promising chemotherapeutic now in clinical trials that selectively kills diseased cells. PACS-2 is similarly required for apoptosis in tumor

cell lines and transformed mouse embryonic fibroblasts. Using cells derived from *PACS-2*$^{-/-}$ mice, we demonstrate PACS-2 is a requisite component of TRAIL signaling pathways as PACS-2 is required for the cleavage and activation of Bid as well as caspase-9 and caspase-3 in response to TRAIL. Through biochemical, pharmacological and cellular methods, we demonstrate that Akt phosphorylates PACS-2 at Serine 437 *in vitro* and *in vivo* to block cell death. Using biophysical, biochemical and cellular methods, we demonstrate that PACS-2 phospho-Serine 437 constitutes a binding site for anti-apoptotic 14-3-3 proteins. This chapter concludes that PACS-2 is an Akt substrate that regulates apoptotic induction at the mitochondria in response to TRAIL.

The implications of the results described in this dissertation are discussed in Chapter 5. Given the diversity of functions described for PACS-2, these implications are wide ranging and cover essential pathways of cell death common to cancer and neuronal cell death.

An appendix of this dissertation includes a study of the role of Bid phosphorylation in regulating the sensitivity of hepatocytes to apoptosis *in vivo*. Previous studies, including those described in Chapter 3, suggest that mitochondrial pathways to apoptosis are regulated by CK1 and CK2 phosphorylation of Bid. While such studies show that Bid phosphorylation blocks apoptosis in cultured cells, this work argues that Bid phosphorylation blocks apoptosis *in vivo*. Using bile-duct ligation to induce chronic cholestasis in mice, we show that this model of chronic liver disease confers resistance to Fas-induced apoptosis in the liver. This block in apoptotic activation, like that in PACS-2

depleted cells, occurs downstream of Caspase-8 activation but prior to mitochondrial cytochrome c release. Microarray analysis of gene expression in bile duct ligated mice shows that CK1 expression is upregulated in liver upon disruption of liver homeostasis. Using biochemical methods, I confirm that CK1 activity is upregulated in the livers of these mice. Liver extracts from these mice more readily phosphorylate Bid *in vitro* compared to liver extracts from control mice. Bile duct ligated mice also show higher in vivo levels of phospho-Bid (Serine 61/64). Using *in vitro* methods I also demonstrate that the DNA-damage sensing kinase ATM can phosphorylate Bid in vitro. Interestingly, bile duct ligated *ATM -/-* mice show no difference in apoptosis sensitivity compared to control mice, suggesting that ATM does not have a role in this process in vivo. While this study does not include data describing a direct role for PACS-2 in this process, we discuss a hypothesis that phosphorylation of Bid by CK1 prevents its interaction with PACS-2 to block apoptotic programs. This work was published in *Gastroenterology* in 2006 and also presented at the Keystone Symposium on Cellular Senescence and Cell Death in Keystone, Colorado in March 2005.

## 1.2 The Eukaryotic Endomembrane System

An essential and profound divergence in the evolutionary history of the biological world occurs between prokaryotic and eukaryotic cells (Doolittle and Brown, 1994; Vellai and Vida, 1999; Woese, 2002). Though structurally less complex, prokaryotic organisms are marked by a great diversity in biochemical and metabolic processes, allowing for their survival in extreme conditions, but precluding their rapid adaptation and expansion into dynamic environments. Eukaryotes, however, have a greatly expanded complexity resultant of the presence of intracellular structures and response networks that essentially comprise biological information processing systems (de Bivort et al., 2007; Huang and Ingber, 2000). These features not only allow eukaryotic cells to sense and adapt to environmental changes in real-time, but also establish a mechanism to assemble and homeostatically regulate multicellular tissues and organ systems.

Eukaryotic cells are defined by the presence of a membrane-bound nucleus, a cytoskeleton, mitochondria and a system of functionally connected membrane compartments generally referred to as the endomembrane system (Dacks and Field, 2007; Dacks et al., 2008). This system of organelles and vesicles coordinates biosynthetic-secretory, homeostatic and endocytic pathways to sort, modify and transport intracellular material. The endomembrane system begins with the endoplasmic reticulum, which is contiguous with the nuclear envelope at the point at which nascent polypeptides are inserted into the secretory pathway (Figure 1-1).

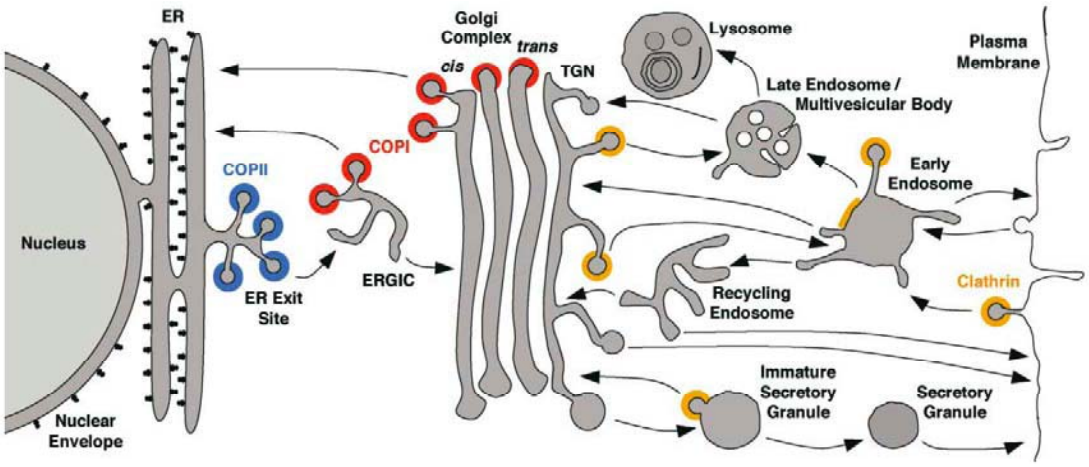


Figure 1-1: Intracellular transport pathways in the eukaryotic endomembrane system.

This schematic diagram shows the essential routes of cargo traversing the secretory pathway. Transport steps are indicated by arrows. Vesicular trafficking steps from the ER to the ERGIC and Golgi mediated by COPII are marked in blue while cargo retrieved from the Golgi by COPI coats are shown in red. Late secretory pathway steps mediated by clathrin are shown in orange. From (Bonifacino and Glick, 2004). © 2004 Elsivier. Used with permission.

Transport vesicles bud from the ER, fusing with other such vesicles or the ER Golgi intermediate compartment (ERGIC). Vesicular intermediates then travel to the *cis* face of the Golgi complex to enter the parallel stacks of cisternae that make up the Golgi complex. Material transported from the ER is modified and sorted for transport to various organelles upon exit from the *trans*-Golgi network (TGN). Upon exit from the TGN, proteins may take a variety of routes. Vesicles may traffic in a retrograde manner back to the ER; anterograde, ultimately to the plasma membrane where vesicles fuse to release their contents to the extracellular milieu or present their membrane bound cargo. TGN-derived vesicles fuse with endocytic vesicles derived from the plasma membrane – so-called “sorting endosomes” - or with pre-existing late endosomes. Late endosomes also

fuse with lysosomes to deliver their contents to these hydrolytic organelles that mediate the turnover, degradation and recycling of intracellular material (Luzio et al., 2007).

The membrane-bound organelles comprising the eukaryotic endomembrane system establish the essential machinery of cellular homeostasis, controlling functions such as the production of proteins and lipids, protein secretion, nutrient uptake, protein and organellar degradation, energy production, the establishment of electrochemical gradients and the induction of programmed cell death pathways (Dacks and Field, 2007). These functions are linked through communication and transport processes amongst organelles which take place via vesicular transport, organelle maturation or direct organelle-to-organelle contacts (Bonifacino and Glick, 2004; Levine and Loewen, 2006). Vesicular transport amongst organelles generally occurs through four basic steps of vesicular budding, transport, docking and fusion (Bonifacino and Glick, 2004). Vesicular transport processes and their ultimate control of cellular homeostasis and cell fate are controlled and modulated by an array of factors, including GTPases, specific lipids such as phosphoinostitides and ceramide, motor proteins, kinases and phosphatases and adaptor complexes which allow for the formation of vesicular coats for vesicular transport amongst organelles. The mechanisms by which these factors control vesicular trafficking is a rich topic of investigation, however, the manner in which such factors signal cell death remains unexplored.

## 1.3 Itinerant Membrane Proteins

The exact course traversed by proteins and lipids throughout the compartments of the endomembrane system varies depending on the intracellular cargo in question and its function. For example, upon delivery to the cell surface, transferrin receptors constitutively recycle between the plasma membrane and recycling endosomes through early endosomal intermediates (van Dam et al., 2002); growth factor receptors such as the epidermal growth factor receptor (EGFR) segregate into early endosomal compartments for sorting and delivery to lysosomes where they are hydrolytically degraded (Gruenberg and Stenmark, 2004; Raiborg et al., 2003); and ion channels such as IP3 receptors localize to the ER to maintain calcium homeostasis (Foskett et al., 2007). The trafficking itinerary of membrane proteins is dynamic and reflects the biological functions of such proteins in the maintenance of cellular homeostasis. Classic examples include the cation-dependent and independent mannose-6-phosphare receptors (CD-MPR and CI-MPR) which sort lysosomal hydrolase enzymes such as Cathepsin D to lysosomes before returning to the TGN (Griffiths et al., 1988). Similarly, the endoprotease furin localizes to the TGN, cycling between the TGN, cell surface, immature secretory granules and endosomes. Furin passes through the secretory pathway, becoming autocatalytically activated to cleave and activate precursor peptide hormones (Molloy et al., 1999). In general, the sorting and intracellular transport of such proteins relies on canonical sorting motifs within the cytosolic domains of the itinerant membrane protein. Such sorting motifs act as “address labels” that are recognized by specific components of the cellular sorting machinery that act to sort and deliver cargo proteins to specific subcellular compartments.

Cytosolic sorting signals such as tyrosine (YXXΦ, where Φ is a hydrophobic residue) and dileucine ([D/ExxxL[L/I]) motifs establish an interaction between iterant proteins and adaptor protein (AP) complexes that direct the assembly of lattice-like vesicular coats comprised of clathrin, hence concentrating cargo in clathrin coated vesicles. Membrane adaptor complexes such as AP-1, AP-2, AP-3 and AP-4 are each composed of four subunits that form a "mickey-mouse"-like structure with two "ear" domains (Robinson and Bonifacino, 2001). APs govern the mechanics of vesicular traffic process that carry out endocytic and biosynthetic processes as clathrin coated vesicles mediate protein transport between the TGN and endosomes. APs connect clathrin to itinerant proteins such as furin and the MPRs to promote their concentration into clathrin coated pits and vesicles to mediate sorting amongst components of the late secretory pathway (Robinson, 2004). Trafficking in the early secretory pathway between the ER and Golgi, however, typically takes place through clathrin-independent vesicular coats based on coatomer COPI and COPII complexes which recognize KKXX and FF motifs (McMahon and Mills, 2004).

Canonical sorting motifs are often found near stretches of acidic amino acids which contain serine or threonine residues that can be phosphorylated by the acidic-directed kinase CK2 and less often CK1 (Bonifacino and Traub, 2003; Gu et al., 2001; Thomas, 2002). Such acidic motifs direct the sorting of several itinerant proteins such as furin throughout the secretory pathway (Thomas, 2002). The phosphorylation state of a specific serine residue within the furin acidic cluster (EECPp$S_{773}$DpSEED) controls the

sorting of furin between the TGN, endosomes and cell surface and the removal of furin from immature secretory granules in neuroendocrine cells. Dephosphorylation of furin, by contrast, by protein phosphatase 2A (PP2A) directs the transport of furin from sorting endosomes to the TGN. While it was known that furin bound APs, it was not understood how phosphorylation of furin could direct its sorting itinerary.

## 1.4 The PACS Family of Proteins

An analysis of candidate sorting factors associated with the phosphorylated acid cluster sorting motif of furin uncovered the phosphofurin acidic cluster sorting, or PACS family of proteins (Thomas, 2002; Wan et al., 1998; Youker et al., 2008). Work in the lab of Gary Thomas at the Vollum Institute first identified PACS-1 in a yeast-2-hybrid screen through its interaction with the phosphorylated acidic cluster motif on the cytosolic domain of furin (Wan et al., 1998). These studies ascribed the first known function of the PACS proteins as "sorting connectors" as PACS-1 connects CK2-phosphorylated furin to the cytosolic coat protein AP-1 and is a requisite component for the subcellular localization of furin to the TGN (Crump et al., 2001).

Genomic analyses of the *PACS-1* gene led to the discovery of a related gene, *PACS-2*, whose protein product also binds to acidic clusters of itinerant proteins (Chapter 3; (Kottgen et al., 2005)). Rather than connecting cargo to clathrin adaptor proteins, PACS-2 connects acidic cluster-containing cargo proteins to COPI coatomer to mediate vesicular, clathrin-independent, Golgi-to-ER transport in the early secretory pathway (Kottgen et al., 2005). This was first demonstrated in studies of the subcellular trafficking of the ER-localized TRP channel polycystin-2 (PKD2), whose intracellular itinerary and

function are regulated by the phosphorylation of an acidic cluster in a cytosolic domain that binds to both PACS-1 and PACS-2 (Kottgen et al., 2005). PKD-2 acts with IP3 receptors and ryanodine receptors at the ER to regulate intracellular calcium concentrations (Cai et al., 1999; Fu et al., 2008). PKD-2 also localizes to the primary cilium and acts with PKD-1 to detect fluid flow in tubules and modulate signaling pathways to promote cell cycle arrest and tubulogenesis (Boletta et al., 2000; Low et al., 2006; Nauli et al., 2003). Interestingly, mutations or deletions of the acidic cluster region of PKD-2 lead to the development of autosomal dominant polycystic kidney disease (ADPKD), a chronic disease state characterized by the formation of fluid-filled cysts in the kidney epithelium that to leads to renal failure (Cai et al., 1999; Delmas et al., 2004; Sutters and Germino, 2003).

PKD2 serves as a model PACS cargo protein, localizing between the ER, Golgi and cell surface (Figure 1-2). PACS-2 is required to localize PKD-2 to the ER in a COPI dependent manner as disruption of PACS-2 function redistributes PKD-2 from the ER to the Golgi/TGN. Dual disruption of PACS-1 and PACS-2 redistributes PKD-2 to the cell surface as PACS-1 localizes PKD-2 to the Golgi/TGN. Such studies using dominant-negative interfering mutants as well as PACS siRNAs demonstrate that PACS-1 is required to localize such PACS cargo proteins from endosomes to the TGN while PACS-

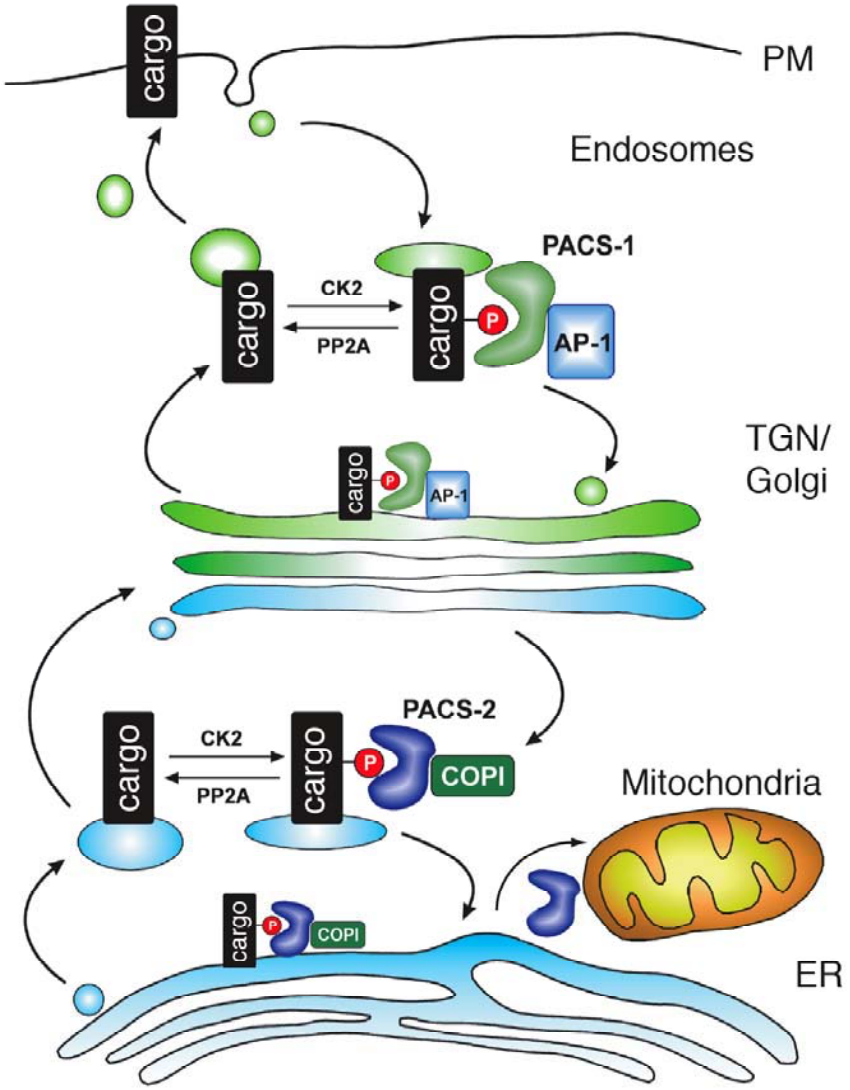


Figure 1-2: PACS-1 and PACS-2 direct the sorting of acidic cluster containing cargo throughout the secretory pathway.

The phosphorylation state of acidic cluster-containing cargo is controlled by CK2 phosphorylation and PP2A dephosphorylation of cargo to modulate interactions with the PACS proteins. PACS-1 connects cargo such as PKD-2 to clathrin adaptors such as AP-1 to direct late secretory pathway trafficking from endosomes to the TGN. PACS-2 connects cargo to the clathrin-independent coat adaptor COPI to mediate TGN to ER retrieval of cargo. PACS-2 also has a role at the mitochondria, regulating ER-mitochondria communication as well as apoptotic programs.

2 delivers cargo from the Golgi to the ER. Together, studies of the PACS proteins have established a paradigm of PACS sorting function whereby the CK2/PP2A-controlled phosphorylation state of cargo control the complex intracellular trafficking itineraries of acidic cluster containing cargo (Youker et al., 2008).

The 889 amino acid human PACS-2 protein shares 54% sequence homology to PACS-1, a protein of 963 amino acids. Regions of homology between PACS-1 and PACS-2 divide the PACS proteins into four regions (Figure 1-3). These include an Atrophin-1 related region (ARR) found only in PACS-1 which displays homology to the Atrophin-1 transcriptional repressor (Wood et al., 2000). The cargo, or furin binding region (FBR) of the PACS proteins are 81% homologous and interact with cargo as well as and AP-1 and AP-3 and GGA adaptor complexes on PACS-1 (Crump et al., 2001; Scott et al., 2006) and COPI coatomer on PACS-2 (Kottgen et al., 2005). The PACS-1 FBR also serves as a scaffold for the kinase CK2 through an association with a regulatory subunit (Scott et al., 2006). The middle region (MR) of the PACS proteins contain an acidic cluster much like that of PACS cargo proteins that acts as an autoregulatory domain (Scott et al., 2006). The C-terminal regions (CTR) of the PACS proteins to date remain uncharacterized.

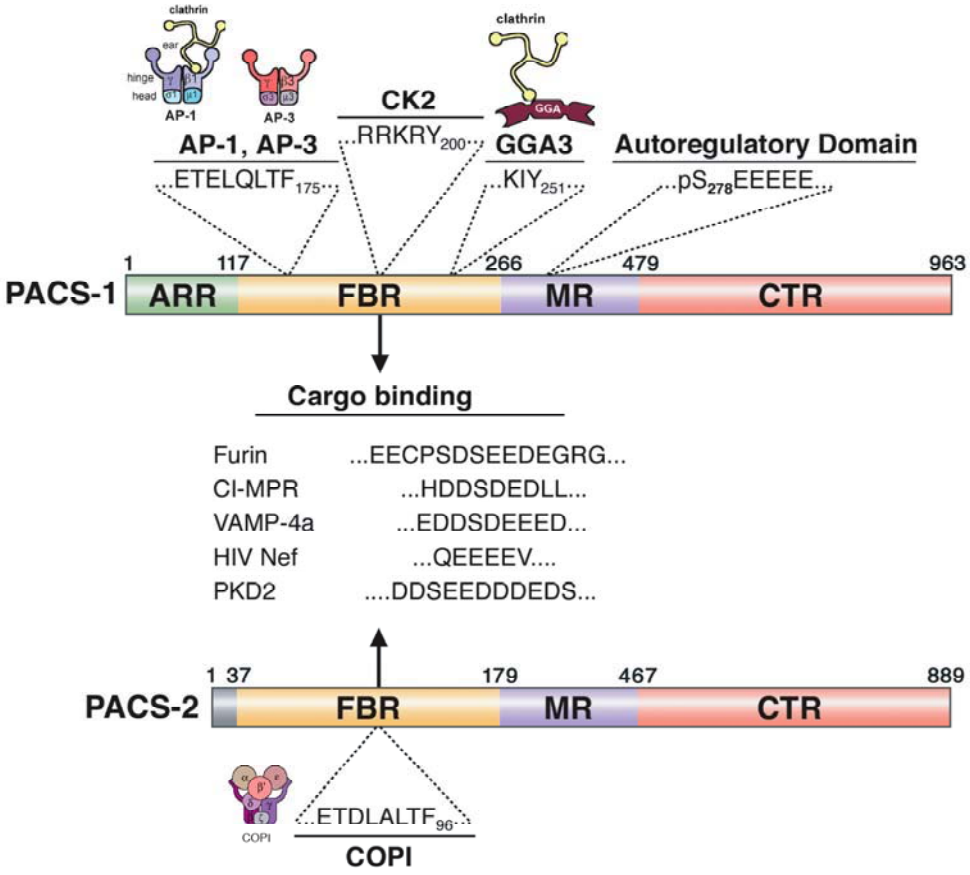


Figure 1-3: Domain structure of the PACS-1 and PACS-2 proteins.

A schematic diagram illustrating the locations of the Atrophin-1 related region (ARR); cargo, or furin binding region (FBR); middle region (MR); and C-terminal region (CTR). Residues critical to AP-1 and AP-3 binding on PACS-1 as well as the residues responsible for binding COPI on PACS-2 are indicated. PACS-1 also binds to a regulatory subunit of the kinase CK2 as well as GGA adaptors (Scott et al., 2006). The autoregulatory acidic cluster found in the PACS-1 MR resembles the acidic clusters found in PACS cargo proteins such as furin, Nef and PKD-2 (Scott et al., 2003; Youker et al., 2008).

Since the initial discovery of PACS-1 as a furin sorting protein, several other itinerant proteins with acidic cluster motifs have been shown to use PACS proteins to regulate their sorting itineraries. These include other processing enzymes such as carboxypeptidase D (Kalinina et al., 2002) and protein convertase 6B (Xiang et al., 2000); transporters such as the vesicular monoamine transporter 2 (VMAT2) (Waites et al., 2001); receptors and ion channels such as the metabotropic glutamate receptor mGluR5 (Farr et al., 2004), TRPV4 and CLC7 (Kottgen et al., 2005); and a number of viral pathogenic factors such as varicella-zoster virus VZV gE (Wan et al., 1998) and human cytomegalovirus HCMV gB (Crump et al., 2003) as well as the human immunodeficiency virus 1 negative factor HIV-1 Nef (Piguet et al., 2000). Indeed, pathogenic viruses have developed ways to exploit components of their host endomembrane machinery, including the PACS proteins, to promote replication and counteract host anti-viral responses. The herpes virus envelope glycoprotein HCMV gB, essential for viral envelope formation, contains a CK2 phosphorylatable acidic cluster that direct binding of gB to PACS-1 (Crump et al., 2003). Disruption of PACS-1 function mislocalizes gB from the TGN to endosomal compartments and reduces viral progeny while overexpression of PACS-1 can enhance viral titer. Most notably, HIV-1 Nef usurps both PACS-1 and PACS-2 sorting pathways to result in host MHC-I downregulation and immunoevasion by directing the assembly of a multikinase cascade (Figure 1-4) (Atkins et al., 2008; Blagoveshchenskaya et al., 2002; Hung et al., 2007). PACS proteins also mediate the trafficking of the Karposi sarcoma herpes virus K5 protein, a ubiquitin ligase that localizes to the ER to direct the turnover of host factors such as CD31 and MHC-1 to block antigen presentation to promote immunoevasion (Mansouri et al., 2006).

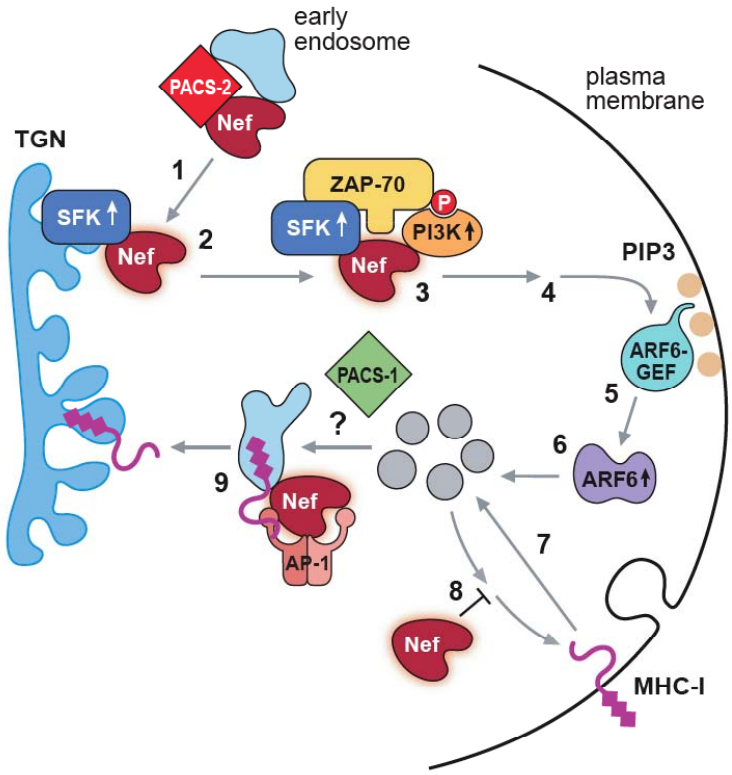


Figure 1-4: PACS-2 assembles a multikinase cascade to direct MHC-I downregulation and immunoevasion upon HIV-1 infection.

(1) PACS-2 binds to the acidic cluster of the HIV-1 immediate early gene product Nef ($EEEE_{65}$) and is targeted to the TGN. (2) At the TGN, a TGN-localized Src family kinase (SFK) binds to and is activated by Nef $PXXP_{75}$. (3) The Nef/SFK complex recruits and activates ZAP-70/Syk. Tyrosine-phosphorylated ZAP70/Syk then binds to a class I PI3 kinase. (4) Nef-stimulated PI3K generates $PIP_3$ on the inner leaflet of the plasma membrane to recruit and ARF6-GEF (5), which then activates ARF6 (6). (7) MHC-I is endocytosed from the plasma membrane to endosomal compartments (Atkins et al., 2008; Hung et al., 2007). Internalized MHC-I is then sequestered to the Golgi through an interaction with AP-1 that may be regulated by PACS-1 (Blagoveshchenskaya et al., 2002).

### 1.4.1 PACS-2 coordinates communication between the ER and mitochondria

The roles of PACS-2 at the ER are not limited to trafficking cargo in the early secretory pathway; PACS-2 also controls an intimate relationship between the ER and mitochondria at MAMs to control lipid metabolism, ATP production and calcium signaling (Chapter 3; (Myhill et al., 2008)). As demonstrated in Chapter 3, these close contacts between the ER and mitochondria are lost upon depletion of PACS-2, leading to a mislocalization of the MAM-localized lipid biosynthetic enzymes FACL-4 and PSS-1 and a disruption of histamine-inducible calcium release from the ER. The integrity of MAMs may be mediated by the localization of the chaperones to the ER as calnexin, calreticulin and ERp44 interact with ER-calcium pumps IP3R and SERCA2b which are enriched in MAMs (Higo et al., 2005; John et al., 1998; Roderick et al., 2000). Like other PACS cargo proteins, the cytosolic tail of calnexin contains a CK2 phosphorylatable acidic cluster that mediates an interaction with PACS-2, localizing calnexin amongst the ER, MAMs and a small pool at the cell surface (Myhill et al., 2008). In addition to folding intermediates, ribosomes and SERCA2b, calnexin also associates with BAP31, a multi-functional sorting chaperone and sensor of intracellular stress (Breckenridge et al., 2003; Zuppini et al., 2002). Depletion of PACS-2 mislocalizes calnexin from the ER to the cell surface, but also leads to the caspase cleavage of BAP31 and a fission of mitochondria into punctate or donut-like structures characteristic of apoptotic cells (Chapter 3; (Myhill et al., 2008)). Interestingly, despite this stress phenotype, PACS-2 depleted cells fail to execute apoptotic programs as described in Chapters 3 and 4.

### 1.4.2 PACS-2 regulates organellar pathways to cell death induction

In Chapter 3, we show that under conditions of cellular stress, PACS-2 can redistribute from the cytosol/ER to the mitochondria. The role of PACS proteins as acidic cluster binding proteins and the apoptotic redistribution of PACS-2 to mitochondria suggested that PACS-2 shuttles a pro-apoptotic factor to mitochondria upon the induction of stress. As the pro-apoptotic Bcl-2 family member Bid contains a CK2- phosphorylatable acidic cluster typical of PACS cargo (Degli Esposti et al., 2003a; Desagher et al., 2001), we assayed PACS-2 for Bid sorting activity. As demonstrated in the data chapters of this thesis, we found that PACS-2 plays a requisite role in mitochondrial cell death pathways in part by delivering Bid to mitochondria.

Prior to our characterization of PACS-2, the *PACS-2* human EST, KIAA0602, was reported to be lost in 15-40% sporadic colon cancer cases (Anderson et al., 2001). In Chapter 4 we confirm that the expression of the PACS-2 protein is lost in a significant portion of human colon cancer specimens. The requisite role of PACS-2 in apoptosis and its loss in cancer establish an argument that PACS-2 is a candidate tumor suppressor and a possible biomarker of chemotherapeutic sensitivity to anti-tumor agents such as TRAIL. These ideas are presented and discussed in Chapter 4 of this thesis, which describes the regulation of PACS-2 by the pro-survival kinase Akt and the 14-3-3 proteins (Figure 1-5).

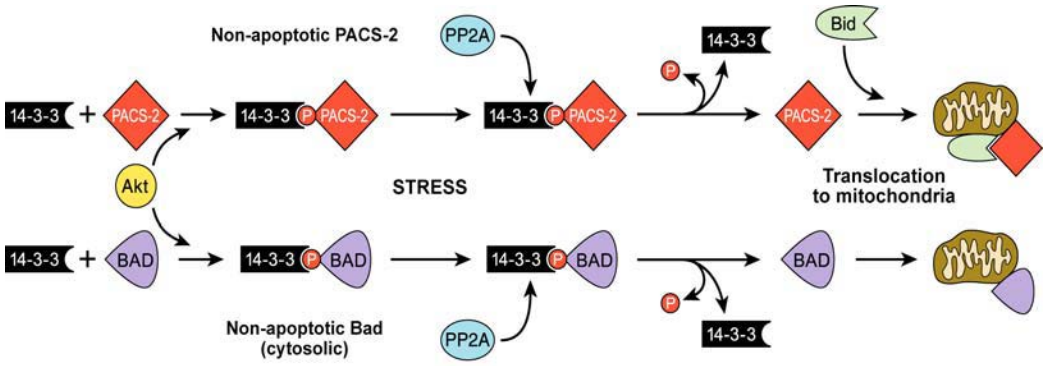


Figure 1-5: Model of apoptotic regulation of PACS-2.

As described in Chapter 4, under basal conditions, PACS-2 is phosphorylated at Serine 437 and held in a non-apoptotic state by 14-3-3 proteins. Upon the induction of apoptosis, Akt signals are dampened as PP2A activity increases, resulting in dephosphorylation of PACS-2, release of 14-3-3 and the induction of mitochondrial cell death pathways. This mechanism of apoptotic activation resembles the previously described activation and translocation of the pro-apoptotic Bcl-2 protein Bad (Datta et al., 1997; Masters et al., 2001).

In the next chapter, a literature review builds a hypothesis of apoptotic interorganellar communication whereby Bid localizes to the mitochondria or to an interface between the mitochondria and lysosomes to drive an essential step of apoptotic programs. As PACS-2 coordinates the intracellular distribution of endocytosed material in endosomes (Atkins et al., 2008), PACS-2 may drive endo-lysosomes and mitochondria together in a step required to cleave Bid and permeabilize mitochondria. These hypotheses and their relation to current paradigms of protein and organellar trafficking and apoptosis are reviewed in the following chapter.

# Chapter 2. Death by committee: organellar trafficking and communication in apoptosis.

Joseph E. Aslan and Gary Thomas*

Vollum Institute, Oregon Health & Science University,
3181 SW Sam Jackson Park Road, Portland OR, 97239, USA

*Corresponding author; Tel: (503) 494-6955; Email: thomasg@ohsu.edu

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**Summary**

Apoptosis proceeds through a set of evolutionarily conserved processes that coordinate the auto-elimination of damaged or unneeded cells. This program of cell death is carried out by organelle-directed regulators, including the Bcl-2 proteins, and ultimately executed by proteases of the caspase family. While the biochemical mechanisms of apoptosis are increasingly understood, the underlying cell biology orchestrating programmed cell death remains enigmatic. In this review we summarize the current understanding of Bcl-2 protein regulation and caspase activation while examining cell biological mechanisms and consequences of apoptotic induction. Organellar contributions to apoptotic induction include death receptor endocytosis, mitochondrial and lysosomal permeabilization, endoplasmic reticulum calcium release and fragmentation of the Golgi apparatus. These early apoptotic events are accompanied by a stabilization of the microtubule cytoskeleton and a translocation of organelles to the microtubule organizing center. Together, these phenomena establish a model of apoptotic induction whereby a cytoskeletal-dependent coalescence and "scrambling" of organelles in the paranuclear region coordinates apoptotic communication, caspase activation and cell death.

## 2.1 Introduction

Apoptosis, the process whereby cells die through an orchestrated self-destruction, occurs in response to environmental or developmental cues, cellular stresses, and specific cell death signals. This self-inflicted death, named for a characteristic rounding and "falling off" of cells, involves a number of evolutionarily conserved biochemical pathways that have been intensively studied for over two decades (reviewed in (Degterev and Yuan, 2008)). Apoptotic cell death is generally characterized by an inward collapse of organelles, a "blebbing" of the plasma membrane into vesicular apoptotic bodies, and the destruction of genetic material. The molecular events that drive such apoptotic processes were uncovered through genetic studies of the nematode *C. elegans*, which demonstrated the central importance of the *ced-3*, *ced-4* and *ced-9* genes in the control of an efficient cell death program. A search for the mammalian counterparts of *ced-3*, *-9* and *-4* identified *ced-9* as a homologue of the "B-cell Lymphoma" Bcl-2 oncogene and the more than 20 related Bcl-2 family members. Investigations of *ced-3* unveiled a family of 18 cysteinyl aspartate proteases, coined "caspases," that regulate and execute apoptosis through the cleavage of over 400 identified substrates with essential roles in cellular, metabolic and developmental processes, as well as inflammation, degenerative diseases and cancer (Degterev and Yuan, 2008; Eckhart et al., 2008; Luthi and Martin, 2007).

The molecular events regulating apoptosis are dependent on cell type as well as the context of death induction. Nonetheless, key molecular milestones are common to many of modes of cell death (Figure 2-1). "Executioner" caspases such as caspase-3 carry out the final, committed steps of the apoptotic program after activation of upstream, apical

"initiator" caspases, such as caspase-8, which activate apoptosis through two generalized pathways. In cells that employ a type I pathway, initiator caspases directly cleave and activate executioner caspases independent of actions at the mitochondria (Peter and Krammer, 2003). In type II cells, however, initiator caspases trigger the activation of executioner caspases through Bcl-2 proteins such as Bid, which promote the release of cytochrome c from mitochondria into the cytosol via mitochondria membrane permeabilization (MMP) (Peter and Krammer, 2003). Upon release, cytosolic cytochrome c binds to the apoptotic protease activating factor Apaf-1 to establish a multimeric "apoptosome" complex that activates caspase-9 to amplify the activation of executioner caspases.

The signaling cascades that ultimately activate cell death through caspases are initiated and regulated by organelle-specific events (Ferri and Kroemer, 2001). Death pathways, however, have only recently begun to be integrated with basic cell biological paradigms of intracellular protein and organellar trafficking. In this review, we describe the emerging links between the intracellular signaling events of apoptosis and trafficking at the molecular and organellar level. On the basis of classic as well as more recent data, we present a model of death receptor- and stress-induced apoptosis in which apoptotic events promote a coalescence of organelles and proteins *en route* to the paranuclear region to coordinate apoptotic interorganellar communication among lysosomes, mitochondria, the endoplasmic reticulum (ER), Golgi and nucleus.

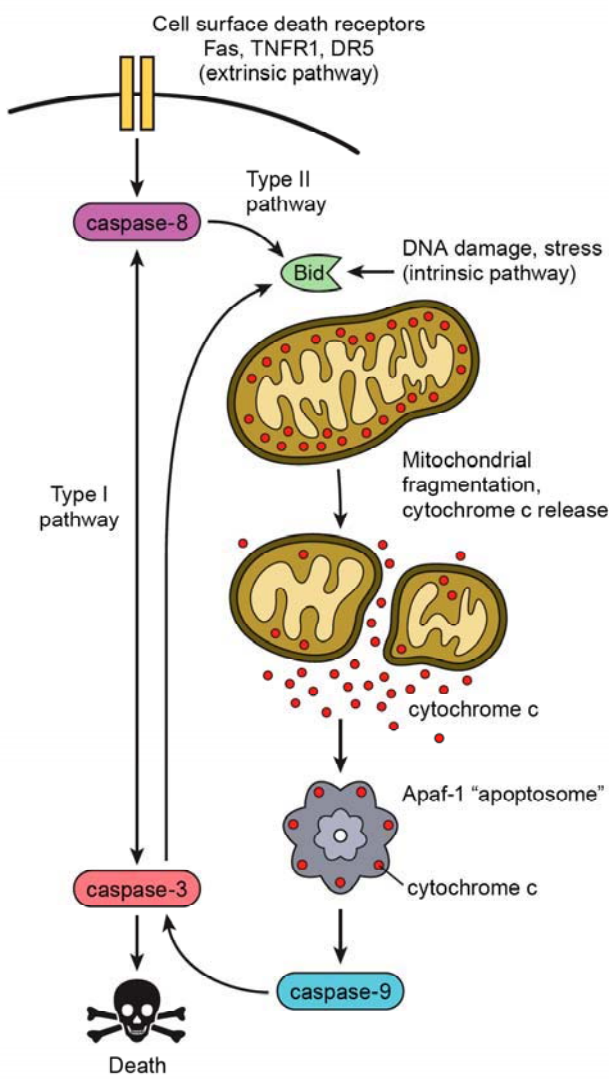


Figure 2-1: Essential pathways to caspase activation and cell death.

Apoptosis is initiated by internal cellular stress or extracellularly through the binding of ligands to cell surface death receptors. Type I pathways directly activate executioner caspases through initiator caspases to result in death. In Type II pathways, death signals are routed through the Bcl-2 proteins such as Bid and the mitochondria to control the release of cytochrome c. Cytosolic cytochrome c binds Apaf-1 to activate the apoptosome and caspase-9 to result in executioner caspase-3 activation and cell death.

## 2.2 Bcl-2 proteins regulate organellar "life or death" decisions

The Bcl-2 proteins regulate pro- and anti-apoptotic signaling processes at distinct organellar centers to modulate apoptotic communication, caspase activation and the ultimate decision to carry out cellular suicide (Figure 2-2). While the members of this diverse protein family are primarily noted for roles in regulating the release of apoptogenic factors such as cytochrome c from mitochondria (reviewed in (Kroemer et al., 2007)), they also perform a number of daily regulatory functions in healthy cells. This includes roles at the endoplasmic reticulum where Bcl-2 proteins maintain ER homeostasis (Hetz and Glimcher, 2008), in the nucleus to control genetic integrity (Zinkel et al., 2006), at synapses to modulate neurotransmission (Li et al., 2008) as well as at the mitochondria where they regulate mitochondrial division and cellular metabolism (Danial et al., 2008; Karbowski et al., 2006).

Bcl-2 protein family members are categorized on the basis of containing up to four functional Bcl-2 homology "BH domains" (reviewed in (Youle and Strasser, 2008)). "Multi-BH domain" proteins can participate in both pro- and anti- apoptotic functions while to date, single "BH3-domain only" proteins are strictly pro-apoptotic. The multi-BH domain members Bax, Bak and Bok localize in part to the mitochondrial outer membrane and are requisite for the formation of pores in the mitochondria to permit the release of proapoptotic factors such as cytochrome c, the second mitochondrial activator of caspases SMAC/DIABLO, the apoptosis initiating factor AIF, which activates caspases in the nucleus and Endonuclease G, an apoptotic DNase that degrades nuclear

DNA (Kroemer et al., 2007) (Figure 2-2). Pro-apoptotic BH3-domain only proteins such as Bid, Bim and PUMA, induce Bax and Bak activation upon apoptotic induction to promote MMP. Such permeabilization is regulated by anti-apoptotic multi-BH domain proteins such as the Bcl-2 protein itself, Bcl-xl, Bcl-w, Mcl-1 and A1 that prevent Bax and Bak activation by BH3-only pro-apoptotic relatives. While it is presumed that BH3-only proteins such as Bid and Bim directly interact with Bax and Bak to promote their activation and MMP, recent studies suggest that BH3-only proteins only act indirectly to activate MMP by binding to and interfering with anti-apoptotic Bcl-2 family members (Adams and Cory, 2007).

## 2.3 Activation and sequestration of Bax and Bad

Bax activation and translocation to mitochondria is regulated by Bax dimerization/oligomerization and dephosphorylation (Kroemer et al., 2007) and amplified by a feed-forward wave of caspase activation (Lakhani et al., 2006). Non-apoptotic Bax is sequestered in a monomeric state in the cytosol or loosely attached to mitochondria or the ER (Scorrano et al., 2003) (Figure 2-2). Upon apoptotic activation, Bax oligomerizes and exposes its C-terminal segment to form a hydrophobic protrusion that inserts into the outer mitochondrial membrane in a requisite step to induce cytochrome c release (Kroemer et al., 2007). Bax is phosphorylated at serine 184 by pro-survival kinases such as Akt/PKB which block Bax activation and translocation to mitochondria (Yamaguchi and Wang, 2001). This phosphorylation of Bax is influenced through sphingolipid signaling pathways that coordinate inter-organellar communication amongst the Golgi,

ER and mitochondria. A key sphingolipid in apoptotic signaling is ceramide which is synthesized *de novo* at the ER as well as at apoptotic mitochondria (Morales et al., 2007). Ceramide is also enzymatically formed through an apoptotic upregulation of sphingomyelinase activity at lysosomes (Bionda et al., 2004) (Figure 2). Ceramide directly binds to and activates diverse apoptotic enzymes including protein phosphatase PP2A (Morales et al., 2007), which dephosphorylates Bax at serine 184 to increase its association with mitochondria *in vitro* and *in vivo* (Birbes et al., 2005; Xin and Deng, 2006).

In healthy cells, Bax is held in a non-apoptotic, soluble, monomeric state by cytosolic retention factors. This is in contrast to some Bcl-2 family members such as Bim and Bmf which are sequestered to distinct compartments and cytoskeletal structures through associations with motor protein subunits (Puthalakath et al., 1999; Puthalakath et al., 2001). Bax cytosolic retention factors include humanin, a mammalian anti-apoptotic peptide (Guo et al., 2003); Ku70, a Bax deubiquitinylation protein and subunit of the Ku DNA-repair complex (Amsel et al., 2008); and the 14-3-3 proteins (Nomura et al., 2003). The 14-3-3 proteins bind more than 200 “client” phosphoproteins *in vivo* to mediate cell survival as well as cell cycle and cell division events (Pozuelo Rubio et al., 2004). 14-3-3 proteins sequester Bax in an unconventional Bax-phosphorylation independent manner (Nomura et al., 2003). More typically, 14-3-3 proteins bind and inactivate clients at specific phosphorylation sites. This includes Bad, which is phosphorylated by Akt/PKB at serine 136 to establish a 14-3-3 binding site (Datta et al., 2002). Upon loss of Akt survival signals or apoptotic activation of PP2A, Bad is dephosphorylated and releases

bound 14-3-3 proteins. Dephosphorylated Bad subsequently translocates to mitochondria to interact with and inhibit the anti-apoptotic effect of Bcl-2, thus activating Bax and MMP (Chiang et al., 2001; Datta et al., 2002). In addition to dephosphorylation, clients such as Bax, Bad and c-Abl are released from 14-3-3 proteins upon apoptotic Jnk phosphorylation of 14-3-3 itself which allows for client translocation to mitochondria or the nucleus upon apoptotic induction (Sunayama et al., 2005; Yoshida et al., 2005).

## 2.4 Bid translocates to mitochondria to activate Bax and MMP

Unlike Bax and Bad, activation of the pro-apoptotic "BH3 interacting domain death agonist" Bid occurs through its cleavage and myristoylation, which drive an association with mitochondria where Bid is required for Bax activation and MMP (Li et al., 1998; Luo et al., 1998). Upon death receptor ligation, Bid is cleaved at aspartate 59 by both initiator and executioner caspases, yielding a potently apoptotic truncated "tBid" (Gross et al., 1999b; Li et al., 1998; Luo et al., 1998). While both the full-length and truncated forms of Bid localize to mitochondria, tBid displays higher affinity for mitochondria and releases cytochrome c from isolated mitochondria *in vitro*. These observations of Bid regulation initially grounded a caspase cleavage-based model to explain Bid mitochondrial translocation (Gross et al., 1999b). In this classic model, cytosolic, full-length Bid is cleaved by caspase-8 and subsequently N-terminally myristoylated at the newly exposed amino terminus of glycine 60 (Zha et al., 2000). Myristoylated tBid is then inserted into mitochondrial membranes to activate Bax and drive apoptosis. Proteases other than caspases, however, cleave and activate Bid, including granzyme B, a

protease released from cytotoxic lymphocytes which cleaves Bid at aspartate 75 (Waterhouse et al., 2005). Lysosomal cathepsins also promote Bid cleavage and apoptosis (Heinrich et al., 2004). Cathepsins cleave Bid within an unstructured loop between two alpha helicies at tyrosine 47, glutamine 57, arginine 65 and arginine 71, generating tBid species that localize to mitochondria and induce apoptosis despite the lack of a myristoylatable glycine (Cirman et al., 2004). This suggests that myristoylation is not absolutely required to target Bid to the mitochondria.

## 2.5 Bid cleavage follows lysosomal permeablization and cathepsin release

The activation and release of lysosomal cathepsins has a substantial role in MMP and cell death as cathepsin inhibitors and gene knock-outs prevent apoptosis at the level of Bid cleavage (Cirman et al., 2004; Heinrich et al., 2004). This is evident in cultured fibroblasts from inclusion-cell disease (ICD) patients deficient in lysosomal hydrolase activity which are unresponsive to TNFα death pathways, failing to cleave Bid and activate caspases (Tardy et al., 2004). Likewise, mannose-6-phosphate receptor null mice which fail to deliver cathepsins to lysosomes have an ICD phenotype and do not respond to TNFα and CD95/FasL (Tardy et al., 2004). The mechanisms by which apoptotic pathways target cathepsins and lysosomes are not well understood but involve initiator caspase activation of lysosomal acidic sphingomyelinase (A-SMase) and its production of ceramide, which directly activates lysosomal cathepsins (Rotolo et al., 2005) (Figure 2). Active cathepsins are released to the cytosol upon an apoptotic Jnk-dependent translocation of Bax and Bim to lysosomes, a step inhibited by the Bax sequestering

protein Mcl-1 (Werneburg et al., 2007). As death receptor-mediated apoptosis promotes a co-localization of cathepsins and Bid (Heinrich et al., 2004), as well as an association of lysosomes with mitochondria (Ouasti et al., 2007), it has been suggested that cathepsin release and Bid cleavage take place at an interface between mitochondria and lysosomes (Terman et al., 2006).

## 2.6 PACS-2 translocates full-length Bid to mitochondria

The hypothesis that cleavage of Bid occurs along a mitochondria-lysosome axis suggests that Bid localizes to an interface between these organelles prior to cleavage (Terman et al., 2006). Consistent with such a model, FRET-based assays demonstrate that full-length Bid interacts with Bax upon treatment of HeLa cells with TNFα and that Bid is not cleaved until late into the apoptotic program after the activation of a caspase feedback amplification loop (Pei et al., 2007). In addition, full-length Bid itself translocates to mitochondria during the onset of cellular stress through a mechanism not explained by the cleavage/myristoylation model (Esposti et al., 2001; Sarig et al., 2003; Tafani et al., 2002). Bid is kept inactive through CK1 and CK2 phosphorylation of serine and threonine residues proximal to Bid's caspase cleavage site which block protease access and cleavage (Degli Esposti et al., 2003a; Desagher et al., 2001). Accordingly, CK1 phosphorylation of Bid is upregulated in pre-cancerous models of liver disease that block hepatocyte apoptosis (Appendix A and (Vogel et al., 2006)).

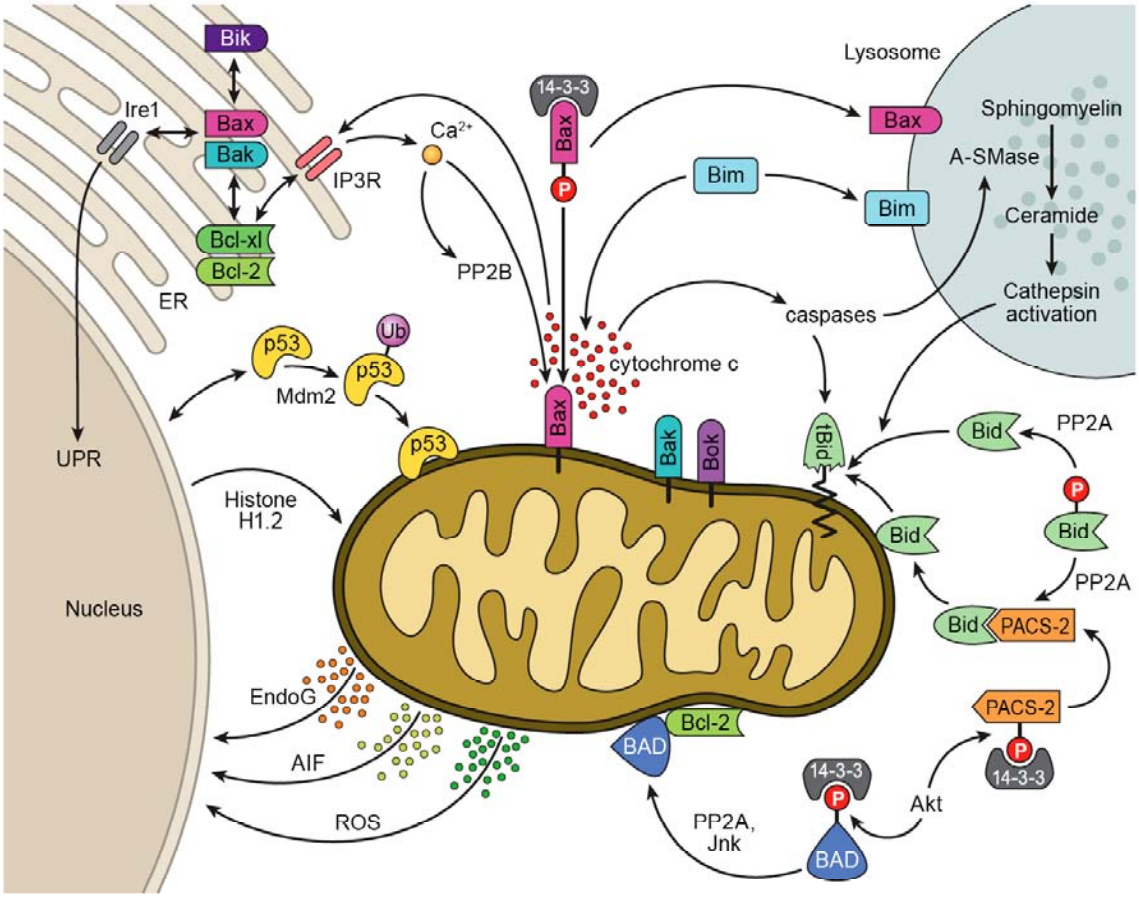


Figure 2-2. Bcl-2 proteins modulate apoptosis at multiple organellar sites.

Bcl-2 proteins converge on the mitochondria to control the release of apoptogenic factors such as cytochrome c and SMAC/DIABLO to enhance caspase activation (Kroemer et al., 2007). Factors such as Endonuclease G, AIF and reactive oxygen species are also released from the mitochondria to the nucleus to promote genome destruction (Ferri and Kroemer, 2001; Li et al., 2004). Mitochondrial Bax/Bak release events are activated by Bid translocation to mitochondria and cleavage of Bid by caspases or lysosomal cathepsins (Cirman et al., 2004; Gross et al., 1999b). Full-length Bid is trafficked to the mitochondria though associations with PACS-2 (Simmen et al., 2005). Active cathepsins are released from lysosomes upon the translocation of Bax and Bim to lysosomes and a caspase activation of A-SMase to produce ceramide which activates cathepsins (Heinrich et al., 2004; Rotolo et al., 2005; Werneburg et al., 2007). Apoptotic proteins such as Bad, Bax and PACS-2 are sequestered by 14-3-3 proteins and become active upon dephosphorylation and 14-3-3 release (Chiang et al., 2001). Factors such as p53 and Histone H1.2 also apoptotically target the mitochondria to modulate Bax activation and apoptosis (Konishi et al., 2003; Mihara et al., 2003). Bcl-2 proteins additionally regulate apoptosis via ER calcium release through IP3Rs to modulate ER-mitochondria crosstalk (Bassik et al., 2004; Li et al., 2007; Mathai et al., 2005) and influence the UPR through interactions with Ire1 (Hetz et al., 2006).

CK2 phosphorylation of Bid also regulates the binding of full-length Bid to the sorting protein PACS-2, a multi-functional acidic-cluster binding protein that integrates membrane traffic with ER-mitochondrial communication and apoptosis (Atkins et al., 2008; Myhill et al., 2008; Simmen et al., 2005). Upon the induction of apoptosis through death receptors or stress, PACS-2 associates with full-length Bid and translocates from the cytosol to the mitochondria. Loss of PACS-2 inhibits apoptotic Bid cleavage and executioner caspase activity but does not inhibit caspase-8, suggesting that Bid cleavage can occur after delivery to the mitochondria (Chapter 3; (Simmen et al., 2005)). The apoptotic activity of PACS-2 is regulated by Akt phosphorylation at serine 437, which binds 14-3-3 proteins (Chapter 4). Upon the induction of apoptosis, PACS-2 is dephosphorylated to release 14-3-3 proteins in a step prior to caspase-3 activation and cell death. These studies suggest a model in which apoptotic induction promotes the binding of full-length Bid to PACS-2 which subsequently translocates to mitochondria or a lysosome/mitochondria interface where Bid is cleaved to tBid to drive MMP, cytochrome c release and cell death.

## 2.7 Drp1-mediated mitochondrial fission recruits Bax and Bid to mitochondria

In respiring cells, mitochondria continuously fuse and divide to organize into reticular networks, exchange metabolites and facilitate mtDNA mixing (Chen et al., 2003). These processes of mitochondrial fusion and fission are also essential to the apoptotic program as apoptotic changes in mitochondrial size and shape regulate MMP and cytochrome c release. Fission and fusion events are regulated by GTPase proteins which alter the

morphology of mitochondrial membranes and cristae to fragment mitochondria and promote the release of cytochrome c and other factors from internal mitochondrial stores (Cerveny et al., 2007). One key GTPase, the dynamin related protein Drp1 (Karbowski et al., 2002), accumulates at fission sites of the outer mitochondrial membrane with a mitochondrial localized binding partner, hFis1, forming chain-like spirals at membrane scission sites (Ingerman et al., 2005).

The apoptotic translocation of Drp1, Bax and Bid to mitochondria appears to be interdependent, as Bax and Bid specifically concentrate at Drp1 generated mitochondrial scission sites (Karbowski et al., 2002) (Figure 4). Likewise, mitochondrial fission and Drp1 recruitment require a Bax/Bak dependent release of the Drp1 binding protein DDP/TIMM8a from inner mitochondrial stores which lead Drp1 to the mitochondria (Arnoult et al., 2005). Modification of Drp1 by the small ubiquitin related modifier SUMO via the SUMO conjugating enzyme Ubc9 also directs Drp1 to the mitochondria (Harder et al., 2004) while desumoylation by SENP5 limits Drp1 recruitment (Zunino et al., 2007). Treatment of cells with Mdivi-1, a specific chemical inhibitor of Drp1 GTPase activity, demonstrates that Drp1 is required to drive both mitochondrial fission and cytochrome c release (Cassidy-Stone et al., 2008). Interestingly, Mdivi-1 treatment of isolated mitochondria prevents the release of cytochrome c following tBid treatment *in vitro*, implicating Drp1 GTPase activity as a requirement for MMP and cell death (Cassidy-Stone et al., 2008).

Drp1 recruitment and mitochondrial fission are additionally under the control of apoptotic ER calcium release. Calcium activates PP2B/calcineurin to dephosphorylate Drp1 at serine 656 which activates mitochondrial fission, cytochrome c release and apoptosis (Cribbs and Strack, 2007) (Figure 4). Apoptotic ER calcium release, mitochondrial fission and mitochondrial fragmentation are controlled by caspases which cleave the integral resident ER 31 kD "B-cell Receptor Associated Protein" BAP31 (Breckenridge et al., 2003). In non-apoptotic cells, BAP31 associates with class I MHC molecules at the ER to regulate their ultimate delivery the plasma membrane (Ladasky et al., 2006). Upon apoptotic induction however, caspase-8 cleaves BAP31 to a $p20_{BAP31}$ fragment that promotes release of calcium from the ER (Breckenridge et al., 2003; Chandra et al., 2004).

## 2.8 Bcl-2 proteins regulate ER calcium release

The $p20_{BAP31}$-evoked release of calcium from the ER and subsequent fragmentation of mitochondria and apoptosis are under the control of ER-localized Bcl-2 proteins (Mathai et al., 2005). ER-based Bcl-2 proteins also modulate the unfolded protein response (UPR) through interactions with Ire1 which control ER communication with the nucleus (Hetz et al., 2006). The mechanisms by which Bcl-2 proteins localize to the ER remain uncharacterized, though ER associated Bcl-2 proteins are anchored through single transmembrane helical domains. ER calcium is released over the course of apoptotic induction through IP3 receptors which are regulated by direct interactions with Bcl-2 and Bcl-xl (Bassik et al., 2004; Joseph and Hajnoczky, 2007; Li et al., 2007).

Phosphorylation of Bcl-2 likely promotes an interaction with the IP3 receptor that blocks receptor phosphorylation (Oakes et al., 2005). Upon the induction of apoptosis, Bcl-2 is dephosphorylated and loses interactions with IP3Rs in favor of association with ER-localized Bax and Bak (Oakes et al., 2005). Loss of the Bcl-2 – IP3R interaction promotes IP3R phosphorylation, calcium release, mitochondrial fragmentation and apoptosis. The mechanism by which $p20_{BAP31}$ communicates with Bcl-2 proteins and IP3Rs is not yet understood; however, the ER-localized Bcl-2 family member Bik is required for the process (Mathai et al., 2005). Upon mitochondrial fragmentation, released cytochrome c translocates to the ER to bind to IP3Rs to increase calcium release through a feed-forward apoptotic mechanism requiring PACS-2 (Boehning et al., 2003; Myhill et al., 2008; Simmen et al., 2005).

Communication between the ER and mitochondria has a role in metabolic and apoptotic processes involving the exchange of ATP, lipids and calcium. Such communication is facilitated not only by the proximity of these two organelles, but by direct membranous contacts between the ER and mitochondria at mitochondria-associated membranes (MAMs) (Levine and Loewen, 2006; Rizzuto et al., 1998). Such contacts facilitate the direct transfer of calcium from the ER through IP3Rs to the mitochondria (Rizzuto et al., 1998). MAMs also serve as sites for the synthesis of sphingolipids and their transfer between the ER and mitochondria (Ardail et al., 2003; Bionda et al., 2004). The apposition of the ER and mitochondria is maintained by the multifunctional pro-apoptotic protein PACS-2, which mediates ER homeostasis in part by localizing the membrane-anchored ER-lumenal calcium-dependent chaperone Calnexin

to the ER (Myhill et al., 2008; Simmen et al., 2005). Apoptotic MAM signaling is further enhanced by an increased co-localization of mitochondria and ER at the paranuclear region to facilitate calcium transfer between these organelles (Darios et al., 2005).

## 2.9 Apoptotic communication between the mitochondria and the nucleus

An apoptotic apposition of the mitochondria to the nucleus facilitates the transfer of AIF, Endonuclease G and reactive oxygen species (ROS) directly from the mitochondria to the nucleus to promote genomic destruction (Ferri and Kroemer, 2001; Li et al., 2004). Mitochondria and nuclear communication may also be mediated by the shuttling of Bcl-2 proteins to and from these organelles to control cell cycle events (reviewed in (Zinkel et al., 2006)). These include Bcl-xl and Bcl-2 which promote cell cycle arrest; Bax, which increases S-phase progression and Bad, which regulates cell cycle transitions upon phosphorylation by Cdc2 (Zinkel et al., 2006). Bid also functions in the nucleus to maintain genomic stability. Bid-null mice display chromosomal abnormalities and develop a chronic myelomonocytic leukemia (CMML)-like phenotype (Zinkel et al., 2003). Phosphorylation of Bid at serine 78 by the DNA-damage sensing kinase ATM regulates an S-phase checkpoint, suggesting that Bid regulates genomic integrity following DNA damage (Kamer et al., 2005; Zinkel et al., 2005), though these results are controversial (Kaufmann et al., 2007). Nonetheless, disruption of the MRE11 complex, a key ATM activator, blocks phosphorylation of Bid serine 78 in response to ionizing radiation (Stracker et al., 2007). Furthermore, there is growing precedence for cytosolic apoptotic regulators participating in genomic maintenance regimens. A recently

described example includes Apaf-1, which translocates from the cytosol to the nucleus in response to DNA damage to promote checkpoint kinase Chk1 activation and cell cycle arrest in response to genotoxic stress (Zermati et al., 2007).

Nucleus-to-mitochondria apoptotic communication is mediated by Histone H1.2 (Konishi et al., 2003) and p53 (Mihara et al., 2003), the tumor suppressor that upregulates apoptotic genes in response cell cycle arrest and stress induced apoptosis. At the onset of apoptosis p53 is trafficked to the mitochondria where it binds Bcl-2 family members to activate Bax and release cytochrome c (Mihara et al., 2003). Monoubiquitinylation of p53 by cytosolic Mdm2, an E3 ligase typically considered responsible for tagging p53 for degradation, directs p53 to the mitochondria, which is subsequently deubiquitinylated by mitochondrial-localized HAUSP (Marchenko et al., 2007). The mechanism by which Histone H1.2 travels from the nucleus to the mitochondria is not understood. However, p53 is required to release Histone H1.2 from the nucleus upon damage induced by ionizing radiation in a process requiring Chk2 phosphorylation and stabilization of p53 (Chen et al., 2005a).

## 2.10 Paranuclear organellar clustering mediates apoptotic communication and cell death

Bcl-2 family members regulate caspase activation and apoptosis in an organelle-specific manner (Ferri and Kroemer, 2001). However, it remains unknown how the distribution of apoptotic organelles modulates the cell death program. While the

proximity and direct contacts of the endoplasmic reticulum and mitochondria have an essential role in apoptotic communication (Levine and Loewen, 2006; Pizzo and Pozzan, 2007) it is enigmatic how other systems such as the nucleus, lysosomes, and Golgi apparatus come together to communicate with one another during apoptosis. Interestingly, in a process reminiscent of mitosis (Sesso et al., 1999), fragmented apoptotic organelles collect near the Golgi apparatus and microtubule organizing center (MTOC) through the action of mircrotubule-associated motor proteins. This co-localization may represent a functional coalescence of organelles at the paranuclear region to control a number of steps at the induction of apoptosis (Ouasti et al., 2007). This may include the direct, vesicle-independent transfer of sphingolipids such as ceramide amongst mitochondria, lysosomes and the Golgi (Hu et al., 2005; Morales et al., 2007) or perhaps the shuttling of apoptogenic molecules between the mitochondria and nucleus (Li et al., 2004; Zinkel et al., 2006). An early apoptotic, microtubule-dependent redistribution of mitochondria to the Golgi-proximal MTOC serves as the best characterized example of this apoptotic paranuclear relocalization, and is observed upon exposure to TNFα (De Vos et al., 1998), the TNFα Related Apotosis Inducing Ligand TRAIL/Apo2L (Thomas et al., 2000), FasL/CD95/Apo1L (Ouasti et al., 2007), ceramide (Darios et al., 2005), oxidative stress (Dewitt et al., 2006) and viral infection (Schepis et al., 2006).

## 2.11 Molecular motors drive apoptotic paranuclear clustering

In non-apoptotic cells, microtubule associated (+)-end directed kinesin motors traffic mitochondria away from the paranuclear region (Frederick and Shaw, 2007; Tanaka et al., 1998). Knock-out deletions of kinesin genes such as KIF5B result in an abnormal paranuclear clustering of mitochondria in embryonic knockout cells (Tanaka et al., 1998) similar to clustering observed in apoptotic cells (De Vos et al., 1998). If paranuclear trafficking of mitochondria were to serve a pro-apoptotic function, inhibition of kinesin activity would likely enhance apoptosis. Indeed, immunoinhibition of (+)-end directed kinesin motors blocks the basal dispersal of mitochondria in healthy cells, resulting in a paranuclear clustering of mitochondria and a synergistic increase in the apoptotic effects of TNFα (De Vos et al., 2000). While kinesin knock-out or siRNA knock-down cells have not yet been explored in studies of mitochondrial clustering and apoptosis, conditional knock-out deletions of KIF3A in photoreceptor cells and renal cells result in apoptotic cell death (Lin et al., 2003; Marszalek et al., 2000). Apoptotic paranuclear clustering of mitochondria appears to be a mechanistic consequence of apoptotic induction as stress activated MAP kinases p38 and Jnk phosphorylate and inactivate the kinesin light chain (De Vos et al., 2000) and release KIF5B from microtubules to halt mitochondrial dispersal (Stagi et al., 2006) (Figure 3).

As the inactivation of kinesin promotes the trafficking of mitochondria to the paranuclear region, retrograde clustering of organelles is likely driven by a net increase in (-)-end motor activity of dynein and dynactin. Dynein and dynactin complexes associate

with mitochondria on microtubules, and disruption of dynein function disperses mitochondria away from the MTOC (Varadi et al., 2004b). Mitochondrial paranuclear clustering is intimately coordinated with mitochondrial fission, as inhibition of fission prevents mitochondrial clustering and apoptosis (Huang et al., 2007). The Drp1 GTPase, together with Bax and Bid, are recruited to dynein complexes at mitochondrial fission sites as mitochondria travel in the (-)-end direction (Varadi et al., 2004b) (Figure 4). Targeting of Drp1 to sites of mitochondrial scission is in part mediated by sumoylation of Drp1, as sumoylated proteins generally cluster at mitochondrial fission sites (Harder et al., 2004). Sumoylation of Drp1 may direct Drp1 specifically to dynein motor complexes as sumoylation promotes the interaction of cargo molecules with dynein to mediate their retrograde transport (van Niekerk et al., 2007). Like other apoptotic effectors, dynein and dynactin complex proteins such as the cytoplasmic dynein intermediate chain CD-IC and p150glued are regulated by caspase cleavage and myristoylation (Lane et al., 2001; Martin et al., 2008). While it remains to be determined if cleavage and lipid modification anchors a functional, truncated CD-IC fragment to mitochondria, GFP-tagged truncated CD-IC localizes in vesicular mitochondrial-like structures to the paranuclear region (Martin et al., 2008).

## 2.12 Organelles cluster at the paranuclear region via stabilized microtubules

In proliferating cells, microtubules radiate from the MTOC as a web-like network that acts as a scaffold for mitochondria and other organelles to disperse throughout the cell (Frederick and Shaw, 2007). Cellular stress, as well as specific cell cycle events,

stabilizes microtubules such that they become more rigid and rod-like and collapse towards the MTOC, forming concentric rings around the nucleus that may act as a net to bring mitochondria and other associated organelles to the paranuclear region. Death-receptor induced apoptosis triggers microtubule stabilization, mitochondrial clustering and apoptosis through p38 phosphorylation and inactivation of the microtubule destabilizing oncoprotein family member Op18/Stathmin (Mizumura et al., 2006; Vancompernolle et al., 2000) (Figure 2-3). This p38-mediated stabilization of microtubules phenocopies the stabilization seen in cells treated with chemotherapeutic agents such as Taxol, which inhibit tumor cell growth by stabilizing microtubules to disrupt mitosis (Jordan and Wilson, 2004). Interestingly, Taxol treatment results in the co-localization of mitochondria with microtubule bundles at the paranuclear region (Karbowski et al., 2001). This redistribution of mitochondria upon the addition microtubule stabilizing agents may explain how these drugs synergize the apoptotic effects of death receptor ligands such as TNFα and TRAIL.

Microtubule stability is regulated by microtubule associated proteins such as MAP1S/C19ORF5, which bind to both mitochondria and stabilized microtubules in stressed or Taxol-treated cells (Liu et al., 2005) (Figure 2-3). MAP1S associates with other microtubule binding proteins such as the tumor suppressor RASSF1A, (Dallol et al., 2004; Liu et al., 2005) and UXT (Ubiquitously Expressed Transcript), a γ-tubulin/centrosome associated protein that induces paranuclear clustering of mitochondria (Moss et al., 2007). UXT, RASSF1A and MAP1S all interact with the leucine-rich protein LRPPRC in microtubule-associated complexes at an interface between

paranuclear mitochondria and the nucleus (Liu and McKeehan, 2002). As LRPPRC and UXT both interact with several components of the RNA polymerase II complex and CBP/p300 (Liu et al., 2002), it has been hypothesized that LRPPRC and UXT link microtubule stabilization and mitochondrial apoptosis to transcriptional as well chromatin remodeling events in the nucleus (Liu et al., 2002).

Microtubule-associated proteins with pathological functions such as tau, a prime suspect in Alzheimer's disease, also cause paranuclear mitochondrial clustering. This occurs in part through an association of tau with motor proteins and microtubules (Ebneth et al., 1998) (Figure 2-3). The mechanism by which tau contributes to clustering is not straightforward, however, as apoptotic CDK5 phosphorylation of tau conversely promotes paranuclear co-localization of mitochondria and the ER through a tau microtubule dissociation (Darios et al., 2005). Other pathogenic factors, such as the pro-apoptotic Tat protein encoded by HIV-1, bind to microtubules to result in a Taxol-like stabilization and cell death (Chen et al., 2002). Microtubule proteins implicated in tumorigenesis such as the adenomatous polyposis coli (APC) protein and the Von Hippel-Lindau VHL tumor suppressor also coordinate nuclear as well as apoptotic events (Brocardo et al., 2008; Dikovskaya et al., 2007; Hergovich et al., 2003). As APC and VHL both localize in part to mitochondria (Brocardo et al., 2008; Shiao et al., 2000) it is enticing to speculate that these tumor suppressors will participate in the apoptotic paranuclear clustering of microtubules and organelles; however, this has not yet been addressed.

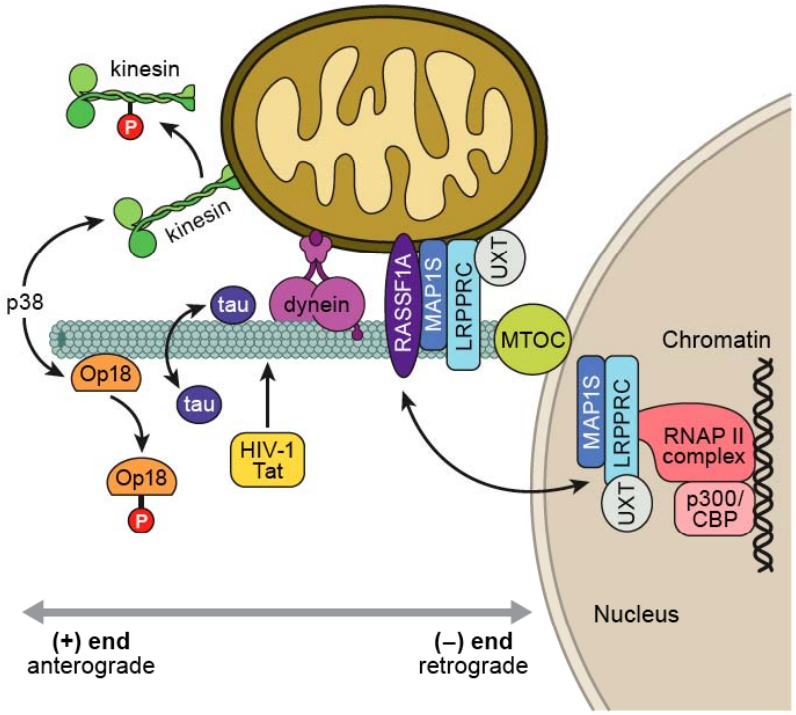


**Figure 2-3. Microtubule associated motors and stabilizing proteins regulate paranuclear mitochondrial clustering and apoptosis.**

Apoptotic p38 phosphorylation of kinesin subunits halts anterograde traffic of mitochondria to promote dynein driven (-)-end accumulation (De Vos et al., 2000). p38 phosphorylation also releases Op18 from microtubules to result in microtubule stabilization and mitochondrial clustering (Mizumura et al., 2006; Vancompernolle et al., 2000). Mitochondrial aggregation and apoptosis are also influenced through microtubules by tau (Darios et al., 2005; Ebneth et al., 1998) and HIV-1 Tat (Chen et al., 2002). MAP1S – LRPPRC – UXT complexes also stabilize microtubules and localize to apoptotic paranuclear mitochondria as well as the nucleus in complex with RNAPII and p300/CBP (Dallol et al., 2004; Lin et al., 2003; Liu et al., 2002; Liu and McKeehan, 2002; Moss et al., 2007). It has been proposed that these microtubule and nucleus associated proteins coordinate both apoptotic mitochondrial clustering and chromatin remodeling events (Liu et al., 2002; Liu and McKeehan, 2002).

## 2.13 Apoptotic signaling proteins cluster at the paranuclear region upon death induction

A number of apoptotic signaling proteins collect in the paranuclear region during the course of apoptosis. Such proteins include p53, which localizes predominantly to the cytosol in non-apoptotic cells through an association with microtubules (Giannakakou et al., 2000). Non-apoptotic p53 is sequestered by a recently identified Parkin-like ubiquitin ligase, Parc, which binds to but does not apparently ubiquitinylate cytosolic p53 (Nikolaev et al., 2003). The mechanism by which p53 is released from Parc and the physiological role of Parc as a ubiquitin ligase are, however, not yet understood. Upon DNA damage, p53 is released from Parc and driven to the paranuclear region by microtubule-associated dynein motors (Giannakakou et al., 2000). p53 associates with microtubules and the dynein motor complex through the dynein light chain LC8, which binds to the p53 binding protein 53BP1, a key cytosolic mediator of the DNA damage response (Lo et al., 2005). A p53 – 53BP1 interaction is required for the paranuclear clustering of p53 upon DNA damage and subsequently apoptosis (Lo et al., 2005). This LC8-driven relocalization of p53 to the paranuclear region may be part of a mechanism to import p53 into the nucleus from the cytosol (Moseley et al., 2007), and may explain how p53 is recruited to mitochondria during apoptosis (Mihara et al., 2003). LC8 additionally binds the Bcl-2 family member Bim and is also a target of the p21 activated kinase PAK1 to promote cell survival and tumorigenesis (Vadlamudi et al., 2004). Whether LC8 or other dynein regulatory factors have roles in bringing additional proteins or organelles to

the paranuclear region to coordinate apoptotic signaling events will be an important line of investigation in uncovering the relation of cytoskeletal motor activity to apoptosis.

## 2.14 Apoptotic fragmentation and membrane scrambling of the Golgi apparatus

The Golgi apparatus neighbors the MTOC at the paranuclear region in an ideal locale to influence microtubule-based apoptotic processes (Hicks and Machamer, 2005; Rios and Bornens, 2003). The cisternal stacks of Golgi membranes are maintained by microtubule and actin cytoskeletal structures (Rios and Bornens, 2003), as well as Golgi-specific structural proteins such as the Golgins and GRASPs (Golgi Reassembly Stacking Proteins) (Rios and Bornens, 2003). Like other membranous organellar systems, the Golgi apparatus undergoes a characteristic fragmentation early in the induction of apoptosis that precedes or coincides with mitochondrial cytochrome c release (Lane et al., 2002; Mukherjee et al., 2007). Golgi fragmentation is triggered by the Golgi-localized initiator caspase-2 cleavage of Golgin-160 followed by the executioner caspase-3 cleavage of GRASP65 (Lane et al., 2002; Mancini et al., 2000) and the Golgi vesicle tethering protein p115 (Chiu et al., 2002). The C-terminal cleavage product of p115 alone is capable of fragmenting the Golgi and relocates to the nucleus to serve a pro-apoptotic role (Chiu et al., 2002). In contrast, cleaved Golgin-160 enters the nucleus to block apoptosis (Hicks and Machamer, 2005).

Although it was originally hypothesized that Golgi fragmentation was the result of an apoptotic breakdown of the cytoskeleton, Golgi fragmentation occurs early in

apoptosis and precedes major apoptotic changes in cytoskeletal structures (Mukherjee et al., 2007). This suggests that an intact cytoskeleton and MTOC are required to traffic factors to the Golgi prior to fragmentation. As mitochondria and other organelles cluster at the Golgi/MTOC in a microtubule-dependent fashion at the induction of apoptosis (Ouasti et al., 2007), the Golgi may serve as a site for organelles to intermingle at the induction of apoptosis. Such a stress-induced co-localization of organelles may promote organellar cross-talk processes, including the transfer of apoptogenic lipids such as ceramide between lysosomes, Golgi and mitochondria. Organellar co-localization is an early consequence of FasL treatment of CEM cells which results in a net redistribution of endosomes, lysosomes and mitochondria to overlapping sucrose density fractions (Matarrese et al., 2008). This organellar convergence may represent a physical coalescence and "scrambling" of organelles as FasL treatment also increases the levels of Golgi, endosomal and lysosomal membrane markers that co-purify with apoptotic paranuclear mitochondria in a step prior to caspase activation, Golgi fragmentation and cytoskeleton breakdown (Ouasti et al., 2007). Interestingly, the global caspase inhibitor zVAD-fmk blocks this stress-induced mixing of Golgi and mitochondrial markers but increases the presence of endo-lysosomal markers associated with apoptotic mitochondria (Matarrese et al., 2008; Ouasti et al., 2007). Together, these experiments suggest a multi-step model in which mitochondria and endo-lysosomes first physically associate to release cathepsins and cleave Bid prior to Golgi-mitochondrial scrambling, Golgi fragmentation and mitochondrial cytochrome c release.

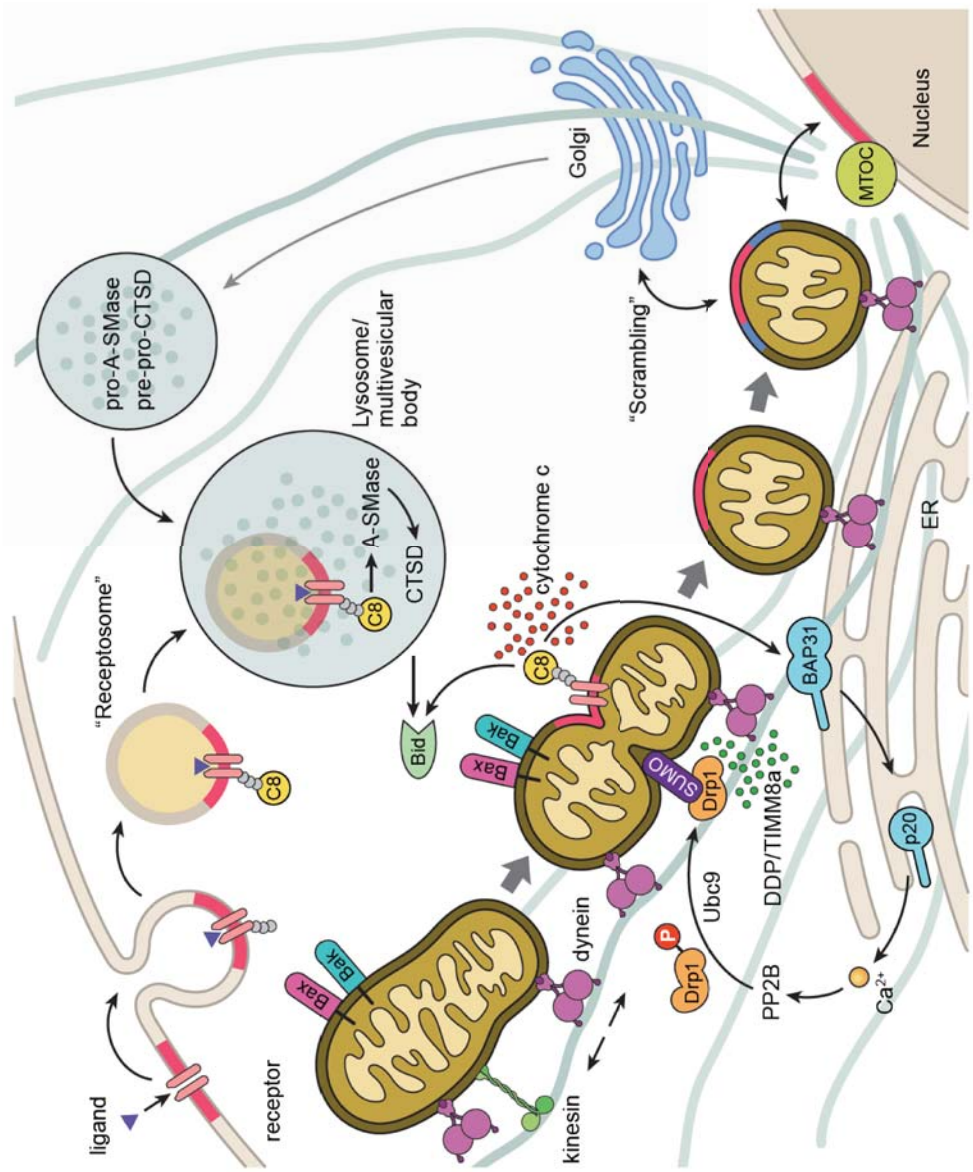
Golgi
Nucleus
MTOC
pro-A-SMase
pre-pro-CTSD
Lysosome/
multivesicular
body
A-SMase
CTSD
C8
"Scrambling"
"Receptosome"
cytochrome c
Bid
Bak
Bax
SUMO
Drp1
BAP31
ER
ligand
receptor
dynein
kinesin
Ubc9
DDP/TIMM8a
p20
PP2B
$Ca^{2+}$
P

**Figure 2-4. Death receptor ligation promotes mitochondrial fragmentation, mitochondrial clustering, and membrane "scrambling".**

Cell surface death receptors concentrate in GD3-containing lipid rafts (in red) (Malorni et al., 2007). Upon death ligand engagement, receptors recruit death adaptors and are internalized with rafts to specific endosomal compartments such as "TNF receptosomes" to promote caspase-8 (C8) activation (Micheau and Tschopp, 2003; Schneider-Brachert et al., 2004). Receptosomes fuse with precursor hydrolase-containing Golgi-derived vesicles to form lysosomal multivesicular bodies that activate A-SMase and cathepsin D (CTSD). Caspases and cathepsins cleave Bid to promote Bax/Bak activation, cytochrome c release and caspase activation (Cirman et al., 2004; Gross et al., 1999b; Heinrich et al., 2004). Mitochondrial-localized caspase-8 cleaves ER localized BAP31 to a p20 fragment which promotes ER calcium release (Breckenridge et al., 2003; Chandra et al., 2004), PP2B activation and Drp1 dephosphorylation (Cribbs and Strack, 2007) and mitochondrial fragmentation. Mitochondrial DDP/TIMM8a release (Arnoult et al., 2005) and Drp1 sumoylation (Harder et al., 2004; Zunino et al., 2007) promote the translocation of Drp1 to mitochondrial scission sites as mitochondria travel in the (-)-end direction (Varadi et al., 2004b). Bax and Bid are also recruited to these scission sites that are rich in GD3 (in red). The accumulation of fragmented mitochondria at the MTOC in proximity to the Golgi results in a caspase dependent fragmentation of the Golgi (Mukherjee et al., 2007) and a "scrambling" of Golgi membranes (in blue) with mitochondria (Ouasti et al., 2007). As GD3-contatining rafts (in red) internalize to endosomes and later localize to mitochondria, it is hypothesized that mitochondria act as "cargo boats" to carry GD3 from the cell surface to the nucleus and adjacent organelles (Garofalo et al., 2007).

## 2.15 GD3 lipid rafts link plasma membrane-to-nucleus signaling and paranuclear trafficking

A mixing of the Golgi apparatus, endosomes, lysosomes, mitochondria and plasma membrane is also evident in the interorganellar apoptotic trafficking of the glycosphingolipid GD3 (Garofalo et al., 2005; Garofalo et al., 2007; Malorni et al., 2007) (Figure 2-4). This ganglioside is produced in the Golgi from ceramide by GD3-synthase and is capable of permeabilizing mitochondria to promote cytochrome c release *in vitro* (Rippo et al., 2000). In healthy hepatocytes and T-cells, GD3 localizes predominantly to the plasma membrane in death receptor-rich lipid rafts. TNFα treatment of hepatocytes, however, redistributes GD3 from the plasma membrane to mitochondria via internalization through endosomal compartments in a process requiring an intact cytoskeleton (Garcia-Ruiz et al., 2002). Upon Fas/TNFR1/DR5 death-receptor engagement, cell-surface-localized death induced signaling complexes (DISCs) internalize through receptor-mediated endocytosis (Lee et al., 2006; Micheau and Tschopp, 2003) to specific endosomal compartments such as "TNF Receptosomes," which subsequently associate with and activate caspase-8 (Schneider-Brachert et al., 2004). Receptosomes may represent DISC-rich GD3 raft-containing endosomes that co-localize with mitochondria to promote cell death (Giammarioli et al., 2001). It has been hypothesized that this co-localization represents a mixing of endosomes with mitochondria, as GD3-containing lipid rafts are found on mitochondria after FasL treatment of CEM cells (Garofalo et al., 2005). These GD3 rafts contain mitochondrial signaling complexes that include the voltage dependent anion channel VDAC-1 and

hFis1 and act as recruitment sites for Bax, Bid and presumably Drp1 (Garofalo et al., 2005). The integrity of these rafts appears to have a role in MMP, as their chemical disruption prevents mitochondrial permeablization in response to exogenous tBid treatment (Garofalo et al., 2005). Like ceramide, GD3 has pleiotropic effects on apoptotic activation at the mitochondria, as well as the Golgi, ER and nucleus (Malorni et al., 2007). As GD3 raft-containing mitochondria and other vesicular structures collect at the paranuclear region upon the induction of apoptosis, such fissured mitochondria may act as "cargo boats" that carry DISC-containing GD3-rafts to multiple organelles at the paranuclear region (Garofalo et al., 2007) (Figure 4). This hypothesis may explain how GD3 traffics to the nucleus upon apoptotic induction by CD95/FasL to modulate histone structures and transcription (Tempera et al., 2008).

## 2.16 Perspectives and Conclusion

The past decade has brought immense progress in identifying key proteins and signaling pathways that initiate and regulate cell death through organelle-specific processes. Although many of these players first gained attention for their roles in cell death, it is now recognized that several organelle-directed apoptotic decision-makers have "day jobs" that relate their apoptotic functions to physiological roles. Future work will undoubtedly aim to describe how the trafficking of such multi-functional players occurs in apoptotic and non-apoptotic contexts. A recent and noteworthy example is provided by the Bcl-2 protein Bad. In addition to promoting cell death, Bad also translocates to mitochondria in a non-apoptotic role to shuttle glucokinase to respiring

mitochondria during high-fat feeding to metabolize glucose and stimulate the release of insulin (Danial et al., 2008). Physiological and apoptotic cross-functionality is, of course, not limited to Bcl-2 family members. The Drp1 GTPase functions in both apoptosis and mitosis to divide mitochondria and also modulates neurotransmission at synapses (Li et al., 2008). Multi-functionality is also seen with the PACS-2 sorting protein which regulates both ER-mitochondrial trafficking and communication as well as apoptosis (Atkins et al., 2008; Myhill et al., 2008; Simmen et al., 2005). Likewise, overlapping functionality is also seen in signaling systems that regulate DNA quality control such as ATM/ATR as well as those that signal cell cycle transitions and cell division events such as Cdc2 and the checkpoint kinases. These and other genetic regulatory kinases phosphorylate apoptotic proteins such as Bad and Bid to control cell cycle and cell division events. Such examples of cross-functionality hint that mitotic cell birth and apoptotic cell death may share fundamental machinery and regulatory mechanisms.

Individual organellar systems such as lysosomes and mitochondria initiate and regulate apoptosis (Ferri and Kroemer, 2001); however, the relation of organellar trafficking and apoptotic signaling pathways is only now coming to light. An apoptosis-induced co-localization of organelles at the MTOC / Golgi / paranuclear region potentially serves a number of functions in cell death to help package and redistribute fragmented organelles, to facilitate nuclear destruction and also to promote a general shrinkage of dying cells. Organellar coalescence also takes place in autophagic organellar destruction as part of a mechanism to traffic degrading mitochondria to lysosomes (Terman et al., 2006). Likewise, autophagosomes as well as misfolded protein aggregates in “aggresomes” are

targeted to paranuclear-localized lysosomes through dynein/dynactin motor activity (Kimura et al., 2008; Rodriguez-Gonzalez et al., 2008). If paranuclear clustering and organellar mixing is indeed a *bona fide* component of the highly regulated apoptotic program, a number of questions regarding the energetics and specificity of this process demand exploration. Interestingly, a subset of Bcl-2 family members, the BNIPs, associate with components of membrane fusion machinery such as the SNARE protein syntaxin 18 to modulate apoptosis in an αSNAP-dependent manner (Nakajima et al., 2004). Future studies relating microtubule structure and motor activity to organellar clustering and the transfer of organellar markers (Degli Esposti, 2008) will prove insightful and clarify the role of scrambling in the induction of cell death.

## 2.17 Acknowledgements

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# Chapter 3. PACS-2 controls ER-mitochondria communication and Bid mediated apoptosis.

Thomas Simmen, Joseph E. Aslan, Anastassia D. Blagoveshchenskaya, Laurel Thomas, Lei Wan, Yang Xiang[1], Sylvain F. Feliciangeli, Chien-Hui Hung, Colin M. Crump[2] and Gary Thomas[3]

Vollum Institute, 3181 SW Sam Jackson Park Road, Portland OR, 97239, USA, [1]Current address, Department of Molecular and Cellular Physiology and Medicine, Stanford University, Stanford CA 94305, [2]Current address, Department of Pathology, University of Cambridge, Cambridge CB2 1QP UK

[3]Corresponding author; Tel: (503) 494-6955; Email: thomasg@ohsu.edu

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In this chapter I performed the experiments in Figures 4D, 6A, and 6D and assisted in the experiments in Figures 2A, 2D, 3C, 4C, and 4E. T. Simmen performed the experiments in Figures 1E, 1F, 2A, 2B, 2C, 2E, 2F, 3, 4A-C, 4E, 5, and 6B. A. Blagoveshchenskaya performed the MAM fractionation (Figure 2D). L. Thomas performed and assisted with immunofluorescence experiments with T. Simmen (Figures 1B, 1E, 5A) and S. Feliciangeli (Figure 1D). L. Thomas and C. Hung performed the Bid-GFP translocation assay (Figure 6C). C. Crump performed the siRNA control experiment (Figure 1C) and was instrumental in identifying and characterizing the PACS-2 gene with L. Wan and Y. Xiang. I also participated in writing and editing the manuscript and addressing reviewer concerns with T. Simmen and G. Thomas.

**Summary**

The endoplasmic reticulum (ER) and mitochondria form contacts that support communication between these two organelles, including synthesis and transfer of lipids, and the exchange of calcium, which regulates ER chaperones, mitochondrial ATP production, and apoptosis. Despite the fundamental roles for ER-mitochondria contacts, little is known about the molecules that regulate them. Here we report the identification of a multifunctional sorting protein, PACS-2, that integrates ER-mitochondria communication, ER homeostasis and apoptosis. PACS-2 controls the apposition of mitochondria with the ER, as depletion of PACS-2 causes the BAP31-depedent mitochondria fragmentation and uncoupling from the ER. PACS-2 also controls formation of ER lipid-synthesizing centers found on MAMs and ER homeostasis. However, in response to apoptotic inducers, PACS-2 translocates Bid to mitochondria, which initiates a sequence of events including the formation of mitochondrial tBid, the release of cytochrome c, and the activation of caspase-3, thereby causing cell death. Together, our results identify PACS-2 as a novel sorting protein that links the ER-mitochondria axis to ER homeostasis and the control of cell fate, and provide new insights into Bid action.

## 3.1 Introduction

The endoplasmic reticulum (ER) controls multiple cellular processes including translocation of soluble and membrane proteins into the secretory pathway, detoxification of metabolites, and biosynthesis of lipids. The ER also serves as the principal internal store of calcium ions that mediate signaling, ATP production, and apoptosis (Voeltz et al., 2002). Extensive biochemical and genetic studies have revealed that communication between the ER, Golgi, and endosome/lysosomes is controlled largely by vesicular traffic mediated by components of the COPII, COPI, and clathrin-based sorting machinery (Bonifacino and Lippincott-Schwartz, 2003). However, high resolution 3D electron tomography reveals that the expansive, reticulated ER forms close contacts with each of these secretory pathway compartments, and with mitochondria (Marsh et al., 2001). Indeed, as much as 20% of the mitochondrial surface is in direct contact with the ER, underscoring the dynamic and highly regulated communication between the ER and mitochondria (Marsh et al., 2001; Rizzuto et al., 1998). The close contacts formed between the ER and mitochondria have led to the model that ER-mitochondria communication may occur by direct transfer rather than vesicular traffic. In support of this model, biochemical studies reveal that the ER also communicates with mitochondria through mitochondria-associated membranes (MAMs), which are ER-contiguous membranes that contain multiple phospholipid- and glycosphingolipid-synthesizing enzymes, including fatty acid CoA ligase 4 (FACL4) and phosphatidylserine synthase-1 (PSS-1), and support direct trasnsfer of lipids between the ER and mitochondria (Piccini et al., 1998; Stone and Vance, 2000).

In addition to supporting lipid transfer, the apposed ER and mitochondria also exchange calcium ions, which regulate processes ranging from ER chaperone-assisted folding of newly synthesized proteins to the regulation of mitochondria-localized dehydrogenases involved in ATP-producing Krebs cycle reactions, and the activation of calcium-dependent enzymes that execute cell death programs (Berridge, 2002). Immunocytochemical studies show that regions of the ER apposed to mitochondria are enriched with IP3 receptors, identifying these zones as "hotspots" of calcium transfer from the ER to the mitochondria (Rizzuto et al., 1998). Interference with calcium homeostasis or calcium communication between the ER and mitochondria, for instance by treatment of cells with thapsigargin, which blocks uptake of calcium by the ER (Hajnoczky et al., 2000), causes a malfunction of ER-localized protein folding, leading to an accumulation of unfolded proteins. As a consequence, ER-localized enzymes that catalyze oxidative protein folding malfunction and unfolded proteins accumulate. This stress induces an unfolded protein response (UPR), which coordinates the suppression of general protein synthesis with the increased expression of ER chaperones in order to reestablish ER homeostasis. However, if ER homeostasis fails to be reestablished, the UPR triggers apoptosis (Rutkowski and Kaufman, 2004).

Apoptosis is executed by caspases, which catalyze the systematic dissolution of structural components, resulting in cell death (Boatright and Salvesen, 2003). The induction of apoptosis is often triggered by initiator caspases, among them caspase-8. Apoptotic signals leading to caspase-8 activation are defined as either extrinsic apoptotic pathways, which are initiated by the binding of Fas ligand or TNF-α to death receptors on

the cell surface, or as intrinsic apoptotic pathways, cued by intracellular signals. The subsequent fission of mitochondria marks an early and key step in apoptotic programs. Caspase-8 promotes mitochondria fission by cleaving the ER cargo receptor BAP31 to form p20, which induces Drp1/Dlp1, a mitochondria-localized dynamin, to fissure mitochondria (Breckenridge et al., 2003). Mitochondria fragmentation promotes the recruitment and activation of proapoptotic molecules that cause mitochondria permeabilization, thereby activating distal steps in the apoptotic program (Karbowski and Youle, 2003).

Mitochondria permeabilization is regulated by a balance between the activities of Bcl-2 proteins, which include anti-apoptotic members such as Bcl-2 and Bcl-xL, or pro-apoptotic members such as Bid, Bak and Bax (Sharpe et al., 2004). Bid is a requisite component of both intrinsic and extrinsic apoptotic pathways. Apoptotic signals induce Bid dephosphorylation, which results in Bid cleavage by caspase-8 to form the potently apoptotic, truncated Bid (tBid) (Desagher et al., 2001; Gross et al., 1999b). Myristoylation of tBid has been proposed to act as a "switch" to target it to mitochondria (Zha et al., 2000), where the tBid BH3 domain interacts with Bak and Bax on the outer mitochondria membrane to form pores that release cytochrome c into the cytosol (Wei et al., 2001). The released cytochrome c activates caspase-3, an executioner caspase that controls the distal stages of the apoptotic program (Tewari et al., 1995). However, recent studies show that full-length Bid can translocate to mitochondria, and subsequently lead to membrane permeabilization and cytochrome c release (Degli Esposti et al., 2003b; Sarig et al., 2003; Tafani et al., 2002). Together with other studies reporting activated

caspase-8 in the cytosol (Micheau and Tschopp, 2003) as well as on mitochondria (Chandra et al., 2004), these studies suggest that Bid cleavage is not required for mitochondria targeting and that tBid formation may occur subsequent to the targeting of full-length Bid to mitochondria.

Previously, we identified a sorting protein, PACS-1, which binds to cargo molecules in a phosphorylation-state-dependent manner and directs their transport from endosomes to the trans-Golgi network (TGN) (Crump et al., 2001; Scott et al., 2003; Wan et al., 1998). Here we report the identification of PACS-2, a multifunctional sorting protein that controls the ER-mitochondria axis, including the apposition of mitochondria with the ER and ER homeostasis. In addition, we show that following induction of apoptosis, PACS-2 binds to dephosphorylated Bid and is required to traffic full-length Bid to mitochondria, where Bid is subsequently cleaved to tBid, leading to the release of cytochrome c, the activation of caspase-3, and cell death.

## 3.2 Results

### 3.2.1 Identification of PACS-2

We previously identified a sorting connector, PACS-1, that localizes membrane proteins to the trans-Golgi network (TGN) by directing their retrieval from endosomal compartments (Crump et al., 2001; Scott et al., 2003; Wan et al., 1998). PACS-1 binds to protein kinase CK2 phosphorylatable acidic-cluster sorting motifs on membrane cargo and links them to the clathrin adaptors AP-1 and AP-3. EST database searches, however, revealed the presence of a second PACS gene. Therefore, we screened a human brain cortex library with DNA probes corresponding to the ESTs and obtained a full-length cDNA encoding a novel PACS family member: PACS-2 (Fig. 3-1a). Sequence alignment showed that the predicted 889aa PACS-2 protein shares 54% overall sequence identity with the 963aa human PACS-1, and shares 81% sequence identity with PACS-1 in the 140aa cargo/adaptor binding region (FBR).

RNA hybridization and immunofluorescence studies suggested PACS-1 and PACS-2 have distinct roles. Northern blot analyses showed that the 4.4 kb PACS-1 and the major 3.8 kb PACS-2 transcripts are broadly expressed, with greatest levels in heart, brain, pancreas, and testis (Fig. 3-1a). PACS-1 is selectively enriched in peripheral blood leukocytes, whereas PACS-2 is selectively enriched in skeletal muscle. Moreover, PACS-1 and PACS-2 show distinct intracellular staining patterns: PACS-1 localized largely to the paranuclear region, where its punctate staining pattern overlapped with that of the AP-1 adaptor, whereas PACS-2 showed a diffuse staining pattern that co-localized

largely with the ER chaperone protein disulfide isomerase (PDI) and COPI coatomer, but not AP-1 (Fig. 1b). Additional analyses showed a limited overlap of PACS-2 staining with mitochondria.

Fig. 3-1: Identification and characterization of PACS-2, a sorting protein found on the ER and mitochondria. **a)** *Top:* Schematic and Kyte-Doolittle hydrophobicity plot of the human PACS-1 and PACS-2 proteins. FBR, cargo/adaptor binding region; ARR, Atrophin-1 related region. Radiation hybrid and genome database analyses mapped the PACS-1 gene to chromosome 11q13.1 (Genbank AY320283) and the PACS-2 gene to chromosome 14q32.33 (Genbank AY320284). *Bottom*: Northern blot analysis of tissue distribution for PACS-1 and PACS-2 transcripts. **b)** Confocal immunofluorescence of endogenous PACS-1 and PACS-2 in A7 cells. PACS-1/-2 were visualized with Alexa488 (Nakamura et al.)(green) and markers were visualized with Alexa546 or mitotracker (red). Scale bar, 10 μm. **c)** A7 cells transfected with PACS-1 or PACS-2 siRNAs were analyzed by western blot 48 hr post-transfection. **d)** A7 cells were transfected or not with PACS-1 or PACS-2 siRNAs. After 48 hr cells were processed for immunofluorescence microscopy using anti-CI-MPR (Nakamura et al.)(green) and anti-TGN46 (red). **e)** A7 cells were transfected with control (scrambled), PACS-1, or PACS-2 siRNAs for 48 hr and processed for confocal immunofluorescence localization of mitochondria (mitotracker, red) and ER (PDI, green). **f)** A7 cells were transfected with the corresponding siRNAs and assayed for cell death by Annexin V/propidium iodide staining and FACS analysis. Treatment of cells with the proapoptotic CtBP siRNA served as a positive control (Zhang et al., 2003). Error for all graphs = SD.

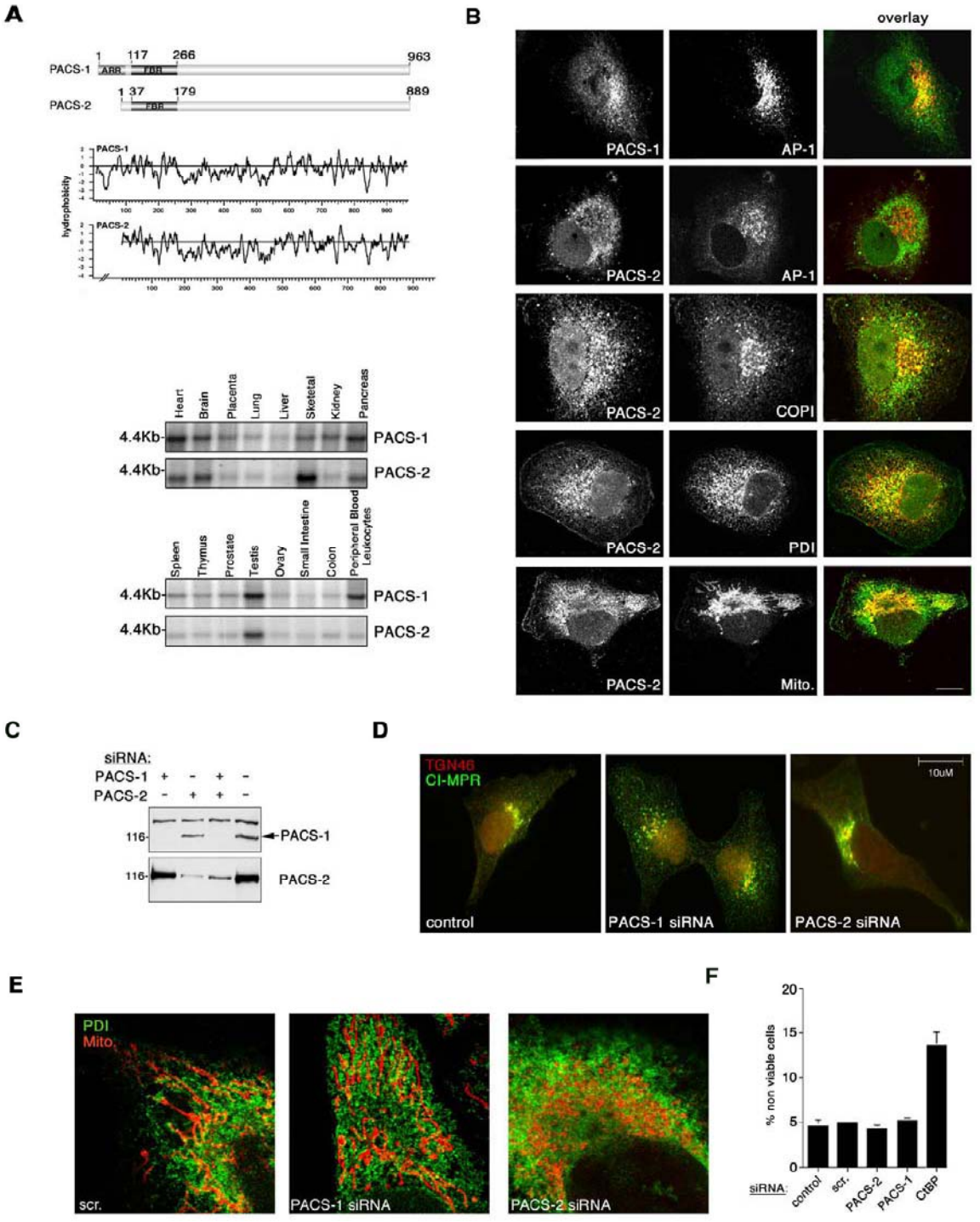


Figure 3-1. Identification and characterization of PACS-2, a sorting protein found on the ER and mitochondria.

### 3.2.2 PACS-2 controls ER-Mitochondria contacts

To identify the role of PACS-2 *in vivo*, we transfected cells with siRNAs that specifically depleted PACS-1 or PACS-2 following a two-day treatment (Fig. 3-1c). In agreement with our earlier studies, we found that depletion of PACS-1, but not PACS-2, caused the cation-independent mannose-6-phosphate receptor (CI-MPR), a PACS-1 cargo protein, to mislocalize from the TGN and accumulate in an endosome population (Fig. 3-1d). Surprisingly, we found that depletion of PACS-2, but not PACS-1 caused extensive mitochondrial fragmentation, and appeared to uncouple the fragmented mitochondria from the ER (Fig. 3-1e). To ensure that the uncoupling of the mitochondria from the ER was not a result of PACS-2 siRNA toxicity, we determined by annexin V/propidium iodide staining that siRNA depletion of either PACS-1 or PACS-2 had negligible effects on cell viability or protein synthesis (Fig. 3-1f and data not shown).

To more rigorously determine the effect of PACS-2 depletion on ER/mitochondria we performed electron microscopic analyses. In PACS-2 depleted cells, we observed an ~2-fold increase in the area of cytosol containing long ER tubules devoid of associated mitochondria, as the mitochondria were found concentrated in the paranuclear region (Figs. 3-2a and b). Despite the extensive fragmentation, and in agreement with the toxicity analysis (Fig. 3-1f), mitochondria integrity was not disrupted in the PACS-2 depleted cells as determined by loading of tetramethyl rhodamine (TMRM), which requires an intact membrane potential ($\Psi_M$, Fig. 3-2c).

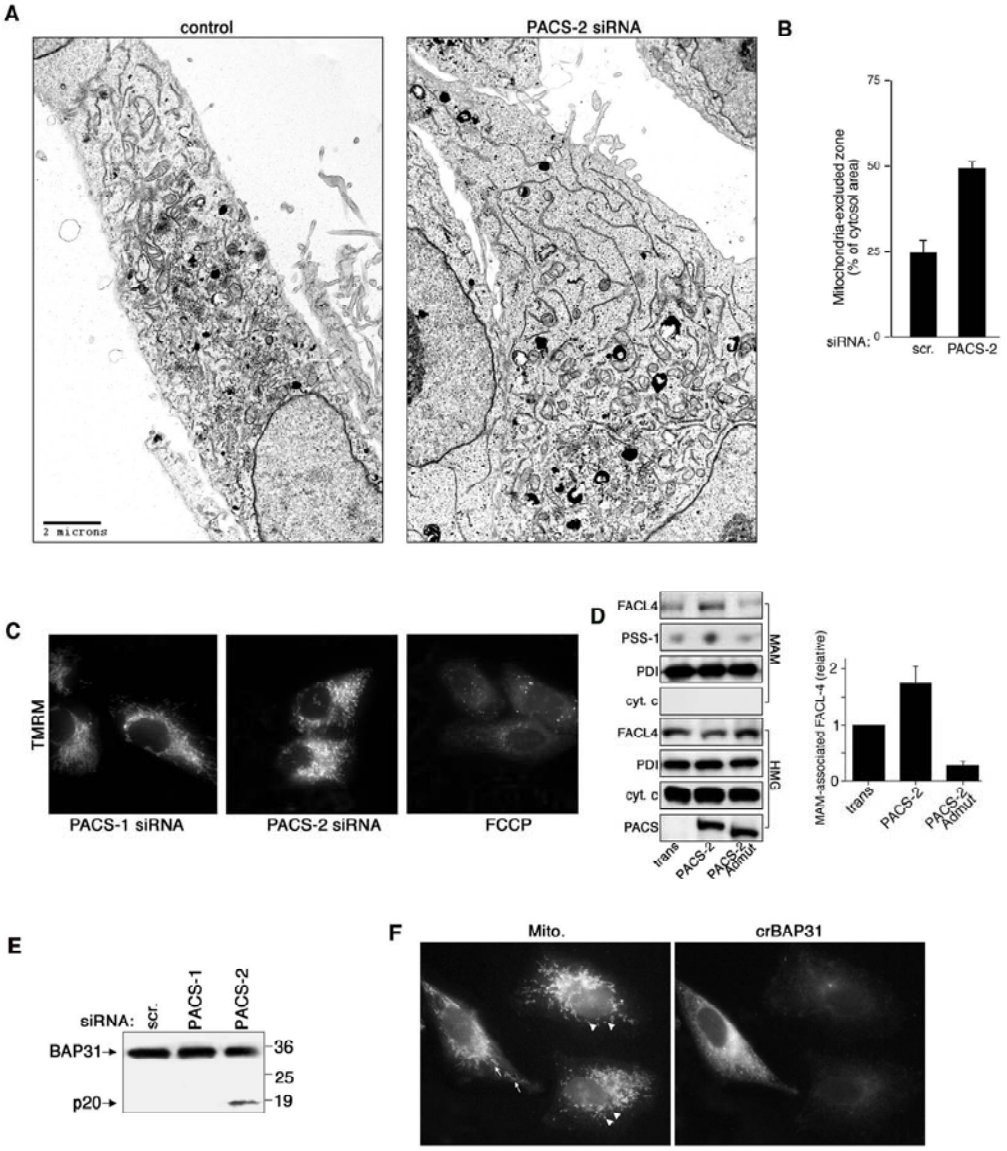


Figure 3-2. PACS-2 depletion disrupts mitochondrial structure.

**a**) A7 cells were transfected or not with the PACS-2 siRNA and processed for electron microscopy. Magnification, 5,800x. **b)** The extent of uncoupling of mitochondria from the ER of either control cells or PACS-2-depleted cells was quantified using morphometric analysis (see Materials and Methods). **c)** Cells were incubated with 100 nM TMRM for 30 min before microscopic analysis. Control cells were treated with 10 μM FCCP for 30 min, which uncouples the $\Psi_M$ and blocks TMRM loading. **d)** MAMs were isolated from crude homogenates (HMG) of control cells (trans) and PACS-2 and PACS-2Admut expressing cells by Percoll gradient fractionation and identified by western blot using an anti-PSS-1 Ab, which is specific for the MAM fraction (Stone and Vance, 2000). The effect of PACS-2 or PACS-2Admut on the localization of MAM-associated FACL4 was determined by western blot. Right: Quantitation of MAM-associated FACL4 (n = 3). **e)** Lysates from control and siRNA transfected A7 cells were analyzed by western blot using anti-BAP31. **f)** A7 cells were transfected with crBAP31-flag and subsequently transfected with the PACS-2 siRNA for 48 h. Cells were then processed for immunofluorescence with anti-Flag mAb to detect crBAP31-expressing cells (right panel) and mitotracker (left panel). Arrows, rod-like mitochondria. Arrowheads, fragmented mitochondria.

Our discovery that the close apposition of mitochondria with the ER requires PACS-2 prompted us to determine whether localization of lipid biosynthetic enzymes to MAMs is also dependent upon PACS-2. PACS-2 overexpression increased the amount of FACL4 associated with isolated MAM fractions (Fig. 3-2d). By contrast, expression of the dominant negative PACS-2Admut (Kottgen et al., 2005) decreased the amount of FACL4 and PSS-1 present in the MAM fraction. Together, these results further support a key role for PACS-2 in maintaining the ER-mitochondria axis.

Our results are in agreement with recent studies suggesting that proteins involved in ER trafficking may also have essential roles in maintaining mitochondria shape and the apposition of mitochondria against the ER, as shown by inactivating yeast COPI or by the caspase-8 catalyzed cleavage of mammalian BAP31 to form p20, either of which induces mitochondria fragmentation similar to that which occurs in PACS-2 depleted cells (Breckenridge et al., 2003; Prinz et al., 2000). Therefore, we tested the possibility that PACS-2 depletion induced the cleavage of BAP31 to p20 and found that depletion of PACS-2 but not PACS-1 induced cleavage of BAP31 to p20, similar to the process observed during apoptosis (Fig. 3-2e). By contrast, expression of a caspase-resistant BAP31 molecule, crBAP31 (Nguyen et al., 2000), blocked the PACS-2 siRNA-induced mitochondria fragmentation, demonstrating that these changes in mitochondrial shape following PACS-2 depletion result from cleavage of BAP31 to p20 (Fig. 3-2f).

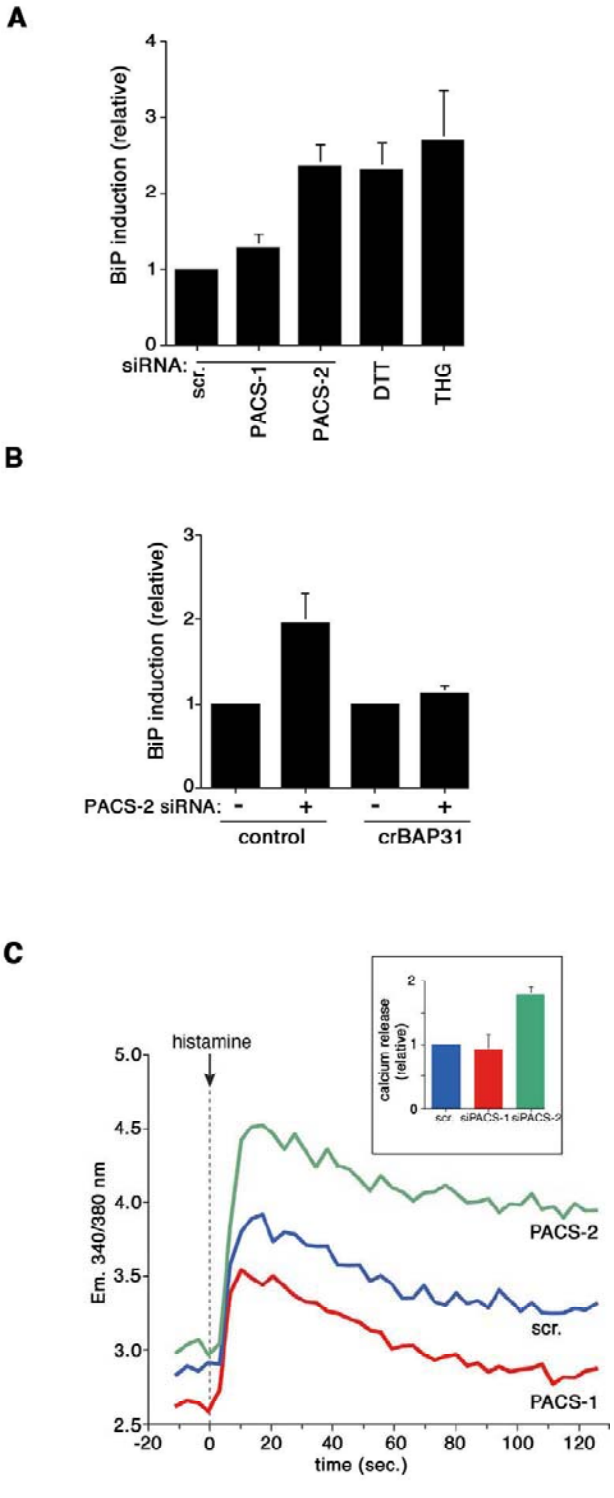


Figure 3-3. PACS-2 depletion disrupts ER homeostasis.

**a)** siRNA-treated A7 cells were lysed 48 hr post-transfection and the amount of BiP was determined by western blot. BiP amounts in cells treated with 1 mM DTT or 5 mM thapsigargin (THG) for 16 hr served as positive controls. All values are normalized to control cells transfected with scrambled siRNA. **b)** Control and HeLa(KB)-crBAP31 cells were transfected or not with PACS-2 siRNA and analyzed for BiP expression by western blot. **c)** A7 cells depleted of PACS-1 or PACS-2 and control cells (transfected with scrambled siRNA) were loaded with Fura-2 and treated with histamine to stimulate calcium release from the ER through $IP_3R$. *Inset:* Relative amounts of histamine-releasable ER calcium (n = 3).

### 3.2.3 PACS-2 mediates ER homeostasis

In addition to promoting lipid transfer between the ER and mitochondria, the juxtaposition of mitochondria against the ER also promotes transfer of ATP to the ER for chaperone-mediated protein folding and allows for calcium-mediated communication between the two organelles (Hajnoczky et al., 2000). Thus, our finding that PACS-2 depletion induced mitochondria fragmentation and uncoupled this organelle from the ER raised the possibility that, in addition to mediating MAM formation, PACS-2 might also influence ER folding and calcium homeostasis. To test this possibility, we first determined whether PACS-2 depletion affected the levels of BiP, an ER chaperone upregulated by ER stressors to maintain efficient folding and export of newly synthesized proteins (Rutkowski and Kaufman, 2004). PACS-2 siRNA induced an ~2-fold increase in BiP levels, similar to the increase elicited by the potent unfolded protein response (UPR) inducers dithiothreitol (DTT) and thapsigargin (Fig. 3-3a). Second, we examined whether the PACS-2 siRNA-mediated BiP induction was a direct response to the BAP31/p20-mediated uncoupling of ER from mitochondria. We found that crBAP31, which blocked the p20-mediated uncoupling of mitochondria from the ER (Fig. 3-2f), also blocked BiP induction (Fig. 3-3b), demonstrating that the mitochondria fragmentation and BiP induction in PACS-2-depleted cells resulted directly from the cleavage of BAP31 to p20. Third, we measured the ability of histamine to elicit IP3 receptor-mediated release of ER calcium in control- or PACS-2-depleted cells loaded with the calcium sensor Fura-2. We found that histamine elicited a 2-fold greater increase in calcium released from the ER into the cytosol of PACS-2 depleted cells compared to PACS-1 depleted or control cells (Fig. 3-3c). Together, these results suggest that the

PACS-2 siRNA-mediated uncoupling of the ER from mitochondria was compensated for by increased levels of the ER protein folding machinery and calcium to reestablish ER homeostasis.

### 3.2.4 PACS-2 depletion blocks apoptotic programs

In addition to causing mitochondrial fragmentation, p20 also induces apoptotic cell death (Breckenridge et al., 2003). Thus, our paradoxical finding that PACS-2 depletion induced both ER stress (Fig. 3-3) and the p20-mediated mitochondrial fragmentation (Fig. 3-2) but not apoptosis (Fig. 3-1), suggested that PACS-2 is somehow required for p20-mediated apoptotic induction. To test this possibility, we treated cells depleted of PACS-1 or PACS-2 with 1.2 μM staurosporine (STS) an additional 24 hr and quantified the amount of apoptotic and necrotic cells after 24 hr (Fig. 3-4a). STS induced death in ~95% of the control and PACS-1-depleted cells. By contrast, PACS-2 depletion conferred a marked resistance to STS, increasing the percentage of viable cells by nearly 4-fold. The anti-apoptotic effects of the PACS-2 siRNA were corroborated by analyzing the STS-mediated caspase cleavage of poly ADP-ribose polymerase (PARP) to generate p85 (Fig. 3-4b). STS treatment of control cells showed a rapid onset of PARP cleavage beginning at 4 hr following the STS addition, with nearly complete cleavage occurring after 6 hr incubation, whereas PACS-2 showed no detectable processing at 6 hr incubation. Significantly, this delay in PARP cleavage was identical to that observed in cells pretreated with the caspase inhibitor zVAD-fmk, suggesting that PACS-2 depletion inhibits executioner caspase-mediated proteolysis. Similar results were obtained when

cells were treated with thapsigargin or Fas Ab, demonstrating the importance of PACS-2 for both the intrinsic and extrinsic apoptotic pathways (data not shown).

We next examined whether PACS-2 depletion blocks the STS-mediated activation of caspase-3, an executioner caspase that cleaves PARP to p85. We found that STS elicited caspase-3 activation in control and PACS-1 depleted cells, whereas PACS-2 depletion blocked STS-mediated caspase-3 activation, similar to that observed in cells treated with zVAD-fmk (Fig. 3-4c). We next determined whether PACS-2 depletion affects caspase-8 activation, an initiator caspase that is cleaved following STS treatment (Joshi and Sahni, 2003). We found that STS stimulated the zVAD-fmk-sensitive activation of caspase-8 irrespective of the presence or absence of either PACS-1 or PACS-2 (Fig. 3-4c). Together these results suggested that the ability of STS to efficiently activate caspase-8 but not caspase-3 following PACS-2 depletion may be due to an inability of PACS-2 depleted cells to stimulate release of cytochrome c from mitochondria in response to an apoptotic signal. To test this possibility, membrane compartments of cells treated with STS were fractionated and analyzed for the release of cytochrome c. We found a marked translocation of cytochrome c from the mitochondrial, heavy membrane fraction to the cytosol by 4 hr following STS addition (Fig. 3-4d). Depletion of PACS-2 however, blocked cytochrome c release under these conditions, thus explaining the failure of caspase-3 to become activated in PACS-2-depleted cells. In addition to activating caspase-3, released cytochrome c increases conductance through the IP3-receptor leading to a massive release of ER calcium, to amplify the apoptotic pathway (Boehning et al., 2003).

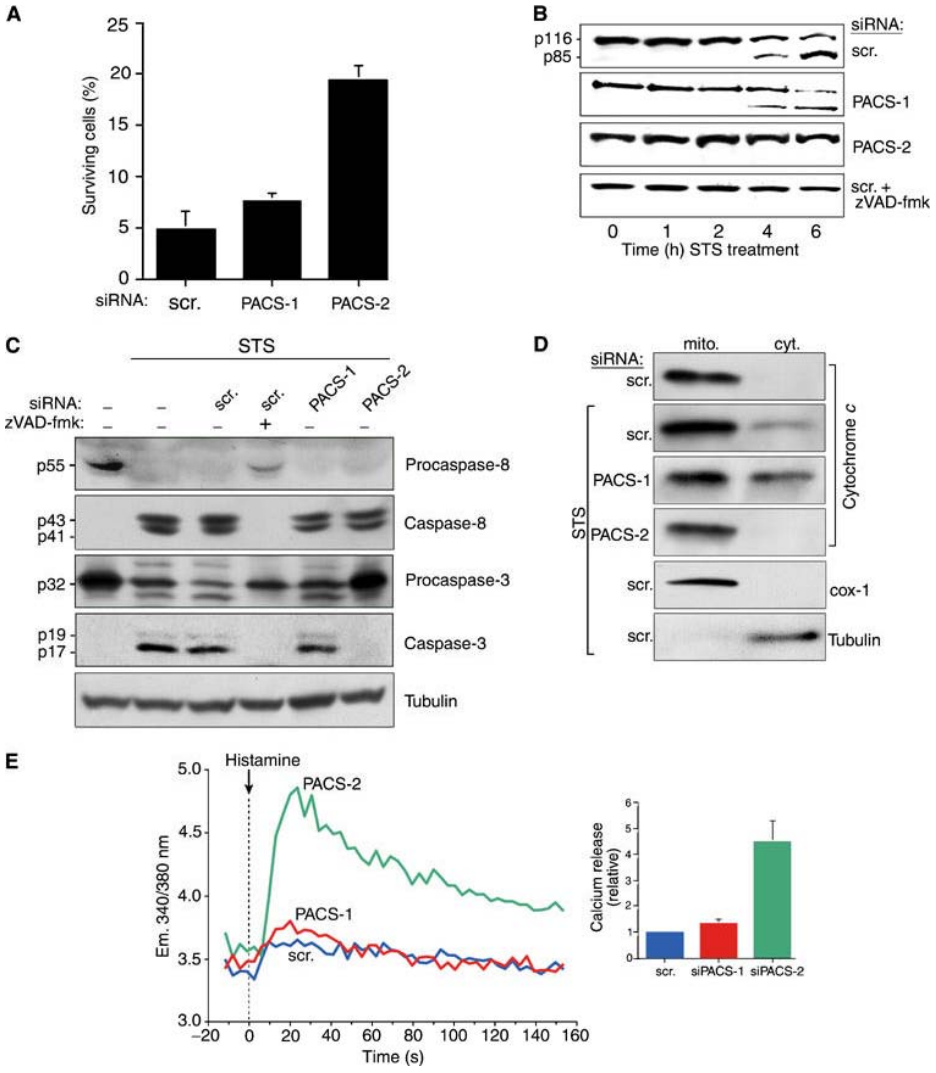


Figure 3-4. STS-induced cell death depends on PACS-2.

**a)** A7 cells transfected with PACS-1, PACS-2 or scrambled siRNAs for 48 hr were incubated with 1.2 μM STS for an additional 24 hr, and the percentage of viable cells was quantified by FACS analysis using anti-annexinV/propidium iodide staining. **b)** A7 cells were transfected with PACS-specific or scrambled siRNAs, or treated with oligofectamine alone (control). Apoptosis was induced with 1.2 μM STS for 0, 1, 2, 4, and 6 h in the absence or presence of 10 μM zVAD-fmk and lysates were analyzed by western blot to detect PARP cleavage to the p85 product. **c)** A7 cells were transfected with siRNAs as indicated, treated with 1.2 μM STS for 4 h in the absence or presence of 10 μM zVAD-fmk, harvested and pro- and activated caspases-8 and –3 or α-tubulin (loading control) were analyzed by western blot. **d)** Post-nuclear supernatants from A7 cells treated with 1.2 μM STS for 4 hr were resolved by sedimentation into cytosol and mitochondria-containing heavy membrane fractions, followed by western blot with anti-cytochrome c, anti-cytochrome c oxidase I (mitochondria marker) or anti-α-tubulin (cytosol marker). **e)** A7 cells depleted of PACS-1 or PACS-2 and control cells (transfected with scrambled siRNA) were incubated with 1.2 μM STS for 4 h and then loaded with Fura-2. Following histamine stimulation, the calcium release was monitored as in Fig. 3c.

Accordingly, we found that the PACS-2 depletion, which blocks cytochrome c release, severely mitigated the ability of STS to invoke depletion of ER calcium stores compared to either a control treatment or PACS-1 depletion (Fig. 3-4e). Together, these results indicate that PACS-2 depletion blocks apoptosis, at least in part, by inhibiting the release of cytochrome c from mitochondria, thereby blocking both caspase-3 activation and the apoptotic release of ER calcium. Thus, in addition to controlling the ER-mitochondria axis, PACS-2 appears to play an essential role in connecting mitochondria to cell death programs.

### 3.2.5 Apoptotic programs induce PACS-2 to target Bid to mitochondria

Despite the importance of PACS-2 for the induction of apoptosis, our results did not explain how this ER sorting protein promotes cytochrome c release from mitochondria in response to apoptotic inducers. We thus investigated the localization of PACS-2 itself during the onset of apoptosis. Surprisingly, we found that apoptotic inducers stimulated the rapid redistribution of PACS-2 from the ER to the mitochondria. In control cells the PACS-2 staining pattern showed a pronounced overlap with the ER marker PDI, and a more limited co-localization with mitochondria, (Fig. 3-5a top, see also Fig. 3-1b). However, treatment of the cells with 1.2 μM STS for one hour stimulated a dramatic redistribution of PACS-2 staining, revealing a pronounced co-localization with mitochondria. Similar results were obtained by treatment of cells with tunicamycin, or Fas Ab (Fig. 3-6c, bottom and data not shown). Lastly, we conducted membrane fractionation of cells to determine biochemically whether apoptotic inducers redistributed PACS-2 from ER to mitochondria.

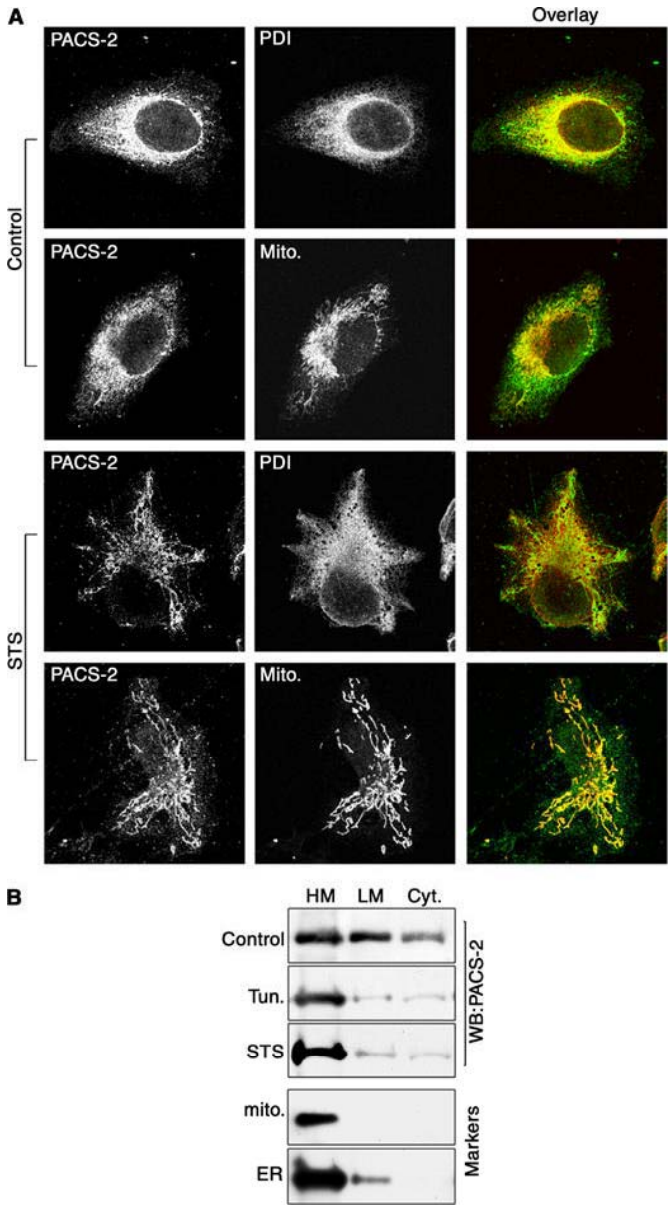


Figure 3-5. Apoptosis induction redirects PACS-2 onto mitochondria.

**a)** *Top;* A7 cells were either untreated (control) or treated with 1.2 μM STS for 1 hr and incubated with primary antibodies and species-specific secondary antibodies to detect PACS-2 (Nakamura et al.)(green) and PDI (red) or stained with mitotracker (red). **b)** Control and tunicamycin (Tun) or STS-treated cells were homogenized and post-nuclear supernatants were resolved by sedimentation into cytosol, light and heavy (mitochondria fraction) membranes followed by western blot with anti-PACS-2, anti-cytochrome c oxidase I (mitochondria) or anti-BiP (ER).

In agreement with the morphological study, apoptotic inducers including STS and tunicamycin stimulated transfer of PACS-2 from cytosolic and ER-enriched light membrane fractions to mitochondria-containing heavy membrane fraction (Fig. 3-5b).

The pronounced redistribution of PACS-2 from ER/cytosol to the mitochondria by apoptotic inducers, together with the requirement for PACS-2 to promote apoptosis (Fig. 3-4) and the established role of the PACS proteins in trafficking cargo molecules to cellular compartments, raised the possibility that apoptotic inducers may direct PACS-2 to recruit one or more proapoptotic factors to mitochondria. One candidate proapoptotic factor is the BH3-only Bcl-2 family member Bid, which is trafficked to mitochondria where its caspase-8 cleavage product tBid promotes release of cytochrome c (Gross et al., 1999b). Like many PACS cargo proteins, Bid contains an acidic cluster and is phosphorylated by protein kinases CK1 and CK2 (Desagher et al., 2001). To determine whether PACS-2 binds to Bid, tBid, or both proteins, we conducted in vitro binding assays. GST-PACS-2FBR, which contains the PACS-2 cargo binding region (Fig. 3-1a), bound to Bid but not to caspase-8-generated tBid, indicating that the intact Bid acidic region is required for PACS-2 binding (Fig. 3-6a, top). Next, we determined whether the phosphorylation state of Bid influences the ability of Bid to bind to GST-PACS-2FBR. We found that PACS-2 bound preferentially to non-phosphorylated Bid suggesting that during the onset of apoptosis, dephosphorylation of Bid promotes binding to PACS-2 (Fig. 3-6a, bottom). Next, we conducted co-immunoprecipitation studies to determine whether PACS-2 associates with Bid *in vivo*, and we found that treatment of cells with STS or Fas Ab increased the association of PACS-2 with Bid (Fig. 3-6b). Consequently,

to determine whether PACS-2 recruits Bid to mitochondria, MCF7 cells stably expressing Bid-GFP were transfected with PACS-2 siRNA or infected with an adenovirus expressing PACS-2 and then treated or not with cycloheximide and Fas Ab to stimulate Bid-GFP recruitment to mitochondria (Zha et al., 2000). In control cells, Fas Ab or thapsigargin markedly increased the recruitment of Bid-GFP and PACS-2 to mitochondria (Fig. 3-6c and data not shown). However, depletion of PACS-2 blocked Bid-GFP recruitment, supporting a role for PACS-2 in the Fas Ab- and thapsigargin-stimulated translocation of Bid to mitochondria. In support of the siRNA studies, we found that overexpression of PACS-2 in Fas Ab-treated cells stimulated Bid-GFP recruitment to mitochondria by nearly 2-fold. By contrast, overexpression of PACS-2 in control cells failed to stimulate Bid-GFP translocation to mitochondria, demonstrating that apoptotic inducers control the PACS-2-dependent trafficking of Bid to mitochondria.

Lastly, we conducted biochemical fractionation studies to determine whether Bid translocation to mitochondria and formation of tBid requires PACS-2. In control cells, Fas Ab stimulated the translocation of full-length Bid and the formation of tBid on mitochondria. However, in PACS-2 depleted cells, Fas Ab failed to induce Bid translocation and tBid formation on mitochondria. Analysis of total cell extracts showed that formation of tBid was markedly reduced in PACS-2 depleted cells demonstrating that PACS-2, which binds Bid but not tBid, is required for formation of tBid in Fas Ab-treated cells (Fig. 3-6d). Together, these data suggest that in response to apoptotic signals PACS-2 recruits full-length Bid to mitochondria where Bid is subsequently cleaved to

form tBid to stimulate cytochrome c release, activate executioner caspases, and commit cells to death.

Fig. 3-6: **Death signals stimulate PACS-2-mediated trafficking of Bid to mitochondria. a)** *Top;* GST-PACS-2FBR was incubated with $His_6$-Bid and tBid and bound Bid molecules were detected by western blot using anti-Bid antibodies. *Bottom*; GST-PACS-2FBR was incubated with either $His_6$-Bid or CK2 phosphorylated $His_6$-Bid and bound $His_6$-Bid was detected by western blot using anti-Bid antibodies. **b)** Control HeLa cells or cells expressing PACS-2-ha were treated or not for 2 hr with 1.2 μM STS or with 0.1 μg/ml Fas Ab and 5 μg/ml cycloheximide. PACS-2-ha was immunoprecipitated from cell lysates and co-immunoprecipitating Bid was detected by western blot using an anti-Bid Ab. **c)** MCF-7:Bid-GFP cells were either transfected with the PACS-2 siRNA for 48 h or infected with Ad:trans (trans, expressing the tet transactivator) alone or together with Ad:PACS-2 for 24 h and then treated with Fas Ab (1 μg/ml) and cycloheximide (20 μg/ml) for 2 hr, followed by a 30 min loading with mitotracker. Fixed cells were scored for Fas Ab-mediated recruitment of Bid-GFP to mitochondria. *Top*; Bar graph normalized to the control (Oligofectamine). *Middle*; Fluorescence microscopy showing Bid-GFP and mitotracker (red) staining under the various conditions. Large fields of the coverslips containing 150 - 450 cells were scored for translocation of GFP-Bid to mitochondria. *Bottom:* Fas Ab stimulates PACS-2 and Bid to translocate to mitochondria. A7 cells treated with Fas Ab for 2 hr were processed for immunofluorescence microscopy. **d**) *Top:* HeLa cells transfected with control (scr.) or PACS-2 siRNAs were treated or not with Fas Ab (1 μg/ml) and cycloheximide (20 μg/ml) for 2 hr. Bid and tBid in mitochondria and cytosolic fractions (top panels) or from the total lysates (bottom panels) were detected by western blot.

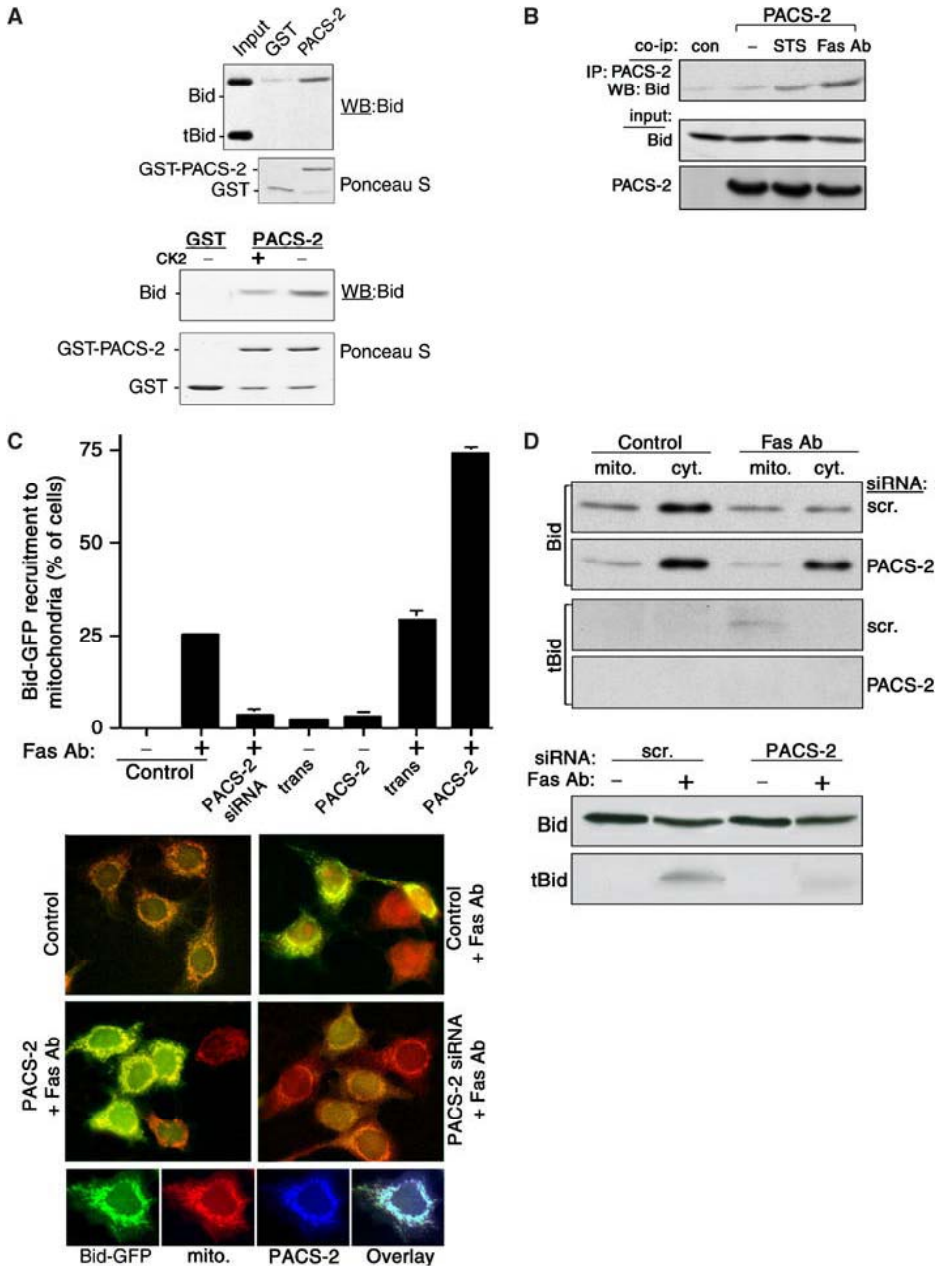


Figure 3-6. Death signals stimulate PACS-2-mediated trafficking of Bid to mitochondria.

## 3.3 Discussion

We report that PACS-2 is a multifunctional sorting protein that controls the ER-mitochondria axis and the role of this axis in cellular homeostasis and apoptosis. Our results show that PACS-2 is required for the intimate association of mitochondria with the ER: absence of PACS-2 induces the caspase-dependent cleavage of BAP31 to yield the proapoptotic fragment p20, causing mitochondria to fragment and uncouple from the ER. This structural uncoupling also disrupts MAMs and induces ER stress, which is compensated for by increased levels of BiP and ER calcium. Moreover, when either cellular- or ER-homeostasis is compromised, or when cell death programs are initiated, PACS-2 is redirected to translocate the apoptosis-inducing protein Bid onto mitochondria. This PACS-2-dependent trafficking of full-length Bid to mitochondria promotes cell death by inducing formation of tBid and release of cytochrome c, which subsequently results in the depletion of releasable ER calcium, the activation of caspase-3, and the cleavage of caspase-3 substrates.

The requirement of PACS-2 for the apposition of rod-like mitochondria to the ER suggests PACS-2 has an essential role in ER-mitochondria communication and influences the dynamic mitochondria fusion/fission events that are coupled with mitochondria homeostasis and inter-mitochondria communication (Szabadkai et al., 2004). We determined that mitochondria fragmentation in PACS-2 depleted cells requires the caspase-generated p20 fragment of BAP31, which promotes mitochondria fission by regulating Drp1/Dlp1 (Breckenridge et al., 2003), a mitochondria-localized dynamin-related GTPase. Thus, our results indicate that PACS-2 depletion induced mitochondria

fragmentation by promoting mitochondria fission rather than by inhibiting of fusion. Our demonstration that PACS-2Admut, which fails to bind COPI (Kottgen et al., 2005), or siRNA depletion of either PACS-2 or COPI (data not shown), uncouples the fragmented mitochondria from the ER, suggests that PACS-2/COPI trafficking is essential for maintaining the ER-mitochondria axis. Our results are consistent with studies in yeast which show that inactivation of COPI similarly uncouples the ER from mitochondria (Prinz et al., 2000).

We extended our morphological studies with biochemical analyses to show that PACS-2 mediates the levels of MAM-associated FACL4, which converts fatty acids to fatty acyl-CoA esters used in the formation of complex lipids (Piccini et al., 1998), and PSS-1, which exchanges the head group of phosphatidylcholine with serine (Fig. 3-2). MAMs are ER-contiguous membranes that, in addition to FACL4 and PSS-1, contain multiple phospholipid- and glycosphingolipid-synthesizing enzymes and support the direct transfer of phospholipids from the ER to mitochondria (Vance, 2003). Our work identifies PACS-2 as the first cellular trafficking protein that regulates MAM formation. However, we do not know whether the reduced the levels of MAM-associated FACL4 and PSS-1 in cells expressing dominant negative PACS-2 result from mistargeting of FACL4 and PSS-1 or whether the MAM itself is redistributed as a result of the uncoupling of the fragmented mitochondria from the ER. Interestingly, we found that the loss of MAM markers in PACS-2-depleted cells was coupled with a pronounced extension of the tubulated, peripheral ER (Fig. 3-2). It is possible that interference with PACS-2 causes the MAM to redistribute into the ER, thereby expanding the ER

membrane fraction while reducing the amount of membrane associated with mitochondria. Consistent with this possibility, expression of a dominant negative Drp1 promotes mitochondria fusion together with a corresponding reduction in the amount of ER membranes (Pitts et al., 1999). Alternatively, the extended tubulation of the peripheral ER observed by electron microscopy in PACS-2-depleted cells may reflect a requirement of PACS-2 for the function of ER modeling proteins (Nakajima et al., 2004; Uchiyama et al., 2002). In addition to contacting the mitochondria, high resolution 3D electron tomography reveals that the ER also forms close contacts with the trans-Golgi and endosomal compartments (Marsh et al., 2001). Whether there are additional roles for PACS-2 in inter-organelle communication remains to be tested, but such an expanded role may explain the high level of PACS-2 expression in skeletal muscle (Fig. 3-1), in which the sarcoplasmic reticulum is apposed with the plasma membrane.

In addition to mediating the ER-mitochondria axis, we found that PACS-2 has a profound role on ER homeostasis as PACS-2 depletion induces a UPR (Fig. 3-3). The UPR is an integrated response to a variety of ER-targeted stressors, which coordinates a repression of cellular protein synthesis by phosphorylating eIF-2α, with an induction of ER chaperone expression to facilitate folding of secreted and membrane proteins (Rutkowski and Kaufman, 2004). We found that PACS-2 depletion leads to a transient increase in phosphorylated eIF-2α , after which ER homeostasis is re-established with a normal rate of protein synthesis by two days following treatment (data not shown). The re-establishment of ER homeostasis coincides with increased levels of BiP and ER calcium (Fig. 3-3). Whereas the importance of the ER trafficking machinery on ER homeostasis is

well established (Belden and Barlowe, 2001), the influence of mitochondrial structure on ER homeostasis is unclear. Thus, our finding that the UPR induced by PACS-2 depletion could be prevented by expression of a caspase-resistant crBAP31, demonstrates that this UPR is a direct result of the p20-mediated uncoupling of the fragmented mitochondria from the ER (Fig. 3-4). Several factors may contribute to the control of ER homeostasis by the apposed mitochondria. For example, mitochondria provide ATP to the ER for oxidative protein folding, which is blocked by hypoxic stress (Koumenis et al., 2002). In addition, the increased levels of calcium-binding ER chaperones require a commensurate increase in ER calcium (Koch, 1990), consistent with our finding that PACS-2 depletion increases by ~2-fold the amount of histamine-releasable ER calcium compared to control cells (Fig. 3-3c).

To what extent the higher ER calcium release in PACS-2 depleted cells is due to promoting ER chaperone activity versus an inability to efficiently transfer calcium to mitochondria requires further investigation and may provide new insight into ER-mitochondria communication. For example, in addition to transferring lipid intermediates, MAMs may participate in calcium transfer between the ER and mitochondria. In support of this possibility, both IP3Rs and ryanodine receptors possess potential PACS-2 binding sites (Kottgen et al., 2005) and may be associated with MAMs (Hajnoczky et al., 2000). Thus, disruption of PACS-2 may cause mislocalization of IP3Rs resulting in reduced calcium transfer from the ER to mitochondria. Recent studies showing that the mere relocalization of mitochondria is not sufficient to affect ER calcium levels (Varadi et al., 2004a), but nonetheless disrupts calcium communication

within the mitochondrial system (Szabadkai et al., 2004). Thus, the observed increase in ER calcium in PACS-2-depleted cells cannot be explained by mitochondria fragmentation alone, but rather by changes in the ER to maintain ER homeostasis.

Ectopic expression of p20 is sufficient to commit cells to apoptosis (Breckenridge et al., 2003). The p20/Drp1-mediated mitochondrial fragmentation primes this organelle for cytochrome c release by promoting the recruitment of Bax/Bak oligomers (Karbowski and Youle, 2003). Thus, we were surprised to find that PACS-2-depleted cells were not only viable, but resistant to apoptosis (Fig. 3-4), despite the p20-mediated mitochondria fragmentation (Fig. 3-1). This block in the apoptotic pathway caused by depletion of PACS-2 led us to determine that apoptotic inducers signal PACS-2 to recruit Bid to mitochondria (Fig. 3-6), which is required for the induction of cell death. In addition, our finding that apoptotic inducers failed to exhaust ER calcium in PACS-2 siRNA-treated cells (Fig. 4), agrees with recent studies showing that cytochrome c released into the cytosol binds to the IP3 receptor to induce release of ER calcium necessary to fully activate cell death proteases (Boehning et al., 2003). Together, our findings suggest that in response to intrinsic and extrinsic apoptotic inducers, ER/cytosolic PACS-2 collaborates with BAP31/p20 to coordinate cell death programs.

Our demonstration that apoptotic inducers promote PACS-2 to bind and translocate full-length Bid to mitochondria (Figs. 3-5 and 3-6), provides new insight into the integration of the ER-mitochondria axis with cell death programs. While full-length Bid can associate with mitochondria and induce the release of cytochrome c, the caspase-8

generated cleavage product tBid possesses a much higher affinity for mitochondria where it accumulates during apoptosis and can more efficiently release cytochrome c (Gross et al., 1999b). N-terminal myristoylation further increases tBid apoptotic activity, supporting the model that this lipid addition acts as a molecular "switch" that targets tBid to mitochondria during apoptosis (Zha et al., 2000). Our results, however, cast new insight into the apoptosis-induced targeting of Bid to mitochondria. We found that PACS-2 depletion blocks the apoptotic translocation of Bid-GFP to mitochondria whereas overexpression of PACS-2 enhances this sorting step (Fig. 3-6). The PACS-2-dependent translocation of Bid to mitochondria appears to occur prior to caspase cleavage of Bid as PACS-2 depletion prevents both the Fas Ab-induced formation of tBid and the accumulation of tBid on mitochondria, but has no effect on cellular caspase-8 activation (Figs. 3-4 and 3-6). Moreover, we found that PACS-2 binds selectively to full-length Bid but not tBid and that apoptotic inducers promote the association of full-length Bid with PACS-2. These findings suggest that PACS-2 first targets full-length Bid to mitochondria where Bid is subsequently cleaved to tBid by cytosolic- or mitochondria-localized caspase-8 (Chandra et al., 2004; Micheau and Tschopp, 2003). Our determination that phosphorylation of Bid by CK2 inhibits binding to PACS-2 suggests Bid phosphorylation has two anti-apoptotic roles; the prevention of binding to PACS-2, which blocks translocation to mitochondria, and the masking of the caspase-8 cleavage site to produce tBid (Desagher et al., 2001). With our discovery of PACS-2 and the role of this novel sorting protein in controlling the ER-mitochondria axis and apoptosis, we propose a revised model of Bid action. In one leg, apoptotic inducers redirect PACS-2 from maintaining the ER-mitochondria axis, which promotes the BAP31/p20-mediated

fragmentation of mitochondria that uncouple from the ER. The fragmentation may "prime" mitochondria by enhancing Bak/Bax recruitment. In the second leg, full-length Bid becomes dephosphorylated, thereby enabling it to bind PACS-2. PACS-2 then targets full-length dephosphorylated Bid to the fragmented mitochondria where Bid can be subsequently cleaved to tBid by mitochondria-localized caspase-8. The cleaved, myristoylated tBid may then combine with Bak/Bax to permeabilize mitochondria, release cytochrome c and commit cells to death. Whether PACS-2 specifically translocates Bid to mitochondria or is involved in the translocation of other proapoptotic proteins that contain potential PACS-2 binding sites (e.g. Bak, Bad, Bim, and Bik) warrants future investigation.

In an accompanying paper, we show that PACS-2 is a COPI connector that controls the ER localization of polycystin-2 and likely many other ER localized membrane proteins that contain PACS-2 binding sites (Kottgen et al., 2005). We show here that the role of PACS-2 extends beyond ER trafficking, as it controls ER homeostasis, the ER mitochondria axis, and, in response to apoptotic inducers, we show PACS-2 is required to translocate Bid to mitochondria to control cell death. Interestingly, genomic analyses show that the PACS-2 gene is localized near the telomere on chromosome 14q32:33, a locus susceptible to chromosomal translocation and loss of heterozygosity in B-cell lymphomas and colorectal cancer. Moreover, and in accord with our discovery that PACS-2 is a proapoptotic protein, the PACS-2 gene is mutated in up to 40% of colorectal cancers (G. Anderson, personal communication and (Anderson et al., 2001)). Based on

our findings, the loss of PACS-2 would likely block apoptosis and may provide an opportunity for subsequent genomic insults that lead to cell immortalization and cancer.

## 3.4 Materials and methods

*Antibodies and reagents*

Reagents were from Sigma except where stated. PACS-1 and PACS-2 antisera (Pocono Farms), antibodies against δ-adaptin (M. Robinson), BAP31 (G. Shore), CI-MPR (S. Pfeffer), PDI (R. Sitia), cytochrome oxidase I (Molecular Probes), α-tubulin (Calbiochem), thioredoxin (Invitrogen), γ-adaptin (Sigma), ß-COP (MaD, Abcam), TGN46 (Serotec), BiP (BD Biosciences), Myc (Santa Cruz), HA (Covance), FLAG (Kodak), GFP (Clontech), Fas (Beckman), PARP, cytochrome c (Apotech), $His_6$ (Qiagen), FACL4 (Abgent), PSS1 (O. Kuge) and Bid, pro/activated caspases-3 and -8 (Cell Signaling), Alexa (546 and 488)-conjugated secondary antibodies (Molecular Probes) and HRP-conjugated secondary antibodies (Southern Biotech) were provided as indicated. Cell lines and plasmids were received as indicated: HeLa KB/crBAP31 (G. Shore), MCF-7:GFP-Bid (S. Korsmeyer), pET15b-Bid (H. Lu), and pFLAG-crBAP31 (G. Shore).

*cDNA cloning, expression vectors, cell lines and virus construction*

The PACS-2 cDNA was isolated by screening lambda ZAPII-hMC human cortex library (Stratagene) with a probe to EST R50031. Thioredoxin(TRX)- PACS-2 FBR (PACS-2 residues 37-179) was expressed using pET32 (Novagen). MCF7:Bid-GFP, HeLa, and A7 melanoma were cultured as described (Crump et al., 2001; Zha et al., 2000). Adenovirus

(Ad) recombinants (tet-off) expressing human PACS-2-ha (wild-type and Admut ($E_{89}$TDLALTF$_{96}$ $\rightarrow$ Ala$_8$)) were generated as described (Blagoveshchenskaya et al., 2002). Adenovirus infections were performed for 24 hr (m.o.i = 10).

*RNA hybridization*

Northern hybridization was performed using human multiple tissue blots (Clontech, Palo Alto, CA). cDNAs for PACS-1, PACS-2 and 1B15 (loading control, not shown) were used to generate random-primed $^{32}$P-probes. Hybridization was done according to the manufacturer's instructions and signals detected by Phosphor-Imager.

*Immunofluorescence, electron microscopy and FACS*

Conventional immunofluorescence microscopy was performed as described previously (Crump et al., 2001; Wan et al., 1998). Confocal microscopy used an Olympus Fluo-View FV300 confocal laser scanning microscope. For the TMRM loading, A7 cells transfected with siRNAs were incubated with 100 nM TMRM (Molecular Probes) for 30 min and then live cells were imaged. Electron microscopy was performed as described (Arvidson et al., 2003). Mitochondrial exclusion zones in electron micrographs were quantified using NIH Image v1.63. Exclusion zones were defined as the total cytoplasmic area minus the area of cytoplasm containing >95% of mitochondria. Using a blind assay, two independent sets of data were recorded for each micrograph (n=7 per condition, $p < 0.0001$). FACS was performed with a FACSCalibur (Becton Dickinson) and using the Annexin V/PI apoptosis staining kit (Oncogene/EMDbiosciences) according to the manufacturer's instructions.

*siRNAs*

siRNAs specific for human PACS-1 (AACUCAGUGGUCAUCGCUGUG and AAUUCUUCGCUCCAACGAGAU), PACS-2 (AACACGCCCGUGCCCAUGAAC and AAGAGGGAAGGCAACAAGCUU), CtBP (AAGGGAGGACCUGGAGAAGUU), and a non-specific scrambled control (Dharmacon) were transfected using Oligofectamine (Invitrogen). Cells were analyzed 2 to 3 days post-transfection.

*Bid translocation, Bid binding to PACS-2, and Bid co-immunoprecipitation*

MCF-7:Bid-GFP cells expressing Bid-GFP were transfected with PACS-2 or PACS-1 siRNAs for 48 hr, or infected with Ad:PACS-2 or Ad:PACS-1 for 24 hr. Apoptosis was induced with Fas Ab and cycloheximide as described (Zha et al., 2000). For Bid binding assays, recombinant Bid (30 μg) was cleaved with 300 U caspase-8 (Calbiochem) for 18 hr at 30°C in cleavage buffer (50 mM HEPES, 100 mM NaCl, 10 mM DTT, 1mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4) to generate an equal mixture of Bid and t-Bid. This mixture was incubated at 4°C for 4 hr with 3μg GST-PACS-2FBR or GST alone, captured with glutathione-agarose and analyzed by western blot. For the Bid co-immunoprecipitation, cells was infected with Ad:PACS-2 and treated for 2 h with either Fas Ab (1 μg/ml, Beckman)/cycloheximide (2 μg/ml) or 1.2 μM STS. Cells were lysed with in m-RIPA (1% NP40, 1% Deoxycholine, 150 mM NaCl, 50 mM Tris, pH 8.0, and protease inhibitors), immunoprecipitated with mAb HA.11 and processed for western blot using an anti-Bid antibody.

*MAM isolation, mitochondria fractionation, cytochrome c release, and Bid translocation*

MAMs were isolated as described (Stone and Vance, 2000), except that cells were broken with a ball bearing homogenizer (18 μm clearance). Mitochondria fractionation was performed as described (Kataoka et al., 2001). For cytochrome c release and Bid translocation, A7 or HeLa cells were washed with cold PBS, scraped into 300 μl mitochondrial fractionation buffer (250 mM sucrose, 10 mM Tris-HCl at pH 7.5, 1 mM EGTA, and protease inhibitors) and lysed with 12 passes through a 27G needle. The post-nuclear supernatant was sedimented at 15000g for 15 min to separate mitochondria and cytosol, followed by western blot with anti-cytochrome c, anti-Bid, anti-cytochrome c oxidase I (mitochondria) or anti-tubulin (cytosol).

*Fura-2 measurements*

A7 cells treated or not with 1.2 μM STS were incubated with 2 μM Fura-2 (Molecular Probes) for 45 min and then in normal medium for another 30 min. Cells were then trypsinized, washed and resuspended in 1.5 ml Tyrode's buffer (10 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) and monitored for light emission at 510 nm after excitation at 340 and 380 nm using a Cary Eclipse Fluorescence spectrophotometer. The emission ratio between the two emissions is directly proportional to the cytosolic [$Ca^{2+}$].

## 3.5 Acknowledgements

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# Chapter 4. Akt and 14-3-3 control a PACS-2 homeostatic switch that integrates membrane traffic with Apo2L/TRAIL-induced apoptosis

Joseph E. Aslan[1,7], Huihong You[1,7], Danielle M. Williamson[1], Jessica Endig[2], Hongjun Shu[3], Yuhong Du[4], Robert L. Milewski[5], Robert T. Youker[1], Laurel Thomas[1], Anthony Possemato[6], Kam Sprott[6], Haian Fu[4], Kenneth D. Greis[3], Douglas N. Runckel[5], Arndt Vogel[2] and Gary Thomas[1,8]

[1]Vollum Institute, Oregon Health & Science University, Portland, OR, USA, [2]Clinic for Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany, [3]Genome Research Institute, University of Cincinnati, Cincinnati, OH, USA, [4]Department of Pharmacology and Emory Chemical Biology Discovery Center, Emory University School of Medicine, Atlanta, and [5]Kaiser Permanente, Portland, OR, USA and [6]Cell Signaling Technology, Danvers MA, USA.

[7]Authors contributed equally to this work

[8]Corresponding Author; email: thomasg@ohsu.edu; Tel: (503) 494-6955

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In this chapter I performed experiments in figures 3B, 3C, 4B, 4C, 5B, 6A, 6B, 7A, 7B, 7C and 9 and part of 3A. I also purified protein for mass spec analyses in figures 4D and 8. Huihong You performed experiments in figure 1A and part of experiment 3A and generated MEF cells used throughout the paper. Danielle Williamson performed experiments in panels 1B, 1C, 5A and 5C and generated rescued MEF stable clones for figure 3A. Jessica Endig and Arndt Vogel performed liver death assays in figures 2D and 2E. Hongjun Shu and Kenneth Greis performed mass spec analyses in figures 4D and 8. Yuhong Du and Haian Fu performed polarization assays in figure 6C and 6D. Robert Milewski and Doug Runckel perfored histochemistry in figure 1C and provided tumor samples for figure 1B. Robert Youker and Laurel Thomas performed trafficking assays in figure 7D. Anthony Possemato and Kam Spratt provided the mAb81 antibody. I also participated by writing the manuscript with Gary Thomas.

## Summary

Apo2L/TRAIL selectively kills diseased cells in vivo without causing harm to healthy cells. However, little is known about the mechanism mediating Apo2L/TRAIL-induced apoptosis. Here we identify the multifunctional sorting protein PACS-2 as an essential Apo2L/TRAIL effector, required for killing colon cancer cells in vitro and virally infected cells in vivo in a viral hepatitis model. PACS-2 is phosphorylated at $Ser_{437}$ in vivo and pharmacologic and genetic studies demonstrate Akt is an in vivo $Ser_{437}$ kinase. Akt combines with 14-3-3 proteins to regulate the homeostatic and apoptotic properties of PACS-2 that mediate Apo2L/TRAIL action. Phosphorylated $Ser_{437}$ binds 14-3-3 proteins with high affinity, which represses PACS-2 apoptotic activity and is required for PACS-2 to mediate trafficking of membrane cargo. Apo2L/TRAIL triggers dephosphorylation of $Ser_{437}$, reprogramming PACS-2 to promote apoptosis. Together, these studies identify the phosphorylation state of PACS-2 $Ser_{437}$ as a molecular switch that regulates cellular homeostasis and Apo2L/TRAIL killing.

## 4.1 Introduction

The tumor necrosis factor (TNF) superfamily directs signaling pathways eliciting diverse responses ranging from tumor surveillance and inflammation to organogenesis and autoimmunity (Aggarwal, 2003). The prototypic superfamily ligand, TNFα, is a principally proinflammatory cytokine that mediates NF-κB signaling. However, when NF-κB is repressed, TNFα signals to the cytosolic death domain of the TNFR1 receptor to trigger apoptosis (Ashkenazi and Herbst, 2008). Two additional TNFα superfamily members, FasL and Apo2L/TRAIL, also trigger apoptosis by binding their death-domain containing receptors. The ability of these three TNFα death ligands to eliminate tumors in vivo has been intensively studied (Ashkenazi et al., 2008). Unfortunately, the severe toxicity elicited by systemic administration of TNFα and FasL prevents their development as cancer therapeutics (Rowinsky, 2005). By contrast, binding of Apo2L/TRAIL to its apoptotic receptors DR4 and DR5 selectively kills cancer cells and virally infected cells in vivo without causing harm to healthy cells (Ashkenazi and Herbst, 2008). This ability of Apo2L/TRAIL to selectively kill diseased cells has spurred clinical studies testing the efficacy of Apo2L/TRAIL as a potential therapeutic.

Apo2L/TRAIL is expressed primarily in lymphoid cells and is a multifunctional ligand important for regulation of T-cell homeostasis, innate immunity and for tumor surveillance (Cretney et al., 2002; Grosse-Wilde et al., 2008; Janssen et al., 2005; Takeda et al., 2001). Virus infection induces DR4 and DR5 expression on the cell surface, which bind Apo2L/TRAIL to trigger the innate immune response leading to the apoptotic

destruction of infected cells (Mundt et al., 2005). Similarly, neoplastic transformation of lung, pancreas and colon induce high levels of DR4 and DR5 (Rowinsky, 2005), which correlates with an improved survival rate of people suffering from metastatic colorectal cancer (Strater et al., 2002). Approximately 50% of tested tumor cell lines are however resistant to Apo2L/TRAIL (Kruyt, 2008), suggesting that the underlying regulation of Apo2L/TRAIL-induced apoptosis is complex. Understanding the molecular basis of Apo2L/TRAIL-induced apoptosis is therefore key to develop new therapeutic strategies for this devastating disease.

Apo2L/TRAIL triggers the extrinsic pathway of apoptosis by binding to DR4 and DR5, which directs recruitment of the adaptor FADD and the initiator caspase, procaspase-8 to assemble a death-inducing signaling complex (DISC). Two types of signaling pathways emanate from the DISC to execute the apoptotic program (Jin and El-Deiry, 2005). In type I cells, the DISC/caspase-8 signal is sufficiently robust to directly activate executioner caspases, including caspase-3. Type II cells require an amplification step to activate executioner caspases. This signal is mediated by the proapoptotic Bcl-2 family protein Bid, which links the extrinsic apoptotic pathway to the intrinsic pathway by triggering mitochondria membrane permeabilization (MMP) and caspase-9 activation, thereby amplifying caspase-3 activation (for review, see Chapter 2 and (Aslan and Thomas, 2008; Jin and El-Deiry, 2005)). The apoptotic activity of Bid is complex and regulated by phosphorylation (Desagher et al., 2001; Vogel et al., 2006), and by proteolysis (for review, see Chapter 2 and (Aslan and Thomas, 2008; Jin and El-Deiry, 2005)). Bid action is also regulated by PACS-2, a multi-functional sorting protein that

integrates secretory pathway traffic with ER-mitochondrial communication and apoptosis ((Atkins et al., 2008; Kottgen et al., 2005; Simmen et al., 2005). Apoptotic inducers switch PACS-2 from a secretory pathway trafficking protein to an apoptotic protein that mediates Bid translocation to mitochondria and Bid cleavage in cultured cell lines (Simmen et al., 2005). But how apoptotic cues trigger PACS-2 to become apoptotic is unknown.

Regulators of Apo2L/TRAIL action include a collection of death-inducing and decoy receptors, and several intracellular molecules, including Bcl-2 proteins, c-myc and Akt (for review see (Jin and El-Deiry, 2005)). The class I PI3K/Akt survival pathway is tumorigenic (Vivanco and Sawyers, 2002) and constitutive activation of Akt promotes resistance of tumors to apoptotic inducers, including Apo2L/TRAIL (Martelli et al., 2003; Shankar and Srivastava, 2004). Akt also combines with 14-3-3 proteins to repress the action of several proapoptotic proteins, including the Bcl-2 proteins Bad, Bim and the proapoptotic FOXO transcriptional regulators (Manning and Cantley, 2007; Porter et al., 2006).

Unlike Bad and Bim, Bid neither binds 14-3-3 proteins nor is it a known Akt substrate. Yet, elevated expression of Akt prevents Apo2L/TRAIL-induced apoptosis at the level of Bid cleavage (Chen et al., 2001; Kandasamy and Srivastava, 2002; Nesterov et al., 2001). Here we report Apo2L/TRAIL-mediated apoptosis in vivo requires PACS-2 and that Akt phosphorylation of PACS-2 at $Ser_{437}$ creates a 14-3-3 binding site that regulates PACS-2 apoptotic activity. Our results point to a novel step in the regulation of Apo2L/TRAIL-

mediated apoptosis and identify the molecular mechanism underlying the switch in PACS-2 from a homeostatic to an apoptotic regulator.

## 4.2 Results

### 4.2.1 Apo2L/TRAIL-mediated apoptosis requires PACS-2 in vivo

To test the possibility that PACS-2 is required for Apo2L/TRAIL-induced apoptosis, we transfected the type II colon carcinoma cell line HCT116 (Ravi and Bedi, 2002) with a control siRNA or with an siRNA that specifically knocked down PACS-2 (Fig. 4-1a, inset). The cells were then treated with increasing concentrations of Apo2L/TRAIL for 16 hr and the amount of apoptosis was quantified by Annexin V/propidium iodide staining (Fig. 4-1a). We found that siRNA knockdown of PACS-2 repressed Apo2L/TRAIL-mediated apoptosis in HCT116 cells 4-fold, resulting in a nearly complete protection of the culture from the death ligand.

Consistent with a proapoptotic role for PACS-2 in HCT116 colon carcinoma cells, the PACS-2 locus, located at chromosome 14q32.33, is highly susceptible to loss of heterozygosity in colorectal cancer (Anderson et al., 2001; Bartos et al., 2007), suggesting that PACS-2 protein expression may be altered during the colon adenoma-carcinoma sequence. We therefore isolated matched cancerous and adjacent normal colon tissue from eight volunteers and the expression of PACS-2 in each sample was measured by western blot (Fig. 4-1b). We found that PACS-2 protein expression was lost in 4 out of 8 samples. Immunohistochemical staining corroborated these studies, demonstrating PACS-2 was detected in the normal colonic epithelium but not in the adjacent carcinoma (Fig. 4-1c).

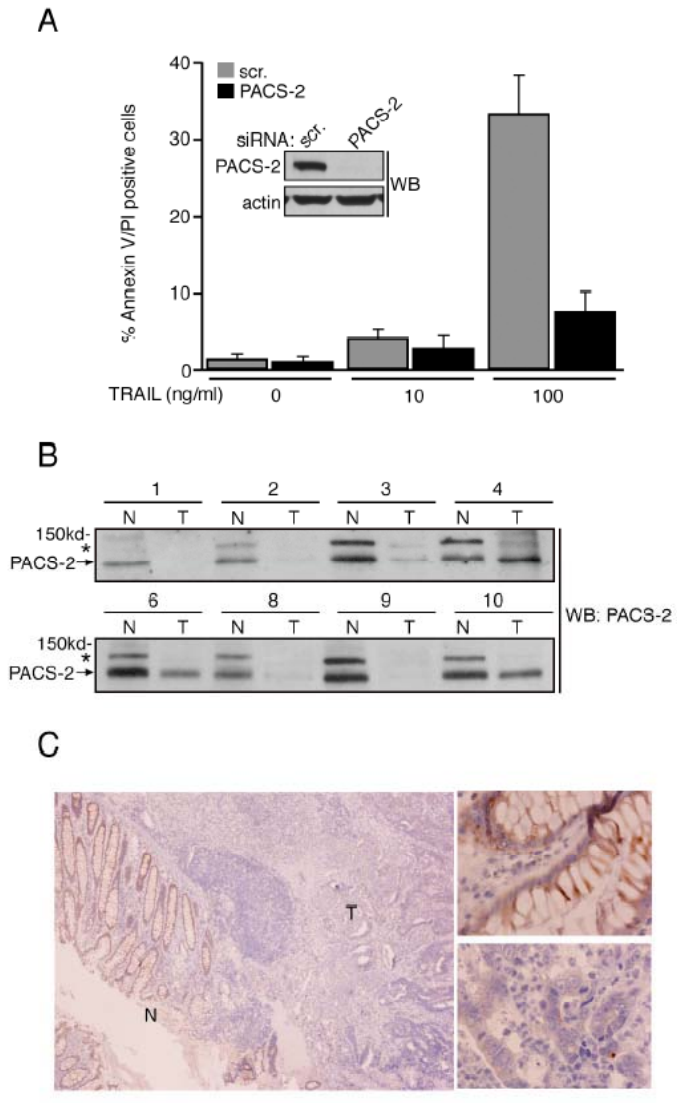



Figure 4-1: PACS-2 is required for TRAIL-mediated tumor cell death.

**(A)** HCT116 colon carcinoma cells were treated with PACS-2 or control (scr.) siRNAs and then treated with 0, 10 or 100 ng/ml Apo2L/TRAIL for 16 hr. The cells were collected and analyzed for apoptosis by Annexin V/propidium iodide staining by flow cytometry. Data are represented as mean +/- SEM from three independent experiments. Inset: Western blot (WB) of PACS-2 in control and PACS-2 knockdown cells. **(B)** Matched colorectal tumor (T) and adjacent normal (N) colonic tissue from eight volunteers was isolated by microdissection. The frozen samples were homogenized and 100 μg of total protein from each sample was resolved by SDS-PAGE and PACS-2 was detected by western blot. Arrow, 110 kDa PACS-2; *, 150 kDa PACS-2 immunoreactive protein of unknown identity. **(C)** A paraffin-embedded block of colonic tumor was sectioned and analyzed by immunohistochemistry using anti-PACS-2 HPA001423 (Atlas). Left, Normal colonic epithelium (N) and adjacent tumor (T). Right, Magnification of normal (top) and tumor (bottom) regions.

To test the physiological role of PACS-2 as a mediator of Apo2L/TRAIL-dependent apoptosis in vivo, we generated PACS-2-gene trapped mice (Fig. 4-2a). These mice were viable but lacked detectable PACS-2 expression in all organs tested as determined by RT-PCR and western blot (Fig. 4-2b and c). The mice were subjected to an experimental paradigm of Apo2L/TRAIL- and DR5-dependent hepatic apoptosis and steatosis using recombinant adenoviruses that mimic the pathology caused by hepatitis C virus (Mundt et al., 2005). Littermate WT and PACS-2 gene trap mice (hereafter called PACS-$2^{-/-}$ mice) were injected in the tail vein with a recombinant adenovirus expressing GFP for 24 hours followed by injection with a second adenovirus expressing Apo2L/TRAIL, which triggers hepatocyte apoptosis. In agreement with others (Mundt et al., 2005), Apo2L/TRAIL induced significant apoptosis in the infected livers of WT mice as determined by TUNEL staining and formation of cleaved caspase-3 (Fig. 4-2d). By contrast, Apo2L/TRAIL failed to elicit apoptosis in livers of PACS-$2^{-/-}$ mice, suggesting PACS-2 is required for Apo2L/TRAIL-mediated apoptosis in vivo. Surprisingly, the absence of PACS-2 did not prevent Apo2L/TRAIL from eliciting pronounced hepatic steatosis, demonstrating that Apo2L/TRAIL-mediated steatosis but not apoptosis can occur in the absence of PACS-2. To determine whether PACS-2 was required for other death ligands to induce apoptosis in the liver, we treated WT and PACS-$2^{-/-}$ with the anti-Fas antibody Jo2 or with LPS/Galactosamine to trigger TNF$\alpha$-induced apoptosis (Fig. 4-2e). The extent of hepatic apoptosis in WT and PACS-$2^{-/-}$ mice treated with these agents was similar, suggesting that Apo2L/TRAIL-mediated hepatic apoptosis selectively requires PACS-2.

Figure 4-2. PACS-2 is required for TRAIL-mediated apoptosis *in vivo*. **(A)** VICTR37 retroviral gene trap inserted into intron 1 of the murine PACS-2 gene (AceView, NCBI). The gene trap contains a promoter-less selectable marker/β-geo fusion gene and produces a truncated protein chimera containing only the first 40 amino acids of the entire 889 amino acid PACS-2. Arrows above exons 1 and 11 depict PCR primers used in panel B. SD, splice donor site; SA, splice acceptor. **(B)** RNA was isolated from PACS-2 WT (PACS-$2^{+/+}$), heterozygote (PACS-$2^{+/\beta\text{-geo}}$, hereafter called PACS-$2^{+/-}$), or PACS-2 gene trap (PACS-$2^{\beta\text{-geo}/\beta\text{-geo}}$, hereafter called PACS-$2^{-/-}$) littermates and analyzed with specific primers to detect PACS-1, PACS-2 and ATP citrate lyase (ACYL, positive control) by RT-PCR. **(C)** Kidney, colon and liver from WT, +/- or -/- mice were harvested and analyzed by western blot for PACS-2 expression. **(D)** WT and PACS-$2^{-/-}$ mice (n = 4 in each group) were injected with $2x10^8$pfu/g and AdGFP and 24h later with $3x10^8$ pfu/g mouse AdTrail. After an additional 24 hr the livers were isolated, fixed, sectioned and stained with H&E or TUNEL or c-caspase 3 antibody (magnification, 200x). TUNEL and c-caspase-3 positive cells were counted in liver sections from WT and PACS-$2^{-/-}$ mice and quantified as the number of TUNEL- and cleaved caspase 3-positive cells per 20X field (graphs). Oil Red O staining was performed to demonstrate Apo2L/TRAIL-induced liver steatosis. Data are representative of three independent experiments. **(E)** WT and PACS-$2^{-/-}$ mice (n = 4 in each group) were injected with the mAb Jo2 (0.7μg/g mouse) to stimulate Fas-mediated apoptosis or with LPS (0.05μg/g)/GalN (700μg/g) to stimulate TNFα-mediated apoptosis and TUNEL-positive nuclei were counted (original magnification 200x).

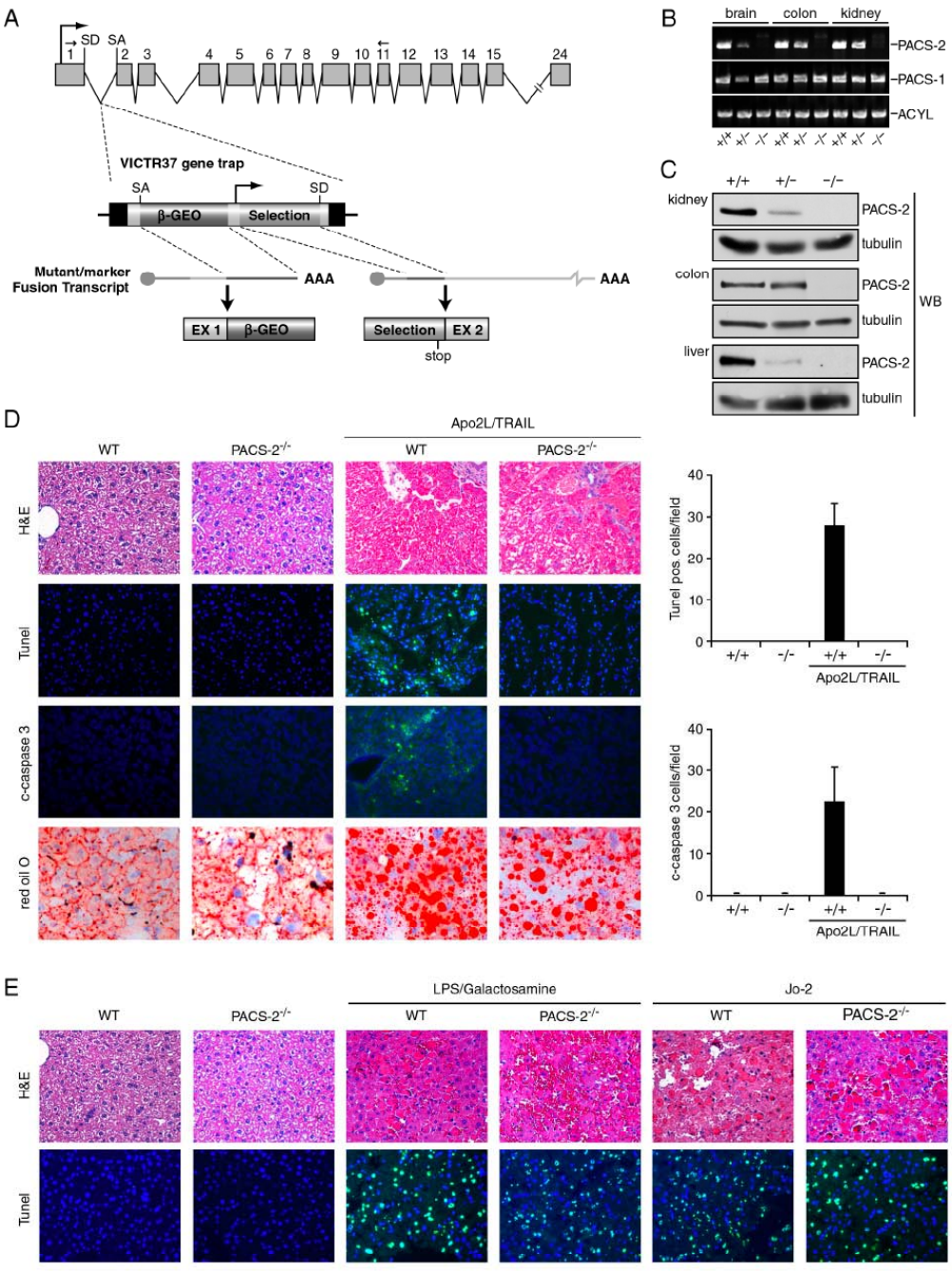



Figure 4-2: PACS-2 is required for TRAIL-mediated apoptosis *in vivo.*

### 4.2.2 Apo2L/TRAIL requires PACS-2 to trigger cleavage of Bid and caspases

To understand the molecular basis underlying how Apo2L/TRAIL induces PACS-2 to become an apoptotic effector, we examined the ability of Apo2L/TRAIL to trigger apoptosis in embryonic fibroblasts (MEFs) from WT and PACS-2$^{-/-}$ mice. In agreement with others (Finnberg et al., 2005; Wiley et al., 1995), primary WT and PACS-2$^{-/-}$ MEFs were resistant to Apo2L/TRAIL (Fig. 4-3a). We therefore transformed the primary MEFs with SV40 large T antigen (TAg), which induced expression of DR5 (Fig. 4-3a, inset). We then treated the TAg-transformed cells with increasing concentrations of Apo2L/TRAIL and measured apoptosis. We found that the TAg-WT MEFs were sensitive to Apo2L/TRAIL. By contrast, the TAg-PACS-2$^{-/-}$ MEFs remained fully resistant to Apo2L/TRAIL at all concentrations examined. To determine whether loss of PACS-2 was causal to Apo2L/TRAIL resistance, we generated a TAg-PACS-2$^{-/-}$ cell line rescued for PACS-2 expression and found that re-expression of PACS-2 restored sensitivity to Apo2L/TRAIL.

**Figure 4-3. PACS-2 is required for Apo2L/TRAIL-induced apoptosis of SV40 large T antigen-transformed MEFs. (A)** Primary WT and PACS-2$^{-/-}$ MEFs, as well as TAg-WT, TAg-PACS-2$^{-/-}$, TAg-PACS-2$^{V}$ or TAg-PACS-2$^{R}$ MEFs were treated with increasing concentrations of Apo2L/TRAIL for 16 hr and then analyzed for apoptosis by Annexin V/ propidium iodide staining using flow cytometry. Inset, western blot of PACS-2 and DR5 expression in each cell culture. Data are represented as mean +/- SEM from three independent experiments. **(B)** Replicate plates of TAg-WT and TAg-PACS-2$^{-/-}$ MEFs were treated with 50 ng/ml Apo2L/TRAIL for the times indicated. The cells were then lysed, the extracts were resolved by SDS-PAGE and the cleavage of each apoptotic factor was determined by western blot. Actin was analyzed as a loading control. Shown is one of four independent experiments. **(C)** Replicate plates of TAg-WT and TAg-PACS-2$^{-/-}$ MEFs were pretreated or not with vehicle (DMSO) or 1 μM PI-103 for 1 hr and then treated with 50 ng/ml Apo2L/TRAIL for the indicated times. The cells were harvested and analyzed as described in panel B. The efficacy of PI-103 was determined by phospho-Akt and phospho-S6 blots. Actin was analyzed as a loading control. Shown is one experiment representative of four independent experiments.

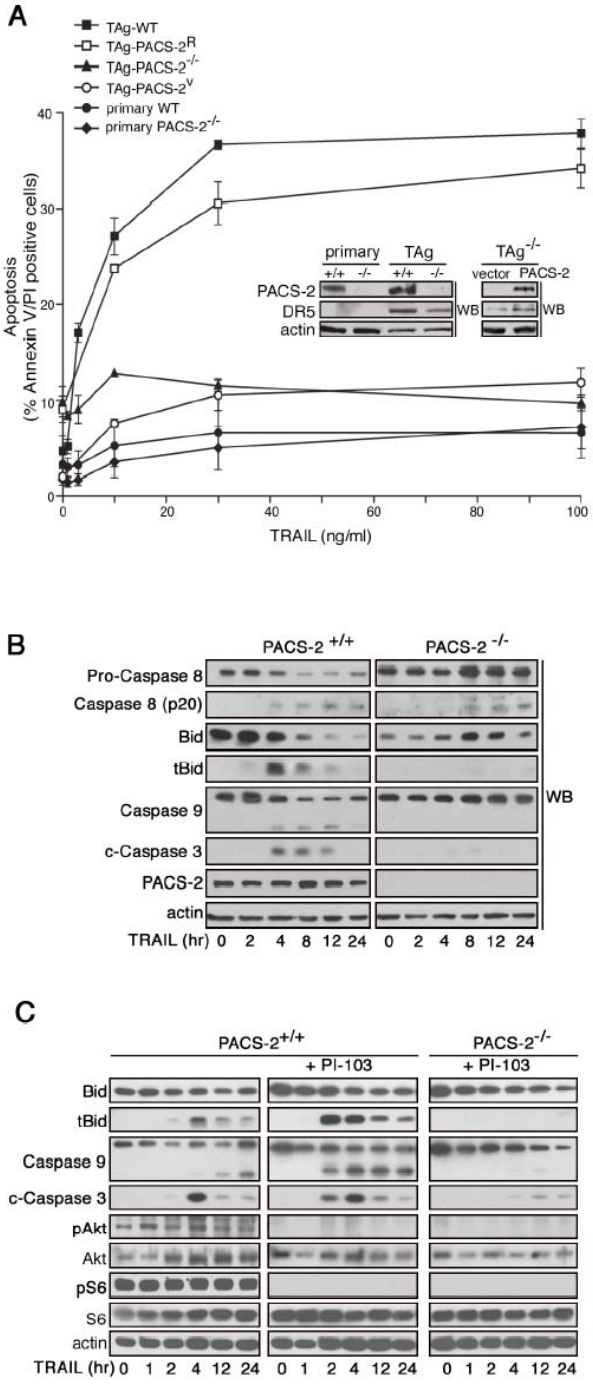



Figure 4-3: PACS-2 is required for Apo2L/TRAIL-induced apoptosis of SV40 large T antigen transformed MEFs.

To better delineate the step by which PACS-2 mediates Apo2L/TRAIL-induced apoptosis, we treated replicate plates of TAg-WT and TAg-PACS-2$^{-/-}$ cells with Apo2L/TRAIL. At increasing times the cells were harvested and the cleavage/activation of initiator and executioner caspases was determined by western blot analysis (Fig. 4-3b). In Apo2L/TRAIL-treated TAg-WT cells, cleaved caspase-8 was detected by 4 hr. The formation of cleaved caspase-8 coincided with the cleavage of Bid and caspase-9 as well cleavage of caspase-3. In TAg-PACS-2$^{-/-}$ cells, Apo2L/TRAIL induced cleavage of caspase-8. However, formation of tBid, and thus subsequent activation of caspases-9 and -3 was inhibited. The failure of Apo2L/TRAIL to induce tBid formation in TAg-PACS-2$^{-/-}$ cells agreed with earlier studies suggesting PACS-2 mediates Bid cleavage.

Previously, it has been shown that Akt represses Apo2L/TRAIL action by interfering with Bid cleavage (Chen et al., 2001; Nesterov et al., 2001; Simmen et al., 2005). We therefore asked whether inhibition of PI3K/Akt would rescue sensitivity of the TAg-PACS-2$^{-/-}$ cells to Apo2L/TRAIL. Parallel plates of TAg-WT and TAg-PACS-2$^{-/-}$ MEFs were treated with Apo2L/TRAIL in the presence or absence of the class I PI3K inhibitor PI-103, which blocked Akt activation and ribosomal protein S6 phosphorylation (Fig. 4-3c). We found that PI-103 markedly enhanced cleavage of Bid to tBid as well as formation of caspases-9 and -3. In TAg-PACS-2$^{-/-}$ cells, however, PI-103 had little effect on the ability of Apo2L/TRAIL to trigger cleavage of Bid or of procaspases-9 and -3.

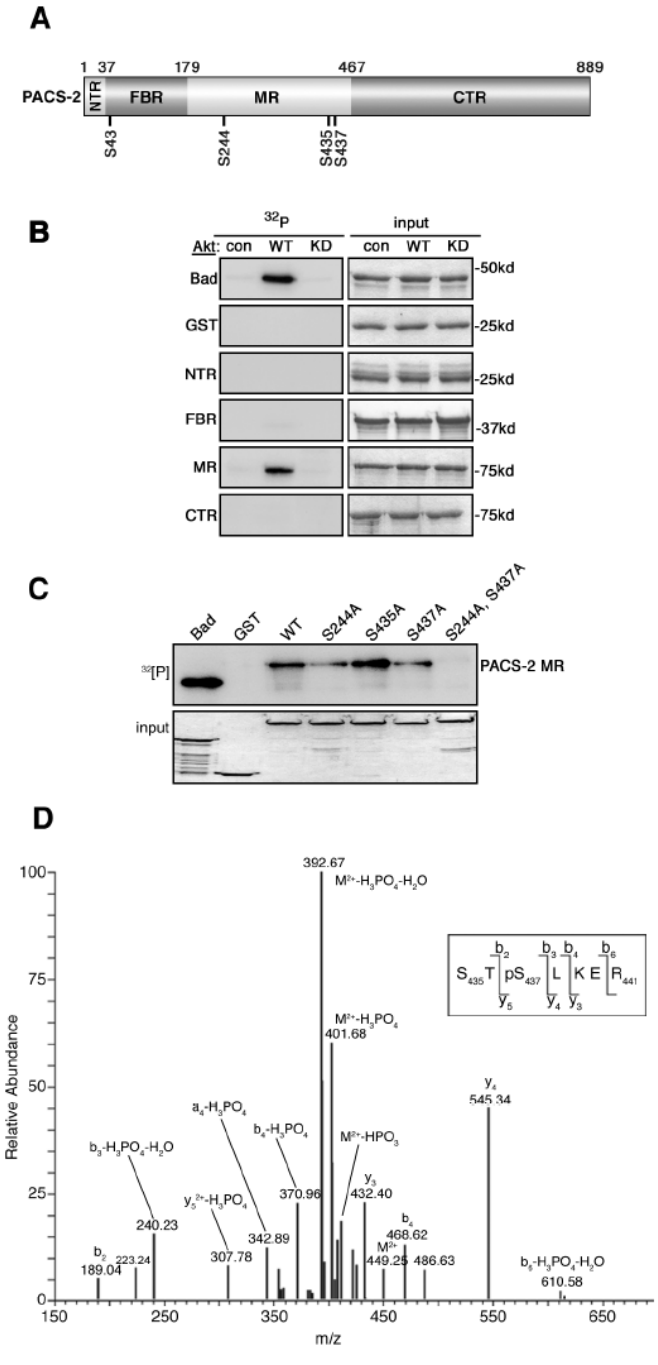



Figure 4-4: Akt phosphorylates PACS-2 $Ser_{437}$.

**(A)** Diagram of PACS-2 showing the N-terminal region (NTR), cargo-binding region (FBR), which contains $Ser_{43}$, the middle region (MR), which contains $Ser_{244}$, $Ser_{435}$ and $Ser_{437}$, and the C-terminal region (CTR). **(B)** The indicated GST-fusion proteins were incubated with [$\gamma$-$^{32}$P]-ATP and constitutively active HA-Akt (WT) or kinase-dead HA-Akt (KD) immunoprecipitates from cells infected with the respective adenovirus recombinants. Con, immunoisolation from mock-infected cells. Left panels, autoradiography; Right panels, input. Data are representative of six independent experiments. **(C)** The indicated GST-fusion proteins were incubated with [$\gamma$-$^{32}$P]-ATP and immunoisolated HA-Akt as described in panel B. Top, autoradiography; Bottom, input. Data are representative of three independent experiments. **(D)** Peptides from in-gel limited trypsin digestion of HA-tagged PACS-2 immunoisolated from BSC-40 cells were enriched for phosphopeptide on $TiO_2$, desalted on a POROS R3 microcolumn and evaluated for candidate phosphopeptide by precursor ion scans in negative ion. A phosphopeptide at [M+2H]=450.7, corresponding to the PACS-2 tryptic peptide $RSTpS_{437}LKER$ with one phosphorylation, was identified by nanoLC-MS/MS on a Thermo LCQ Deca XP quadrupole ion trap mass spectrometer.

### 4.2.3 Akt phosphorylates PACS-2 at $Ser_{437}$

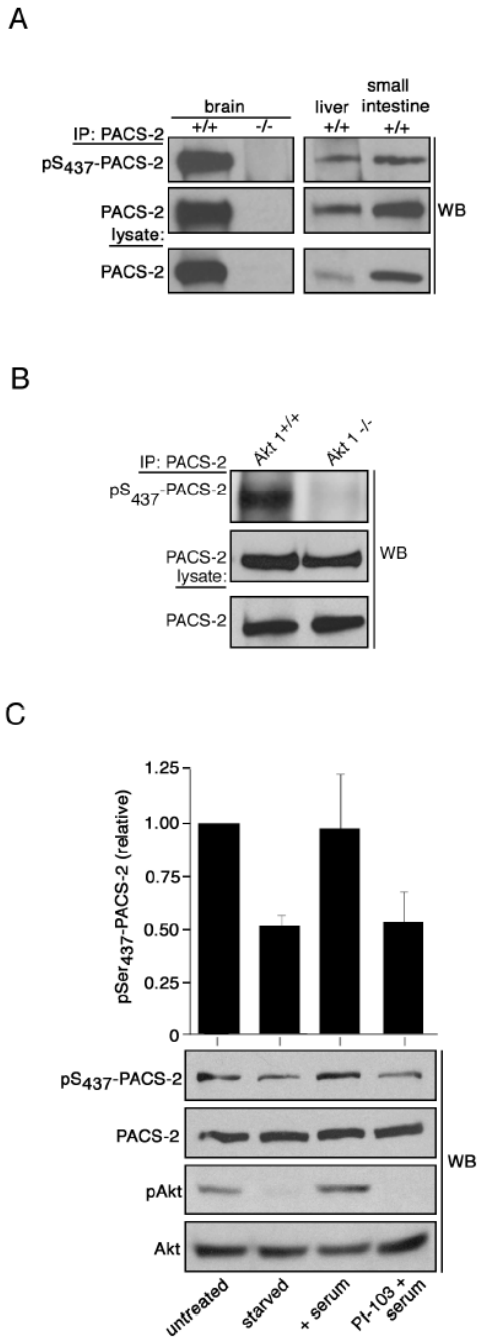
Our determination that inhibition of PI3K/Akt augmented Bid cleavage in TAg-WT cells but not TAg-PACS-$2^{-/-}$ cells raised the possibility that Akt may regulate Bid cleavage by acting directly on PACS-2. Inspection of the 889-amino acid PACS-2 protein sequence (Scansite) identified four candidate Akt phosphorylation sites at $Ser_{43}$, $Ser_{244}$, $Ser_{435}$ and $Ser_{437}$ (Fig. 4-4a). We therefore tested the ability of Akt to phosphorylate in vitro GST-fusion proteins corresponding to the four segments of PACS-2 (PACS-$2_{NTR}$, PACS-$2_{FBR}$, PACS-$2_{MR}$ or PACS-$2_{CTR}$, see Fig. 4-4a), and found that Akt phosphorylated only PACS-$2_{MR}$, which contains $Ser_{244}$, $Ser_{435}$ and $Ser_{437}$ (Fig. 4-4b). To identify which of these sites were required for phosphorylation, we incubated Akt with PACS-$2_{MR}$ mutants containing Ser→Ala substitutions at $Ser_{244}$, $Ser_{435}$ or $Ser_{437}$ and found that the $Ser_{244}$Ala and $Ser_{437}$Ala mutations reduced phosphorylation by ~50%, suggesting Akt phosphorylated both sites *in vitro*. This possibility was confirmed by determining that Akt could not phosphorylate a PACS-$2_{MR}$ mutant containing a $Ser_{244,437}$AlaAla double substitution

To determine if PACS-2 $Ser_{244}$ or $Ser_{437}$ were phosphorylated in vivo, we performed a mass spectrometry analysis of human PACS-2 purified from cultured cells. Purified PACS-2 trypsinolytic peptides were purified over a $TiO_2$ phosphopeptide enrichment column, desalted and sequenced by tandem mass spectrometry (Fig. 4-4d). This analysis yielded the phosphopeptide STp$S_{437}$LKER, demonstrating PACS-2 $Ser_{437}$ is a *bona fide* kinase target *in vivo*. Identification of the phosphopeptide was confirmed by comparing the fragmentation pattern of the peptide released from PACS-2 in vivo with the synthetic peptide ST(pS)LKER (Fig. 4-8).

To examine the regulation of $pSer_{437}$ *in vivo*, we identified an Akt substrate phosphospecific antibody, mAb 81, raised against the sequence (RX)RXXpS that recognizes PACS-2 $pSer_{437}$ (Fig. 4-9). We prepared brain cytosol from WT and PACS-2$^{-/-}$ mice and immunoprecipitated endogenous PACS-2 with a pan-PACS-2 antiserum. The immunoprecipitated material was then analyzed by western blot using the pan-PACS-2 antiserum or mAb 81 (Fig. 4-5a). PACS-2 was immunoprecipitated only from WT mouse brain cytosol and recognized by mAb 81, demonstrating PACS-2 is phosphorylated *in vivo* at $Ser_{437}$. We extended this approach and found that lysates from liver and small intestine contained mAb 81-positive PACS-2, demonstrating that PACS-2 is phosphorylated at $Ser_{437}$ in multiple tissues.

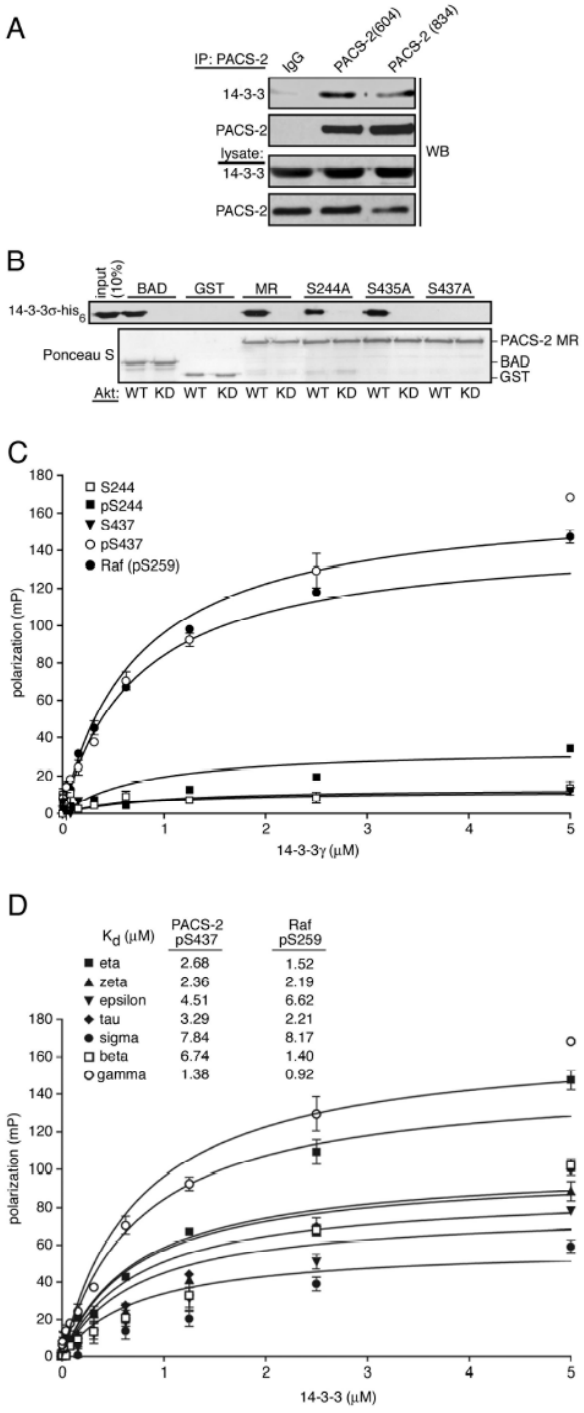
Our determination that Akt phosphorylated PACS-2 $Ser_{437}$ *in vitro* led us to ask whether Akt phosphorylates this site in vivo. We immunoprecipitated endogenous PACS-2 from parallel cultures of WT and Akt1$^{-/-}$ MEFs and then detected $pSer_{437}$ by western blot (Fig. 4-5b). We found that PACS-2 was expressed in both WT and Akt1$^{-/-}$ MEFs. However, PACS-2 $pSer_{437}$ was detected in WT MEFs but not Akt1$^{-/-}$ MEFs, suggesting Akt is an in vivo PACS-2 $Ser_{437}$ kinase. To determine if PI3K/Akt-mediated survival signaling regulates phosphorylation of PACS-2 $Ser_{437}$, HCT116 cells were serum starved overnight before re-feeding with serum in the absence or presence of the class I PI3K inhibitor PI-103 (Fig. 4-5c). Serum starvation decreased PACS-2 $pSer_{437}$ levels in HCT116 cells by 2-fold. Upon addition of serum, $pSer_{437}$ returned to basal levels in control cells but not in PI-103-treated cells. Together, these results suggest PI3K/Akt survival pathways regulate the level of phosphorylation at PACS-2 $Ser_{437}$.

Figure 4-5: *In vivo* phosphorylation of PACS-2 $Ser_{437}$.




(A) Left panel; Brain cytosol from WT and PACS-2-/- mice was incubated with affinity purified anti-PACS-2 (Ab 832) and immunoprecipitated proteins were then analyzed by western blot using anti-PACS-2 (Ab 193) or mAb 81 to detect pSer437. Right panel; Liver and small intestine cytosol from WT mice were analyzed for total PACS-2 and PACS-2 pSer437 as described above. (B) Lysates from WT and Akt1-/- MEFs were incubated with affinity purified anti-PACS-2 (Ab 832) and immunoprecipitated proteins were then analyzed by western blot using anti-PACS-2 (Ab 193) or mAb 81 to detect pSer437 as described in panel A. (C) Replicate plates of HCT116 cells were harvested in proliferative phase (untreated), serum-starved for 16 hr (starved), starved and refed with 20% serum for 2 hr (+ serum), or starved and pre-treated with 1 μM PI-103 for one hr prior to refeeding with 20% serum for 2h (PI-103 + serum). Equivalent amounts of each cell lysate were then analyzed for by western blot using anti-PACS-2 (Ab 193) to detect total PACS-2 and mAb 81 to detect PACS-2 pSer437. The blots were also incubated with anti-Akt and anti-pSer473Akt to detect total and activated Akt, respectively and signals quantified by densitometry using Image J software. The pSer437:total PACS-2 ratio was calculated and normalized to the untreated sample. Data are represented as mean +/- SEM from three independent experiments.

Figure 4-6: 14-3-3 proteins bind PACS-2 phospho-Ser$_{437}$




(A) PACS-2 was immunoprecipitated from rat brain cytosol using Abs 604 or 834 and co-precipitating 14-3-3 was detected by western blot. (B) The indicated GST fusion proteins were incubated with ATP and constitutively active HA-Akt (WT) or kinase-dead HA-Akt (KD), then incubated with 14-3-3σ-his6. Protein complexes were captured with glutathione-sepharose and bound 14-3-3σ-his6 was detected by western blot. (C) 5,6-carboxytetramethylrhodamine (TMR)–labeled 16mer peptides containing pSer259-Raf, PACS-2 Ser244-PACS-2, pSer244-PACS-2, Ser437-PACS-2 or pSer437-PACS-2 (1 nM, final) were incubated in triplicate with increasing concentrations of GST-14-3-3γ in 384-well black microplates with a total volume of 50 μL. Fluorescence polarization signals were recorded and used to calculate Kd values as described in Experimental Methods. Assays were performed in triplicate. (D) GST fusion proteins containing the indicated 14-3-3 isoforms were incubated with (TMR)-labeled pSer437-PACS-2 or pSer259-Raf peptides and fluorescence polarization was measured as described in panel C. Calculated Kd values are shown.

### 4.2.4 14-3-3 proteins bind PACS-2 $pSer_{437}$

The regulation of Bad and FOXO proapoptotic activity by binding of 14-3-3 proteins to Akt-phosphorylated serine residues in these two proteins (Porter et al., 2006), led us to ask whether 14-3-3 binds PACS-2 $Ser_{437}$. We first asked whether PACS-2 is a 14-3-3-client protein in vivo. We immunoprecipitated PACS-2 from brain cytosol with two different PACS-2 antisera and found that 14-3-3 co-precipitated with PACS-2 (Fig. 6a). Next, GST fusion proteins encoding PACS-$2_{MR}$ or the PACS-$2_{MR}$ mutants containing $Ser_{244}$Ala, $Ser_{435}$Ala or $Ser_{437}$Ala substitutions were pre-incubated with Akt and then mixed with $His_6$-14-3-3. The proteins were captured with glutathione agarose and bound $His_6$-14-3-3 was detected by western blot (Fig. 4-6b). We found that $His_6$-14-3-3 bound to the Akt-phosphorylated PACS-$2_{MR}$ and the $Ser_{244}$Ala and $Ser_{435}$A mutants but not the Akt-phosphorylated $Ser_{437}$Ala mutant, suggesting phosphorylation of PACS-2 at $Ser_{437}$ was required for binding 14-3-3 proteins. We then conducted a fluorescence polarization assay to determine quantitatively whether phosphorylated $Ser_{437}$ was sufficient to bind 14-3-3 proteins. Rhodamine-conjugated, 16-mer peptides corresponding to non-phosphorylated or phosphorylated PACS-2 $Ser_{244}$ or $Ser_{437}$ were mixed with increasing concentrations of recombinant 14-3-3γ and the resultant effect on fluorescence polarization was recorded (Fig. 6c). We found only $pSer_{437}$ peptide bound 14-3-3γ and the affinity of this binding was similar to the positive control peptide corresponding to Raf $pSer_{259}$. We then asked whether the $pSer_{437}$ phosphopeptide binds additional 14-3-3 isoforms and found that, similar to Raf $pSer_{259}$, PACS-2 $pSer_{437}$ bound all seven human 14-3-3 isoforms with $K_d$s ranging from 1.34 to 7.84 μM (Fig. 4-6d).

Thus, PACS-2 $pSer_{437}$ binds 14-3-3 proteins with an affinity similar to other 14-3-3 client proteins (Du et al., 2006).

### 4.2.5 PACS-2 $pSer_{437}$ binding to 14-3-3 regulates Apo2L/TRAIL-induced apoptosis

The binding of 14-3-3 proteins to PACS-2 $pSer_{437}$ suggested that apoptotic induction would disrupt binding of PACS-2 to 14-3-3. To test this possibility, we treated HCT116 cells co-expressing PACS-2ha and 14-3-3myc with Apo2L/TRAIL to induce apoptosis. PACS-2ha was immunoprecipitated at increasing times after addition of the death ligand and the status of $Ser_{437}$ phosphorylation and co-precipitated 14-3-3myc were monitored by western blot (Fig. 4-7a). We found that Apo2L/TRAIL induced dephosphorylation of PACS-2 $Ser_{437}$, which was temporally coupled with the release of bound 14-3-3myc and increased cleaved caspase-3.

To determine if 14-3-3 regulates the apoptotic activity of PACS-2, we used a fluorescent protein-based reporter assay of caspase-3 activation (see Materials and Methods). We co-transfected TAg-PACS-$2^{-/-}$ MEFs cells with plasmids expressing the caspase-3 with HA-tagged PACS-2 or PACS-2$Ser_{437}$Ala. The cells were then treated with Apo2L/TRAIL for 24 hr, fixed and the subcellular localization of the eYFP reporter was quantified as a marker of caspase-3 activation (Fig. 4-7b). We found that PACS-2 and PACS-2$Ser_{437}$Ala increased the number of caspase-3-positive cells induced with Apo2L/TRAIL by nearly 5-fold over control cells. Next, we tested the ability of co-expressed 14-3-3 to repress the increased caspase-3 activity and found that 14-3-3myc repressed the ability of PACS-2

but not PACS-2Ser$_{437}$Ala to increase caspase-3 activity. Consistent with these results, co-immunoprecipitation studies showed that 14-3-3myc interacted with HA-tagged PACS-2 but not PACS-2Ser$_{437}$Ala (Fig. 4-7c). These results suggest that the binding of 14-3-3 to PACS-2 Ser$_{437}$ regulates Apo2L/TRAIL-dependent apoptosis.

Figure 4-7. PACS-2 Ser437 and 14-3-3 control a homeostatic switch to apoptosis. (A) Replicate plates of HCT116 cells expressing HA-tagged PACS-2 and myc-tagged 14-3-3ζ were treated with 25 ng/ml TRAIL for the indicated times. Cell lysates were incubated with mAb HA.11 to immunoprecipitate PACS-2ha and immunoblotted with mAb 9E10 to detect co-precipitating 14-3-3ζ, mAb 81 to detect pSer437 and HA.11 to detect total immunoprecipitated PACS-2ha. Western blotting was also performed on total cell lysate to detect c-caspase 3, total PACS-2ha and 14-3-3ζ-myc. (B) Replicate plates of TAg-PACS-2-/- cells were co-transfected with an eYFP-tagged caspase-3 reporter together with pcDNA3.1 (vector) or plasmids expressing HA-tagged PACS-2 or PACS-2S437A alone or in addition to 14-3-3ζ-myc. The cells were then treated with vehicle or 10 ng/ml TRAIL for 16hr, fixed and the number of nuclear eYFP-positive cells indicating activated caspase-3 were counted in 5 random fields. Data are represented as mean +/- SEM from three independent experiments. (C) Replicate plates of TAg-PACS-2-/- described in panel B co-expressing 14-3-3ζ-myc and HA-tagged PACS-2 or PACS-2S437A were lysed, PACS-2 proteins immunoprecipitated with anti-HA (mAb HA.11) and co-precipitating 14-3-3ζ-myc was detected by western blot using mAb 9E10. (D) Replicate plates of HeLa cells were nucleofected (Amaxa) with plasmids expressing myc-polycystin-2 (PKD2, top panels) or Nef-eYFP (bottom panels), together with either empty vector or plasmids expressing PACS-2 or PACS-2S437A. After 36 hr, the cells were fixed and processed for confocal microscopy using anti-myc (mAb 9E10, green) plus anti-Golgin-97 (red, top panels) or anti-Golgin-97 alone (bottom panels) and visualized using Alexa-conjugated secondary antibodies.

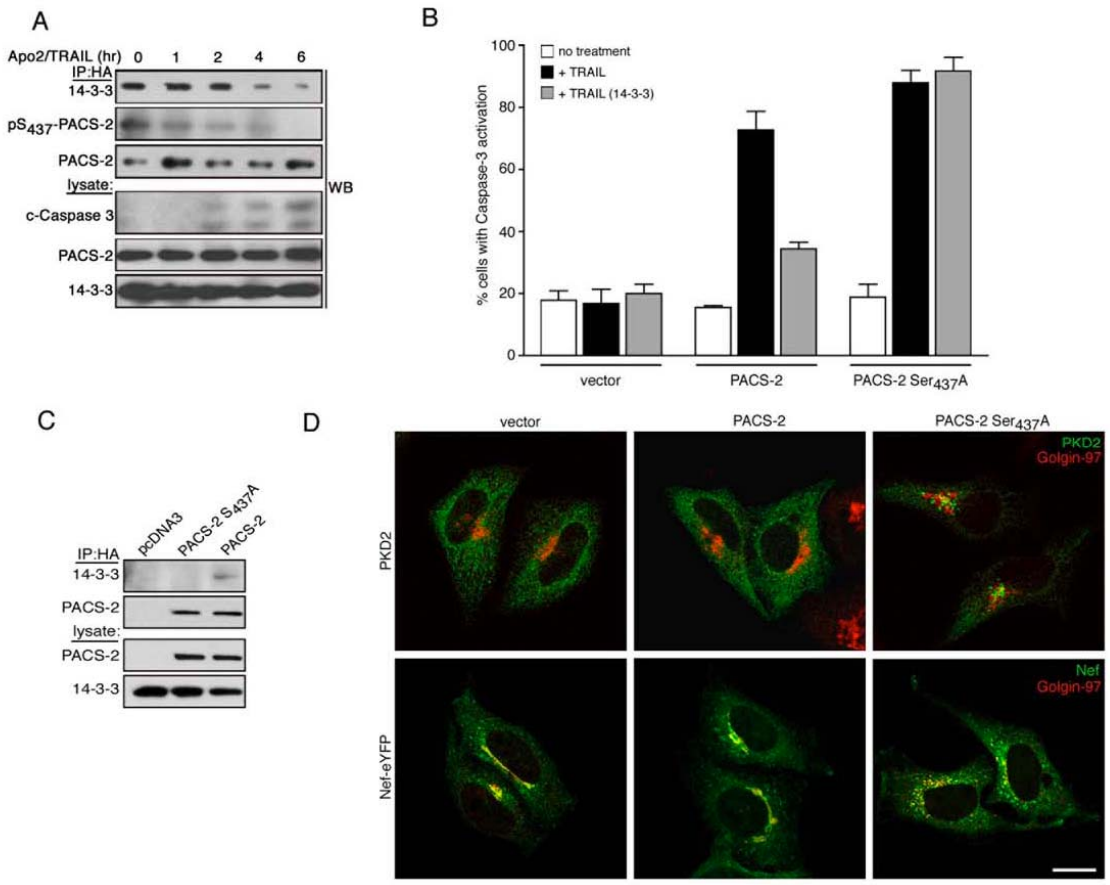



Figure 4-7: PACS-2 $Ser_{437}$ and 14-3-3 control a homeostatic switch to apoptosis.

### 4.2.6 PACS-2 $Ser_{437}$ is required for membrane and protein traffic

In non-apoptotic cells PACS-2 mediates the localization of cellular and viral membrane cargo to secretory pathway compartments. We therefore asked whether phosphorylated $Ser_{437}$ was required for PACS-2 to mediate protein traffic. One PACS-2 cargo protein is the calcium channel polycystin-2, which binds PACS-2 and localizes to the endoplasmic reticulum in a PACS-2-dependent manner (Kottgen et al., 2005). We co-expressed myc-polycystin-2 with either a control vector or with PACS-2 or PACS-2$Ser_{437}$A and monitored the subcellular localization of myc-polycystin-2 by confocal microscopy (Fig. 4-7d). In agreement with earlier studies (Kottgen et al., 2005), we found myc-polycystin-2 localized to the ER in control cells or cells overexpressing PACS-2. By contrast, co-expression of PACS-2$Ser_{437}$A caused myc-polycystin-2 to redistribute from the ER to the paranuclear region where it showed partial overlap with the late Golgi marker Golgin-97. HIV-1 Nef also binds PACS-2 to traffic from endosomal compartments to the paranuclear region where it assembles a multi-kinase complex that triggers MHC-I downregulation (Atkins et al., 2008). We therefore co-expressed Nef-eYFP with PACS-2 or PACS-2$Ser_{437}$Ala and monitored Nef-eYFP localization by confocal microscopy. We found that Nef-eYFP localized to the Golgi region in control cells or cells co-expressing PACS-2. By contrast, PACS-2$Ser_{437}$A disrupted the ability of Nef-eYFP to localize to the Golgi region, causing it instead to accumulate in dispersed compartments that also contained Golgin-97 (Fig. 4-7d). Together, these results suggest the binding of 14-3-3 to $pSer_{437}$ is a key determinant governing the switch of PACS-2 between its roles in membrane traffic and Apo2L/TRAIL-mediated apoptosis.

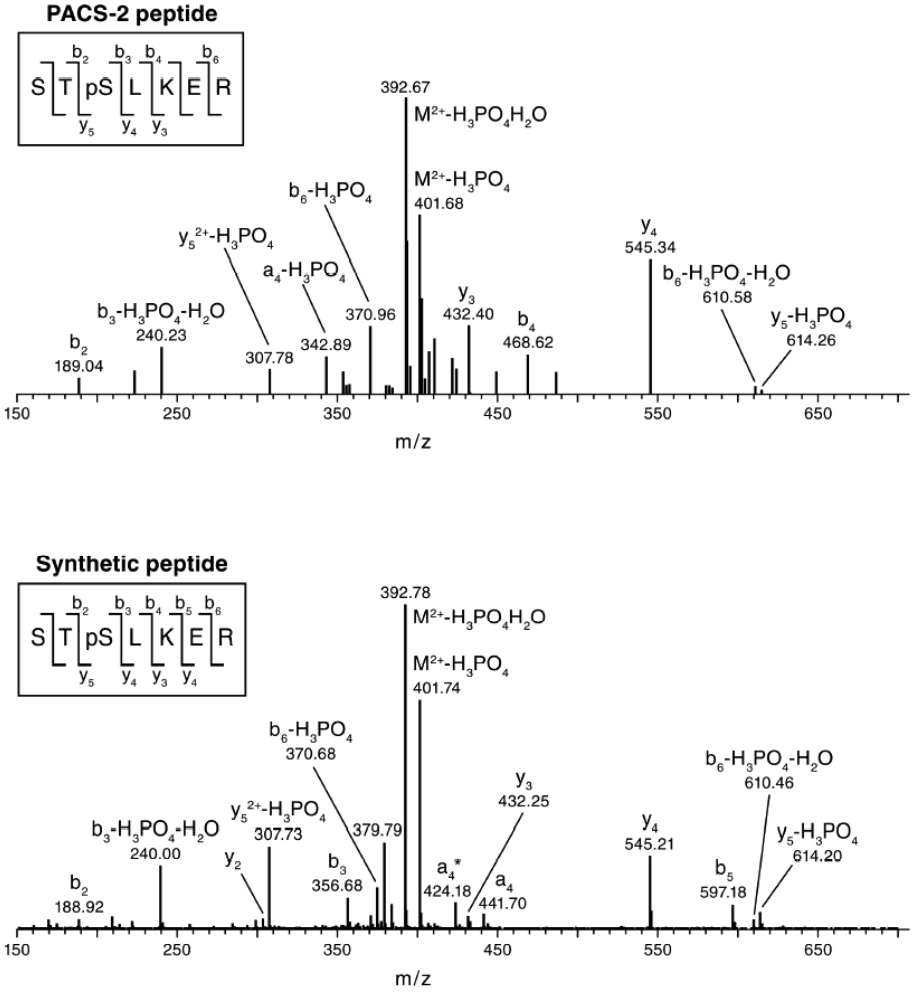



Figure 4-8. Confirmation of $pSer_{437}$ tryptic peptide.

Comparison of the tandemmass spectra from an in vivo PACS-2 phosphopeptide and a synthetic peptide of the determined sequence. Top; PACS-2 tryptic peptide spectrum as described in Fig.4-4d. Bottom, fragmentation spectrum of synthetic RST(pS)LKER.

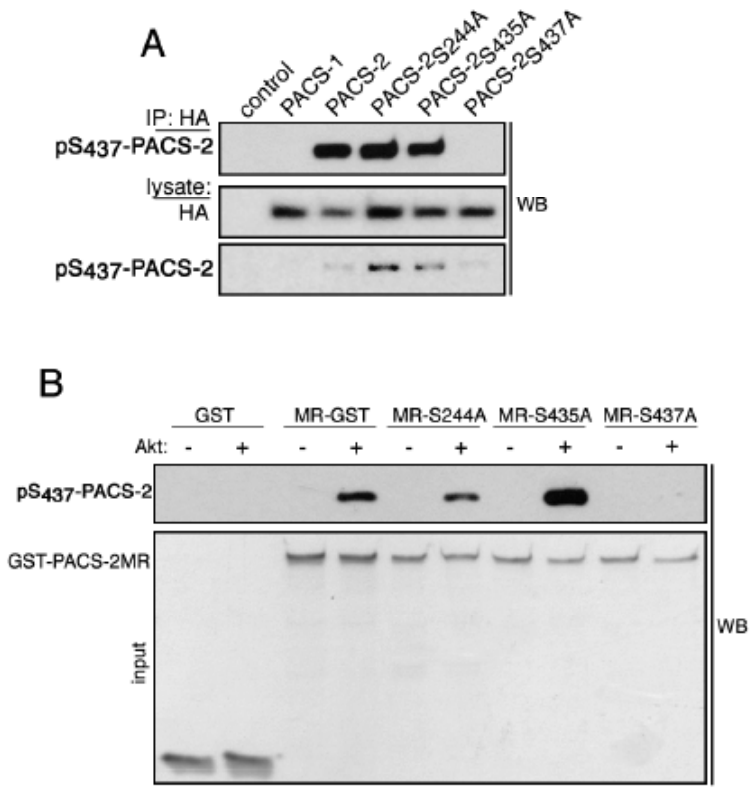



Figure 4-9. Characterization of mAb81.

(A) Western blot analysis of immunoprecipitated PACS proteins from transfected HEK293 cells with the Akt phosphosubstrate specific antibody mAb 81. (B) mAb 81 western blot analysis of in vitro Akt phosphorylated PACS-2 MR-GST proteins.

## 4.3 Discussion

Here we identify phosphorylation state of PACS-2 $Ser_{437}$ as a molecular switch that regulates cellular homeostasis and Apo2L/TRAIL-induced apoptosis. The failure of Apo2L/TRAIL to trigger apoptosis in livers from PACS-$2^{-/-}$ mice using a model of viral hepatitis identifies PACS-2 as a key apoptotic effector in vivo (Fig. 4-2). Viral infection sensitizes cells to Apo2L/TRAIL-mediated apoptosis by inducing DR5 expression as part of the innate immune response (Zender et al., 2005). Despite this increased apoptosis, some pathogenic viruses such as hepatitis C virus persist, leading to steatosis and cytokine-mediated injury and fibrosis (Mundt et al., 2005). Both hepatic apoptosis and steatosis are a direct consequence of Apo2L/TRAIL binding to DR5 (Mundt et al., 2005). In the absence of PACS-2 apoptosis is blocked whereas steatosis appears unabated (Fig. 2), suggesting Apo2L/TRAIL signaling pathways controlling apoptosis and steatosis diverge between the receptor and PACS-2. Moreover, the requirement of PACS-2 for hepatic apoptosis induced by Apo2L/TRAIL but not the TNFα or FasL (Fig. 4-2), suggests that the signaling pathway leading from death receptor activation to Bid cleavage is fundamentally different between the three death ligands. Further analysis of the precise steps regulated by PACS-2 in response to Apo2L/TRAIL will provide new insights into the underlying mechanism of death ligand-induced apoptosis.

The role of PACS-2 in mediating Apo2L/TRAIL action may not be restricted to virally infected cells as siRNA knockdown of PACS-2 prevents Apo2L/TRAIL-mediated killing of HCT116 colorectal cancer cells (Fig. 4-1). Interestingly, the PACS-2 gene, located at chromosome 14q32.33 is lost in sporadic colorectal cancer (Anderson et al., 2001; Bartos

et al., 2007). In agreement with these genomic studies, our immunohistochemical and western blot analysis revealed that the PACS-2 protein is lost in cancer tissues of ~50% of the patients suffering from colorectal cancer (Fig. 4-1). These findings raise the possibility that loss of apoptotic PACS-2 expression may be a contributing factor to the colorectal cancer sequence and suggest PACS-2 may be a biomarker for Apo2L/TRAIL-resistant cancer. To what extent loss of PACS-2 exacerbates the tumorigenesis and whether PACS-2 mediates Apo2L/TRAIL killing in in vivo models of autoimmune disorders warrants further investigation.

Genetic and biochemical analyses have revealed that the PACS proteins are metazoan membrane traffic regulators that mediate organ homeostasis and have important roles in diverse pathologies and disease states (Youker et al., 2008). In healthy cells, cytosolic PACS-2 binds membrane cargo proteins containing acidic cluster sorting motifs and mediates their localization to distinct compartments. In addition, HIV-1 Nef binds PACS-2 to traffic to the Golgi region to assemble a signaling complex that triggers MHC-I downregulation (Atkins et al., 2008). PACS-2 binds an acidic cluster ER-localization motif in the polycystin-2 cytosolic domain and connects this motif to COPI in vitro (Kottgen et al., 2005). Loss of PACS-2 or disruption of the PACS-2—COPI interaction causes polycystin-2 to mislocalize from the ER. Our determination that PACS-2$Ser_{437}$A disrupts the subcellular localization of polycystin-2 (Fig. 4-7), suggests binding of 14-3-3 to p$Ser_{437}$ may be required for PACS-2 to mediate the trafficking of its client proteins in non-apoptotic cells. The mechanism for 14-3-3 binding to PACS-2 required for trafficking cargo proteins, however, is unknown. Interestingly, 14-3-3 and COPI

cooperate to localize other cargo to the ER by binding directly to the cargo protein (O'Kelly et al., 2002). Binding to COPI mediates a Golgi-to-ER trafficking step whereas binding 14-3-3 promotes anterograde movement. Our determination that PACS-2Ser$_{437}$A disrupts ER localization of polycystin-2 suggests a role for 14-3-3 binding to PACS-2 different from mediating anterograde trafficking. Moreover, our finding that PACS-2Ser$_{437}$A also disrupts trafficking of HIV-1 Nef to the Golgi region, a step required for MHC-I downregulation (Atkins et al., 2008), suggests roles or an interaction between PACS-2 and 14-3-3 in mediating endosomal trafficking and HIV-1 immunoevasion.

The combined results of pharmacologic and genetic experiments suggest Akt is an in vivo PACS-2 Ser$_{437}$ kinase (Figs. 4-4 and 4-5). Although more than 100 putative *in vitro* Akt substrates have been described in the literature, only about 20 substrates have been rigorously confirmed as bona fide targets in vivo (Manning and Cantley, 2007). Similar to PACS-2, Akt phosphorylation of FOXO3A and Bad generates a binding site for 14-3-3 proteins (Manning and Cantley, 2007; Porter et al., 2006). PACS-2 Ser$_{437}$ is embedded within an evolutionally conserved consensus Akt phosphorylation site, and kinase studies in vitro and with Akt$^{-/-}$ MEFs and PI-103 demonstrated Akt is a PACS-2 Ser$_{437}$ kinase (and Fig. 4-5). These studies, however, do not exclude the possibility that other kinases may also phosphorylate PACS-2 Ser$_{437}$. Indeed, p70S6K, which is activated by mTOR, phosphorylates Akt consensus sites and phosphorylates Bad Ser$_{136}$ in vivo (Harada et al., 2001). To what extent p70S6K or other kinases may phosphorylate PACS-2 Ser$_{437}$ remains to be determined.

Similar to PACS-2, Bad is a multifunctional protein that integrates metabolic homeostasis with apoptosis that is controlled in part by Akt and 14-3-3 proteins. In healthy hepatocytes and beta cells, Bad directs the assembly of glucokinase-containing complexes at mitochondria to regulate insulin release whereas in apoptotic cells Bad becomes apoptotic and binds anti-apoptotic Bcl-2 proteins to promote MMP (Danial et al., 2003). The switch between these metabolic and apoptotic functions of Bad are regulated by the sequential phosphorylation state multiple serine residues, including Bad $Ser_{136}$, which is phosphorylated by Akt and p70S6K and binds 14-3-3 proteins, and $Ser_{155}$, which is phosphorylated by PKA. Similar multi-modal regulation may occur on PACS-2. In addition to $Ser_{437}$, we found that Akt phosphorylates PACS-2 $Ser_{244}$ *in vitro*. While $Ser_{244}$ does not directly engage 14-3-3 proteins, $pSer_{244}$ and $pSer_{437}$ may combine to regulate whether PACS-2 mediates protein traffic or apoptosis.

The PI3K/Akt survival pathway promotes resistance to chemotherapy, ionizing radiation and Apo2L/TRAIL in solid tumors (Martelli et al., 2003; Shankar and Srivastava, 2004). Conversely, inhibition of PI3K/Akt enhances Apo2L/TRAIL-induced apoptosis in tumor cells with weak response to Apo2L/TRAIL (Kandasamy and Srivastava, 2002; Vaculova et al., 2006). However, the majority of these reports use pan-selective inhibitors such as wortmannin and LY294002, which display similar $IC_{50}$s against all PI3Ks. The concentrations of PI3K inhibitors needed to block PI3Ks are similar to the concentrations that induce cell death—hence preventing their use as chemotherapeutics. The pyridinylfuranopyrimidine PI-103 represents a selective inhibitor of Class I PI3Ks that lacks toxicity of pan-selective inhibitors (Knight et al., 2006). We found that PI-103

enhances Apo2L/TRAIL-mediated cell death in a PACS-2 dependent manner (Fig. 4-3), suggesting that PI-103 may mediate effects in part through inhibition of PACS-2 $Ser_{437}$ phosphorylation. As PACS-2 is required for the enhancement of Bid cleavage and caspase activation in response to PI-103 (Fig. 4-3), PACS-2 $Ser_{437}$ may represent a necessary point of regulation in the apoptotic response. Our finding that 14-3-3 can repress the Apo2L/TRAIL-induced apoptotic activity of PACS-2 but not PACS-2$Ser_{437}$A (Fig. 4-7), supports this model.

While PI3K/Akt regulates Apo2L/TRAIL at the level of Bid cleavage, the underlying mechanism has been elusive. The regulation of Bid cleavage to truncated Bid (tBid) molecules is complex and controlled by multiple proteases. In healthy cells and mouse liver, apoptotic Bid is silenced by CK1 and CK2 phosphorylation of serine and threonine residues proximal to Bid's caspase cleavage site (Desagher et al., 2001; Vogel et al., 2006). Caspase-8 cleaves Bid at $Asp_{59}$ to form myristoylated tBid, which can bind mitochondria membranes and stimulate MMP (see Chapter 2 and (Aslan and Thomas, 2008) for review). However, in Apo2L/TRAIL-treated cells, activation of caspase-8 is necessary but not sufficient for Bid action ((Rokhlin et al., 2002) and see Fig. 4-3). Rather Apo2L/TRAIL triggers lysosome permeabilization to release cathepsins B and D, which cleave Bid at sites other than $Asp_{59}$, and which are required for Apo2L/TRAIL-induced cell death (Werneburg et al., 2007). Thus, the canonical caspase-8/myrisotylation model may not represent the sole mechanism of Bid regulation. The failure of Apo2L/TRAIL to stimulate Bid cleavage in PACS-$2^{-/-}$ MEFs, despite activation of caspase-8, is consistent with this model. PACS-2 binds full-length Bid mediates

translocation of Bid to mitochondria and cleavage (Simmen et al., 2005). The recent determination that PACS-2 mediates ER-mitochondria communication and endosomal traffic (Atkins et al., 2008; Kottgen et al., 2005; Simmen et al., 2005), raises the possibility that Apo2L/TRAIL triggers PACS-2 to alter organellar dynamics to assemble a platform that regulates Bid translocation and cleavage (see Chapter 2 and (Aslan and Thomas, 2008) for review). This possibility is currently under investigation.

## 4.4 Materials and Methods

### Mice, cancer tissue and Reagents.

Mice: A murine ES cell line (129 Sv/Ev strain) containing the VICTR37 retroviral gene trap in intron 1 of the PACS-2 gene (Lexicon Genetics) was injected into 3.5d blastocysts isolated from superovulated C57BL/6 mice and implanted into pseudopregnant ICR mice. Pups were outmated to NIH Black Swiss mice. PACS-2 expression was determined by western blot and RT-PCR of RNA isolated from tissue using Qiagen RNeasy and amplified with Omniscript RT, then Taq polymerase with primers directed to exons 1 and 11 of mouse PACS-2. Exons 4/5 and 15/16 of PACS-1 and exons 5 and 13 of ATP citrate lyase served as controls. Animal care and experiments were in accordance with the Guidelines of the Department of Comparative Medicine, OHSU.

Cell lines: WT and PACS-$2^{-/-}$ primary MEFs were isolated from 13d embryos and were either propagated as primary cultures or immortalized by infection with a retrovirus

expressing SV40 large T antigen (provided by R. Cone, OHSU). TAg-PACS-$2^{-/-}$ cells rescued for PACS-2 expression (TAg-PACS-$2^{R}$) were generated by stable transfection with pcDNA3.1-PACS-2-HA or empty vector (TAg-PACS-$2^{V}$) and pCEP4 to confer hygromycin resistance. MEFs were grown in DMEM supplemented with 10% fetal bovine serum. WT and Akt$1^{-/-}$ MEFs (from N. Hay, UIC), HCT-116 (from B. Vogelstein), A7, BSC-40, HEK293 and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum.

Human Tissues: 200 mg portions of resected, staged colorectal cancer specimens with adjacent normal colonic tissue controls were isolated from consented patients under approval from the OHSU and KP IRB. Tissue samples were quick-frozen in liquid nitrogen and homogenized in mRIPA buffer supplemented with complete protease and phosphatase inhibitors. Extracts were centrifuged at 15,000g (30 min, 4$^{o}$C) and 100 μg of total protein from each supernatant was analyzed for PACS-2 protein by western blot.

Plasmids and siRNA: 14-3-3ζ-pcDNA3.1 was obtained from A. Aitken (U. Edinburgh, UK). 14-3-3σ-pcDNA3 was from H. Piwnica-Worms (Wash. U.). Bad-pGEX-4T1 was from T. Soderling (OHSU). pCaspase-3 Sensor was from BD Biosciences. Plasmids encoding PACS-2 GST and HA-tagged proteins were previously described (Kottgen et al., 2005; Simmen et al., 2005). pTM4-FL encoding myc-polycystin-2 was from S. Somlo (Yale) and pNef-eYFP was described (Hung et al., 2007). Alanine substitution mutations were generated by standard methods. Control (scr)

and PACS-2 siRNAs (Dharmacon) were nucleofected into cells (Amaxa) as described (Atkins et al., 2008).

Antisera: Antibodies used were anti-mouse caspase-8 1G12 (ALX-804-447, Alexis), anti-mouse Bid/tBid (MAB860, R&D Systems), anti-caspase-9 (#9508, Cell Signaling), anti-cleaved caspase-3 (#5A1E, Cell Signaling), anti-DR5 (#28065, Anaspec), anti-$His_6$ (#2365, Cell Signaling), anti-actin (MAB1501, Chemicon/Millipore), anti-tubulin (#2148, Cell Signaling), anti-14-3-3ζ (AB9746, Upstate/Millipore), anti-myc mAb 9E10 (06-340, Upstate/Millipore), goat anti-PACS-2 (HPA001423, Atlas), rabbit anti-PACS-2 604, 832 and 834 (Simmen et al., 2005), rabbit monoclonal antibody #81E12B5X (Cell Signaling) that detects phosphorylated PACS-2 $Ser_{437}$, anti-HA mAb HA.11 (MMS-101R, Covance), anti-Akt (Cell Signaling #9272), anti-pAkt (Cell Signaling #9271), anti-Golgin 97 (#A21270 Molecular Probes), Alexa488 goat anti-rabbit IgG (# A11008 Molecular Probes), Alexa546 goat anti-mouse $IgG_1$ (#A21123 Molecular Probes) and HRP-conjugated secondary antibodies (Southern Biotech and A1949, Sigma). Anti-S6 and anti-pS6 were from G. Thomas (GRI, U. Cincinnati).

**Apoptosis assays.**

*In vivo* induction of apoptosis in mouse liver: To analyze the role of PACS-2 for TRAIL mediated apoptosis, recombinant adenoviruses expressing GFP (Ad-GFP) and Apo2L/TRAIL (Ad-TRAIL) were prepared as described (Mundt et al., 2005). Prior to infection the virus was dialyzed twice against a solution containing 10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 140 mM NaCl at 4°C. Infection of mice was carried out by

administration of 0.25 ml of Ad-GFP virus and of Ad-TRAIL virus solution 24 hours after the first injection into the tail vein with $2x10^8$ pfu/g. 24 hours later mice were sacrificed. To analyze the role of PACS-2 for Fas and TNFα-mediated apoptosis, mice were challenged with intraperitoneal administration of the monoclonal Fas antibody (Jo-2) (BD Pharmingen, San Diego, CA) (0.7 μg/g) and of LPS (0.5 μg/g) + D-galactosamine (700 μg/g, Sigma), respectively. Mice were sacrificed when they became moribund or after 6 hours. At the time of sacrifice, a small fragment of each liver was fixed in buffered formalin. The remaining liver tissues were quick-frozen in liquid nitrogen and stored at -80 °C until analysis.

Flow cytometry assays: HCT116 cells or MEFs were treated with recombinant mouse or human Apo2L/TRAIL (R&D Systems). For biochemical studies, cells were pretreated or not for 1 hour with 1 μM PI-103 (Calbiochem) and harvested directly into heated SDS sample buffer. Apoptosis assays conducted by flow cytometry using Annexin V-APC (Pharmingen) and propidium iodide (Calbiochem) using a FACSCalibur (BD) cytometer and CellQuest acquisition/analysis software (BD) as previously described (Simmen et al., 2005).

pSensor reporter assay: TAg-PACS-$2^{-/-}$ MEFs cells were Lipofectamine 2000 transfected with pSensor and pcDNA3 or pcDNA3.1-PACS-2 constructs with or without 14-3-3ζ-myc-pcDNA3. Cells were treated with 10 ng/ml mouse TRAIL for 16 hours before paraformaldehyde fixation. Five 20x fields of each sample were visualized Zeiss

microscope using Openlab software (Improvision) and scored for eYFP localization in triplicate.

**Phosphorylation and binding assays.**

GST-tagged PACS-2 proteins were prepared as previously described (Simmen et al., 2005; Tureckova et al., 2001). HA-tagged active and kinase-dead (KD) Akt were expressed using recombinant adenoviruses as described (Tureckova et al., 2001) and Akt phosphorylation was conducted as described (Tureckova et al., 2001). [$\gamma$-$^{32}$P]ATP was from Perkin Elmer. Akt phosphorylated GST proteins (1 μg) were captured on Glutathione Sepharose, washed in 14-3-3 binding buffer (20 mM Tris pH 7.4, 1% NP40, 1 mM EDTA), and incubated with 10 μg 14-3-3$\sigma$-$His_6$ for one hr at RT, washed and then separated by SDS-PAGE and visualized by autoradiography. Endogenous PACS-2 was immunoprecipitated from the indicated tissue- or cell-cytosol by incubating 10 μg affinity purified anti-PACS-2 overnight and then captured with 50 μl Protein G agarose (Zymed).

**Fluorescence polarization.**

FP assays for 14-3-3 interactions were performed as previously described (Du et al., 2006). TM-Rhodamine-conjugated peptides for $Ser_{244}$-PACS-2 (TMR-RRSIVRTTSMTRQQN), $pSer_{244}$-PACS-2 (TMR-RRSIVRTTpSMTRQQN), $Ser_{437}$-PACS-2 (TMR-GRRGRSTSLKERQA) and $pSer_{437}$-PACS-2 (TMR-GRRGRSTpSLKERQA) were from Anaspec. The fluorescence polarization (FP) signals were recorded after 1 hr of incubation using the Analyst HT. The assay window was defined by subtracting free peptide polarization values from values of bound peptide

recorded in the presence of specified protein concentrations of GST-14-3-3. The data were fit to the model FP = ([14-3-3] • Bmax)/([14-3-3] + $K_d$) using nonlinear regression analysis, where $B_{max}$ is the maximal binding (Prism 4.0; Graphpad).

**Mass Spectrometry.**

PACS-2-HA immunoisolated from BSC40 cells was subject to partial in-gel trypsin digestion as described (Shevchenko et al., 2006). Phosphopeptides were enriched as described (Larsen et al., 2005), dissolved in formic acid, desalted on a POROS Oligo R3 reversed-phase column (ABI), and eluted into a nanoelectrospray needle with either ammonium hydroxide/acetonitrile for precursor ion scanning in negative ion mode or acetic acid/acetonitrile for tandem mass spectrometry (MS/MS) in positive ion mode. Precursor ion mass spectrometry analysis was performed on a QSTAR XL quadrupole time-of-flight mass spectrometer equipped with a nanoelectrospray ion source (MDS Proteomics). After identification of candidate phosphopeptides, the remaining sample was used to identify sites of phosphorylation by nanoscale LC-MS/MS sequencing on an LC-Packings (Dionex) nanoLC system coupled to a Thermo LCQ Deca XP MAX quadruple ion trap mass spectrometry system.

**IHC and Confocal Microscopy**.

Immunohistochemistry: formalin-fixed, paraffin embedded tissue samples from consented patients were cut in 3 micron sections, placed on poly-lysine-coated slides, then deparaffinized and antigen was retrieved with heated citric acid (Ventana CC10). The samples were stained with Ab HPA001423 and visualized with diaminobenzamidine

(Ventana). The slides were counterstained with hematoxylin and images were captured with a Nikon Eclipse 80i microscope equipped with a Nikon Digital Sight DS-L1 camera.

Confocal microscopy: HeLa cells were nucleofected (Amaxa) with pTM4-FL encoding myc-polycystin-2 or pNef-eYFP. After 36 hr, cells were fixed with paraformaldehyde and processed for confocal microscopy as described and cells were visualized with a 60x oil immersion objective on an Olympus Fluo-View FV300 laser scanning confocal microscope as described (Atkins et al., 2008). As indicated in the figure legends, following incubation with anti-Myc and anti-Golgin 97 antibodies, staining was visualized using species and subtype-specific Alexafluor secondary antibodies (Invitrogen).

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# Chapter 5. Discussion

## 5.1 Summary

The results presented in this dissertation demonstrate that PACS-2 directs intracellular trafficking and interorganellar processes that mediate cellular homeostasis and apoptotic cell death. Under basal conditions, PACS-2 localizes primarily to the ER and cytosol to mediate the sorting of cargo such as PKD2 to mediate their sorting itineraries. PACS-2 also controls communication processes between the ER and mitochondria, likely through a sorting function that localizes the folding chaperone calnexin to the ER and MAMs. In response to stress, PACS-2 localizes to the mitochondria to drive Bid-dependent cell death. PACS-2 binds to the full-length form of Bid and siRNA depletion studies in Chapter 3 show that PACS-2 is required to cleave and deliver Bid to the mitochondria to promote mitochondrial cytochrome c release, caspase activation and death in response to staurosporine and Fas-Ab/CD95. Studies of tumor cells as well cells from knockout mice in Chapter 4 further confirm that PACS-2 is required for Bid action in response to the chemotherapeutic agent TRAIL. Furthermore, PACS-2 is required for TRAIL-mediated cell death in vivo as PACS-$2^{-/-}$ mice show no detectable apoptosis in a hepatitis model of TRAIL sensitivity. PACS-2 is phosphorylated by the pro-survival kinase Akt in vitro and in vivo at $Ser_{437}$ to establish a binding site for 14-3-3 proteins which inhibit apoptosis in a PACS-2 $Ser_{437}$-dependent manner. Apoptotic induction leads to PACS-2 $Ser_{437}$ dephosphorylation, which may act as a switch to change the function of PACS-2 from a homeostatic sorting protein to an inducer of apoptosis. These results have many implications for future research directions concerning the function of PACS-2 as well as

PACS-1 and bring up a number of novel insights in the future pursuits of studies of cell biology, apoptosis, cancer and disease.

## 5.2 Implications for Bcl-2 protein function

In Chapter 3 we show that that PACS-2 controls the apoptotic trafficking of Bid to the mitochondria in response to the potent kinase inhibitor staurosporine and Fas-Ab/CD95. While initial studies of Bid action argue that Bid is first cleaved to a tBid species with affinity for mitochondria (Gross et al., 1999b), our studies allude to a mechanism in which PACS-2 first binds to Bid in an apoptotic manner to traffic full length Bid to the mitochondria which is subsequently cleaved to tBid. Interestingly Bid translocation is under the control of both Akt and Jnk (Chen et al., 2001; Deng et al., 2003), yet no direct phosphorylation of Bid by these kinases has been reported. Likewise, we have been unable to demonstrate that PACS-2 is a Jnk substrate in vitro (J. Aslan and G. Thomas, unpublished results). Our studies allude to a mechanism in which Akt prevents Bid translocation while Jnk promotes translocation of full length Bid to the mitochondria through phosphorylation of PACS-2 and 14-3-3 proteins. While 14-3-3 release from apoptotic clients such as bad and PACS-2 is hypothesized to take place by client dephosphorylation, 14-3-3 proteins themselves are targets of apoptotically active Jnk kinases that phosphorylate 14-3-3 proteins, leading to their release from such as Bad, Bax and c-Abl (Sunayama et al., 2005; Tsuruta et al., 2004; Yoshida et al., 2005). Future studies will aim to describe how these apoptotic signaling systems coordinate both Bid and PACS-2 activation. Additionally, future studies will examine whether or not the PACS proteins can direct the apoptotic function of additional Bcl-2 family members such

as Mcl-1, Bax, Bad, Bim, Bcl-2 and Bik. Protein-protein interaction studies examining potential interactions between the PACS proteins and such Bcl-2 proteins are currently in progress in the Thomas lab.

## 5.3 A lysosomal pathway to Bid activation

Apoptotic pathways mediated by death ligands such as FasL, TNFα and TRAIL classically route from cell surface receptors to the mitochondria (Peter and Krammer, 2003). As discussed in Chapter 2, a number of studies now report that lysosomes may act as intermediates in this apoptotic signal transduction pathway (Cirman et al., 2004; Heinrich et al., 2004; Werneburg et al., 2007). Interestingly, these studies argue that Bcl-2 proteins such as Bid, Bax and Bim have roles in permeabilizing lysosomes to release cathepsin proteases that may act on Bid in a requisite step to drive caspase activation and apoptosis. As described in Chapter 2, cell death signals initiated at the cell surface through death receptors are transduced from the cell surface into an endosomal "receptosome" compartment that triggers downstream caspase activation (Micheau and Tschopp, 2003), perhaps through an activation of cathepsins in lysosomes, or possibly late endosomes or multivesicular bodies (Schneider-Brachert et al., 2004; Schutze et al., 2008). Interestingly, in addition to working in the early secretory pathway, PACS-2 also regulates CD8 internalization from the cell surface and is required to recycle CD8 positive endosomes (Atkins et al., 2008). Current projects in the Thomas lab are working to determine if lysosomal trafficking, morphology and signaling pathways are altered in PACS-2 deficient systems in a manner that would explain the phenomena described in Chapters 3 and 4.

## 5.4 PACS-2 as a 14-3-3 client protein

14-3-3 proteins act as tumor suppressors in part for their role in blocking apoptotic programs through the sequestration of apoptotic client proteins to the cytosol to block apoptotic activities (Hermeking, 2003). From a structural perspective, 14-3-3 proteins function as homodimers or heterodimers to regulate client activity (Fu et al., 2000). This was first demonstrated in the case of Raf, as 14-3-3 dimerization is requisite for Raf kinase activity (Tzivion et al., 1998). This dimeric nature of 14-3-3 protein function led to initial hypotheses that 14-3-3 dimers may regulate apoptosis through binding to multiple phosphorylated sites on pro-apoptotic proteins such as Bad which contains multiple phosphorylation sites including $Serine_{112}$ and $Serine_{136}$. Bad $Serine_{112}$ has, however, recently been shown to be dispensable for apoptotic regulation by 14-3-3 proteins while $Serine_{136}$ is now known to be an Akt target that binds to 14-3-3 upon phosphorylation (Masters et al., 2001). Similar to apoptotic 14-3-3 client proteins such as Bad, PACS-2 contains a number of potential sites of phosphorylation which may regulate PACS-2 function and apoptotic activity through simultaneous binding to multiple binding sites. In Chapter 4, I show that Akt phosphorylates PACS-2 $Serine_{244}$ and $Serine_{437}$ in vitro and provide evidence that $Serine_{437}$ is phosphorylated in vivo to control a threshold to TRAIL-mediated apoptosis. While fluorescence polarization analysis shows that phosphorylation of PACS-2 $Ser_{244}$ alone is not sufficient to establish 14-3-3 binding (Figure 4-6), mutation of $Ser_{244}$ does have a small but consistent inhibitory effect on 14-3-3 binding to phosphorylated PACS-2 in vitro (Figure 4-6). This $Ser_{244}$A mutation, however has no effect on the modulation of caspase-3 activity in response to TNFα upon

PACS-2 overexpression in HEK293 cells (Figure A-6). Nonetheless, $Ser_{244}$ may be a kinase target in vivo controlling an as of yet identified function of PACS-2 in trafficking or apoptotic regulation.

Interestingly, the 14-3-3 binding site identified on PACS-2 at $Ser_{437}$ ($RGRSTS_{437}LKER$) does not conform to classic mode 1 or mode 2 (RSXpSXP and RXXXpSXP） motifs that use a proline to bind 14-3-3. This site is, however, nearly identical to the Akt phosphorylation and 14-3-3 binding site of the tuberous sclerosis protein TSC-2 ($RARSTS_{939}LNER$) (Li et al., 2002). TSC-2 also has a role in maintaining genomic stability and is a target of the mTOR pathway that regulates cellular growth and tumor development (Huang and Manning, 2008). Future studies may reveal a connection between mTOR/TSC2 signaling and PACS-2 function. Such a connection may be found through p70S6K, an mTOR kinase that shares substrate specificity with Akt. Indeed, p70S6K phosphorylates Bad at $Ser_{136}$ to block apoptotic programs (Harada et al., 2001). Whether p70S6K or other arginine-directed serine/threonine kinases target PACS-2 $Ser_{437}$ will be an important and interesting future line of investigation.

14-3-3 proteins represent one of the most abundant proteins in mammalian cells, comprising about 1% total protein from human brain (Fu et al., 2000). Despite the great quantity of 14-3-3 proteins present in mamallian tissues and their relative simple structure, little is known of how the seven human isoforms of 14-3-3 regulate cellular function. Future studies will determine which isoforms bind to PACS-2. No study to date has demonstrated the stoiciometry of binding of 14-3-3 clients. Whether 14-3-3 proteins

bind to sequester clients in a non-active form or if the on-off rate of binding is rapid, serving as a simple switch also remains to be determined.

## 5.5 PACS-2 and disease

The PACS proteins function to maintain the proper intracellular location of cargo such as the ion channel PKD2 and nephrocystin (Kottgen et al., 2005; Schermer et al., 2005). PACS mediated sorting of such factors is likely relevant to pathological states such as polycystic kidney disease as mutation or deletion of the acidic cluster of PKD2 leads to this devastating disease (Cai et al., 1999; Delmas et al., 2004; Sutters and Germino, 2003). In Chapter 4 we show that PACS-2 $Ser_{437}$ in part mediates the homeostatic sorting of the PKD2 channel in addition to having a role in apoptosis. This dual functionality is not unique to PACS-2; other pro-apoptotic proteins such as Bad are emerging as important intracellular regulators as Bad directs glucokinase to mitochondria in response to high fat feeding to stimulate insulin secretion (Danial et al., 2003; Danial et al., 2008). As discussed in Chapter 2, a number of apoptotic players such as other Bcl-2 proteins and Drp1 have homeostatic as well as apoptotic roles. The manner in which these proteins "switch" from homeostatic regulators to inducers of death is not understood. Likewise the manner in which the intracellular location of these factors influences their apoptotic functions is not known. Our results, like those for Bad hint that Akt may not only mediate cell proliferation and survival, but may act to maintain cellular homeostasis by setting the function of targets such as Bad and PACS-2.

Our results in Chapter 4 suggest that pro-inflammatory pathways mediated by TRAIL may not involve PACS-2 as PACS-2 deficient mice, like wt controls, also display steatosis of the liver in response to TRAIL. This result suggests that DR5-mediated steatosis and apoptosis signaling pathways diverge at some point before PACS-2. These results, however, do show that PACS-2 could have a role in mediating apoptotic response to TRAIL in response to hepatitis virus infection. As discussed in the introduction to this dissertation, the PACS proteins are commonly usurped by viral signaling pathways to counter host immune responses. Whether or not hepatitis virus has a mechanism to prevent host cell apoptosis that specifically targets PACS-2 remains to be determined. PACS-2 may however have a role in cytomegalovirus (CMV) infection as the UL37x1 gene product vMIA, like gB and Nef, contains an acidic cluster. vMIA binds PACS-2 but not PACS-1 (G. Thomas et al., preliminary results). vMIA targets mitochondria to block apoptotic programs (Goldmacher, 2002). vMIA specifically localizes to MAMs (Bozidis et al., 2008). Interestingly, it has recently been proposed that PACS-2 may mediate the localization of vMIA to the MAMs or mitochondria where it exerts anti-apoptotic effects to halt Bax activation (Bozidis et al., 2008). Other notable examples of viral inhibition of host cell apoptotic pathways include HIV-1 Nef. In addition to taking advantage of a host PACS-2 sorting mechanism to direct downregulation of MHC-I to block host immune response (Atkins et al., 2008), Nef also upregulates the activity of the p21 activated kinase PAK2 which, like Akt, blocks apoptotic programs (Wolf et al., 2001). This occurs in part through PAK2 phosphorylation Bad $Ser_{136}$. As PAK2 and Akt have overlapping substrate specificity, PAK2 as well as other PAK family members may phosphorylate the Akt/14-3-3 motif of PACS-2 to block apoptosis. Future studies will

determine if PAKs can phosphorylate PACS-2 in vitro and determine if Nef expression leads to PACS-2 $Ser_{437}$ phosphorylation and a block of host apoptotic programs.

## 5.6 PACS proteins and cancer

The multifunctional nature of the PACS proteins has implications for several pathological states, perhaps most notably tumorigenesis and cancer. Previous reports demonstrate that the PACS-2 locus, located at human chromosome 14q32.33, is deleted through a loss of heterozygosity mechanism in colon cancer (Anderson et al., 2001; Bartos et al., 2007). In Chapter 4 we confirm that PACS-2 protein expression is completely downregulated in approximately half of human colon cancer tissues. The previously described examples of PACS-2 gene is lost on cases of colon cancer driven by genomic instability (Anderson et al., 2001). Interestingly, recent studies of Bid function demonstrate that Bid also localizes to the nucleus where it controls S-phase transition to promote proper maintenance of the genome (Kamer et al., 2005; Zinkel et al., 2006; Zinkel et al., 2005). Similarly, p53, the paradigmatic tumor suppressor, “guardian of the genome” and transcriptional activator now plays a role in permeabilizing mitochondria (Mihara et al., 2003). This noteworthy relationship between mitochondrial permeabilization, genomic stability and may extend to include a role for the PACS proteins as PACS-2 may mediate not only Bid signaling, but pathways used by p53 as well as NF-κB (H. You, D. Williamson and G. Thomas, unpublished results). Whether or not PACS proteins localize to and have a role in the nucleus remains to be determined.

The roles of PACS-2 in cancer extend beyond colorectal cancer as many models of TRAIL-resistant cancers are from tumors of the prostate. These TRAIL-resistant cancers show upregulated Akt which leads to a block in apoptosis at the level of the cleavage of Bid (Nesterov et al., 2001). As discussed in Chapter 4, PACS-2 may serve as a "missing link" between Akt and Bid function in apoptosis in such TRAIL-resistant cancers specifically. Future studies will undoubtedly attempt to identify PACS-2 not only as a tumor suppressor gene, but also specifically as a biomarker for TRAIL sensitivity. This is of clinical relevance as PACS-2 immunostaining of tumor biopsies could be a valuable diagnostic tool to determine if TRAIL is a viable therapy in cancer patients as loss of PACS-2 expression would indicate TRAIL resistance.

Many of the results in this dissertation also have implications for PACS-1, which may compliment PACS-2 by serving an anti-apoptotic function (T. Simmen, H. You and G. Thomas, unpublished results). Interestingly, as PACS-2 is deleted in cancer, PACS-1 has been detected to be upregulated in cervical cancer (Srivatsan et al., 2002; Zainabadi et al., 2005). The potential role of PACS-1 as an oncogene is further supported by studies that have recently identified PACS-1 as a target gene of the β-catenin transcriptional coactivatior (Yochum et al., 2007), a key transducer of Wnt signaling that is misregulated in 90% of human colon cancers (McElhinny et al., 2008). Future studies will determine if PACS-1 is an oncogene and if the pathological states of PACS-1 and PACS-2 overlap in various cancers. While the PACS proteins share over 50% homology, there is no homologous Akt phosphorylation or 14-3-3 binding motif on PACS-1, raising a number of questions as to how PACS-1 apoptotic function could be regulated.

## 5.7 Implications for neuronal function and disease

In Chapter 4 we show that phosphorylation of PACS-2 $Ser_{437}$, presumably in cooperation with 14-3-3 proteins, mediates the retrieval of PKD2 from the Golgi to the ER. This result implicates Akt and 14-3-3 as not only having roles in the pathophysiology of polycystic kidney disease, but also as directing the proper trafficking of ion channels in general. PACS-2 likely routes other channels through the secretory pathway to ciliary compartments as several other cilia-targeted ion channels contain CK2-phosphorylatable acidic clusters that may mediate their sorting. These include the rod and olfactory cyclic nucleotide gated (CNG) channels CNGA1 ($EDDDSAS_{50}TSEES$) and CNGB1 ($NEEAET_{48}ESESMP$). Given the similarity of the acidic cluster on CNG to the olfactory and rod channel to other PACS cargos, it is possible that PACS-2 will mediate the localization of CNG to olfactory cilia or cilia-like rod outer segments. Whether or not PACS proteins play a part in localizing cargo to other ciliary compartments such as hair cell stereocilia is likewise an intriguing possibility. Interestingly, putative hair cell transduction channels localized to stereocilia (Corey, 2006), including a number of TRP and chloride channels contain CK2-phosphorylatable acidic clusters and have been shown to bind to the PACS-2 ((Kottgen et al., 2005) and Table 5-1).

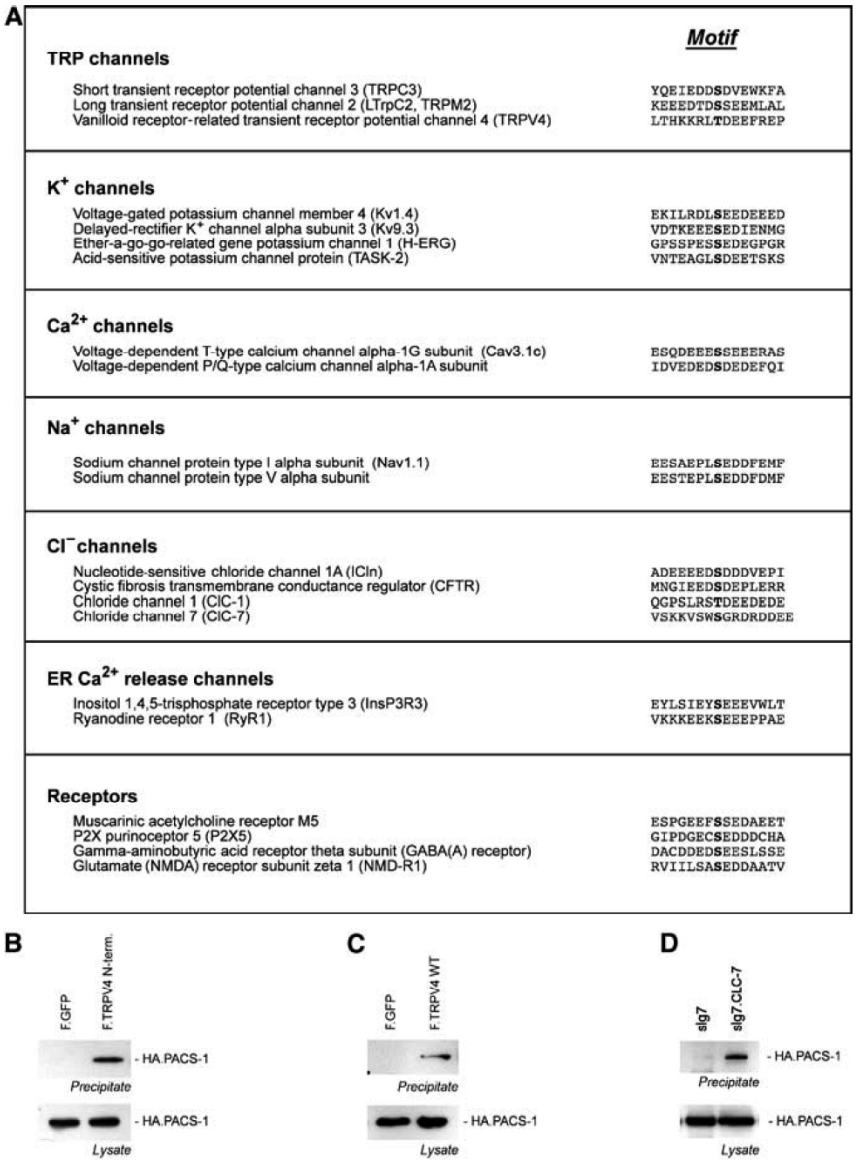


Figure 5-1. PACS interacts with acidic clusters of other ion channels.

(A) Database search for ion channels with acidic clusters. (B) Flag-tagged TRPV41–486 and TRPV4 WT (C) interact with HA-tagged PACS-1. (D) CLC-71–123 fused to an immunoglobulin tag (sIg7.CLC-7) co-precipitates HA-tagged PACS-1. From (Kottgen et al., 2005). © 2005 Nature Publishing Group. Used with permission.

The homeostatic role of PACS proteins are therefore likely to extend beyond roles for only PKD2. As mentioned in Chapter 4, 14-3-3 proteins regulate ion channel traffic, specifically by blocking ER-retrieval via an association TASK1/KCNK3 with COPI to favor forward transport to Golgi (O'Kelly et al., 2002). In Chapter 4 we provide evidence that 14-3-3 proteins may also have a role in mediating retrieval as mutation of the 14-3-3 binding site on PACS-2 blocks retrieval, leading to a concentration of PKD2 as well as Nef in the Golgi.

As shown in Figure 3-A, the PACS proteins are abundant in neural tissues. PACS-2 is highly expressed in GAD65 positive interneurons from rat hippocampus (J. Aslan and G. Thomas, unpublished results). Whether or not PACS-2 specifically plays a role in trafficking in interneurons or whether this increased expression sensitizes interneurons to cell death remains to be addressed. Interestingly, interneurons are sensitive to cell death in response to ischemia and contribute neuronal cell death in a feedback mechanism as loss of their inhibitory activity drives excitatory cell death of target neurons (Wang, 2003). While the role of PACS proteins in ischemic cell death remains to be characterized, preliminary studies in artery occlusion models of stroke and ischemia in mice show a reduction in overall death in response to ischemic stress similar to those in $Bid^{-/-}$ mice (C. Farr, J. Saugestead, G. Thomas, preliminary results and Figure 5-1).

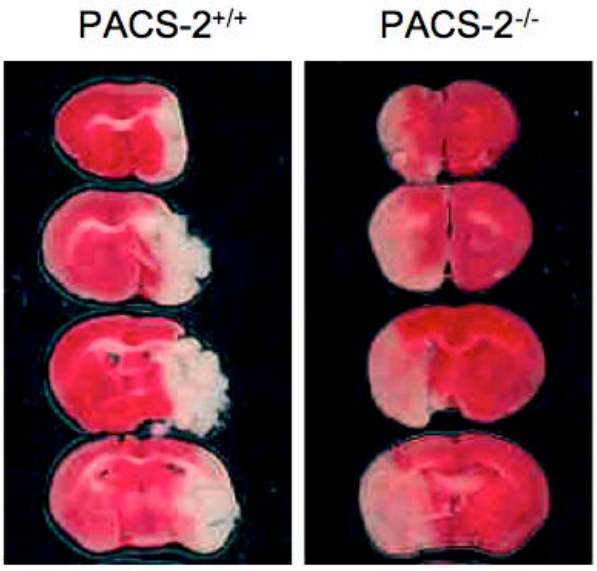


Figure 5-2. PACS-2$^{-/-}$ mice show reduced cerebral damage in response to ischemic stress.

Coronal sections from littermate matched wt and PACS-2$^{-/-}$ mice subject to cerebral artery occlusion as a model of stroke to induce ischemic cell death. Contralateral hemispheres serve as a negative control. Triphenyltetrazolium chloride (TTC) stains normal tissues in red while areas of infarcted brain tissue stain white. (C. Farr, J. Saugstad and G. Thomas, unpublished results).

Fluorescence polarization analysis of 14-3-3 isoforms with a PACS-2 $Ser_{437}$ phosphopeptide reveals that like the phosphorylated Raf, 14-3-3γ shows the highest affinity of all human isoforms for PACS-2 in vitro (Du et al., 2006). 14-3-3γ is primarily expressed in brain where it plays a role in controlling neuronal apoptosis through Bad in response to ischemia (Chen et al., 2005b). Interestingly, neuronal apoptosis in response to ischemia requires Bid and takes place through the translocation of full length Bid to the mitochondria (Konig et al., 2007; Sarig et al., 2003). Our study of the role of PACS-2 in apoptosis in Chapter 3 demonstrates that PACS-2 binds to full-length Bid upon the induction of apoptosis and is required to bring full length Bid to the mitochondria in response to Fas-Ab/CD95 or staurosporine. As discussed above, PACS-2 is primarily expressed in the brain, PACS-2 may play a specific role in controlling neuronal apoptosis in response to ischemic stress. As cell death in response to ischemia requires Bid, this pathway of Bid-mediated cell death may specifically involve the translocation of full length Bid to the mitochondria, alluding a mechanism in which PACS-2 delivers full length Bid to the mitochondria to drive ischemic cell death.

In addition to ischemic stress, neurons also undergo apoptosis in response to inflammatory processes involving TRAIL, specifically in cases of HIV-1/AIDS encephalitis and dementia (Ryan et al., 2004). As ER homeostasis is also heavily involved in other neurodegenerative states, including Alzheimer's and Huntington's diseases (Xu et al., 2005) the possibility that PACS-2 has a role in maintaining ER and mitochondria homeostasis in these disease states warrant investigation.

## 5.8 Conclusion

This dissertation utilized biophysical, biochemical, cellular and in vivo methods to characterize PACS-2 as a sorting protein with homeostatic functions that also has an essential role in mediating cell death programs. The results described herein are of wide and applicable interest to studies of the cell biology of disease as PACS-2 not only functions in times of both health and stress, but mediates pathogenic processes in response to viral infection, polycystic kidney disease and cancer. While the data in this dissertation make a strong case demonstrating a role for PACS-2 in apoptotic and homeostatic processes, an understanding of PACS-2 function is anything but complete. Future studies must determine how exactly PACS-2 carries out apoptotic programs through interactions with key apoptotic mediators and provide an explanation of how PACS-2 coordinates specific organellar events to drive apoptosis and how misregulation of these processes ultimately results in disease.

# Appendix A. Preliminary and Supplementary Results

This appendix contains preliminary and supplementary results demonstrating a role for phosphorylation of furin in binding to PACS-1 (Fig. A-1); the potential role of the stress activated protein kinase Jnk in mediating the translocation of PACS-2 to the mitochondria in response to TNFα/ActD (Fig A-2); the amount of death observed in PACS-2-/- MEFs upon treatment with TRAIL (Fig. A-3); the phosphorylation of PACS-2 peptides by Akt (Fig. A-4); the apoptotic response of PACS-2-/- MEFs to TNFα/CHX (Fig. A-5); and the ability of 14-3-3 proteins to prevent apoptosis in response to PACS-2 Ser437A overexpression (Fig. A-6).

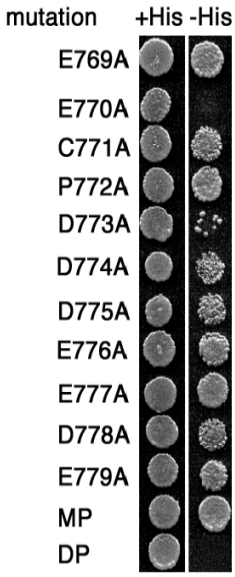


Figure A- 1. Yeast-2-hybrid analysis of the furin acidic cluster binding to the PACS-1 FBR domain.

Phospho-mimic (MP), wildtype dephosphorylated (DP), and point mutants of the acidic cluster region of the furin tail were cloned into LexA DNA-binding domain-containing vector pLexA and co-transformed into a L40 yeast strain with the activation domain-containing vector VP16 encoding the FBR domain of PACS-1. Upon the removal of a histidine source, MP-furin promotes the transcription of genes needed for histidine synthesis though transactivation mediated by binding to the PACS-1 FBR. Furin tail mutants E770A and D773A (S773A in non-phosphomimic) fail to interact with PACS-1 to drive genes needed for histidine biosynthesis.

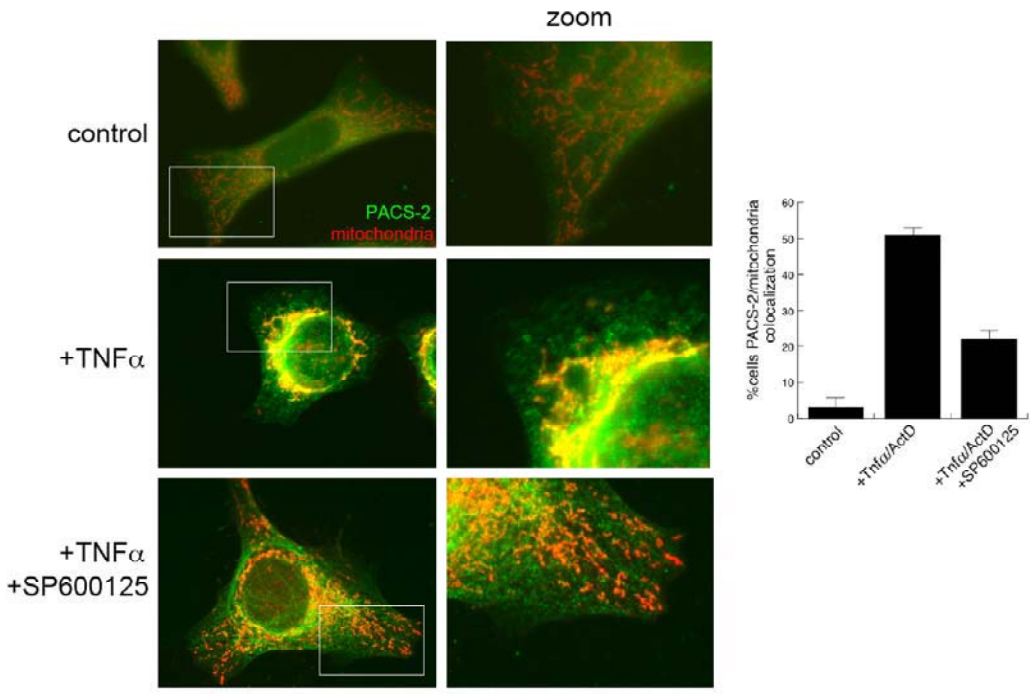


Figure A- 2. The Jnk inhibitor SP600125 blocks the apoptotic translocation of PACS-2 to the mitochondria in response to TNF/ActD in HeLa cells.

HeLa cells were pre-treated with the Jnk kinase inhibitor SP600125 (from Calbiochem) prior to apoptosis induction by TNFα (50 ng/ml) and Actinomycin D (1 μM). Cells were stained with anti-PACS-2 antisera #604 and Mitotracker-CMXRos (Invigtrogen) and analyzed by immunofluorescence microscopy for PACS-2 and mitochondrial localization. As shown on the graph on the right, pre-treatment with SP600125 blocks the apoptotic overlap and perinuclear translocation of PACS-2 and mitochondria characteristic of apoptosis in HeLa cells.

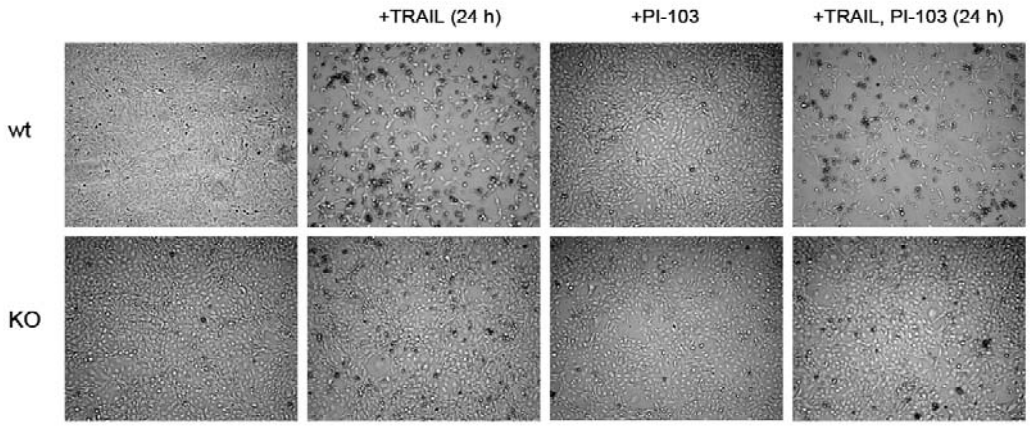


Figure A- 3. PACS-2 -/- MEFs (KO) are resistant to TRAIL-mediated apoptosis.

Prior to FACS analysis in Fig. 4-3A, MEF cells were photographed to demonstrate live vs. dead/floating morphologies.

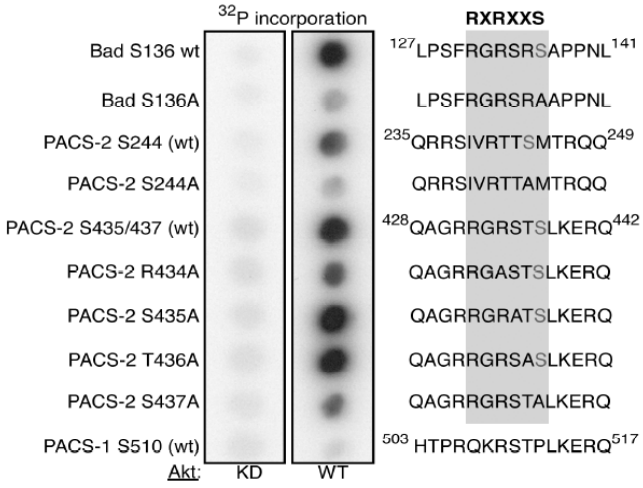


Figure A- 4. *In vitro* Akt phosphorylation of PACS-2 peptides.

To test whether the $Ser_{244}$ or $Ser_{437}$ sites are sufficient for Akt phosphorylation, I phosphorylated an array of 15mer peptides corresponding to the two sites and containing a $Ser_{244}$Ala or $Ser_{437}$Ala substitution. The Akt phosphorylation site of Bad serves as a positive control. I found that Akt phosphorylated both native $Ser_{244}$ and $Ser_{437}$ peptides but not peptides containing the $Ser_{244}$Ala or $Ser_{437}$Ala. Phosphorylation of a peptide lacking a requisite docking arginine, $Arg_{434}$, is also reduced upon phosphorylation by Akt. A homologous region of PACS-1 does not contain a consensus Akt site and is not phosphorylated by Akt. Immobilized peptide arrays were synthesized in triplicate on cellulose paper by D. Beene/ J. Scott (Vollum) as previously described (Carlson JBC 2006). Peptide arrays were cut into 15 cm x 0.5 cm strips, activated in EtOH, washed in MilliQ $H_2O$ and washed with kinase buffer. The peptide array strip was then transferred to a drinking straw sealed with dialysis membrane clips and filled with kinase buffer, 250 µl HA-Akt beads, 100 µM ATP and 10 µCi [$\gamma$-$^{32}$P]-ATP and incubated with rocking at room temperature for four hours. After incubation the strip was washed three times in PBS, two times in 1% phosphoric acid, two times in MilliQ $H_2O$, two times in EtOH and then air dried. Strip was exposed to XAR film to visualize [$^{32}$P] incorporation by autoradiography.

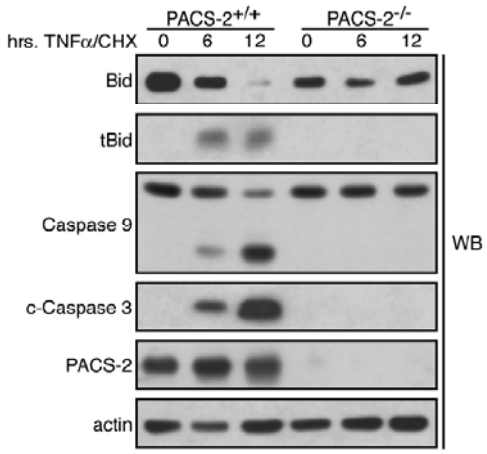


Figure A- 5. PACS-2 -/- MEFs are resistant to apoptosis induced by TNFα/CHX.

SV40 transformed wt and PACS-2 -/- MEFs were seeded into 35mm$^2$ plates one day prior to experiment. Cells were treated with 100 ng/ml recombinant mouse TNFα (R&D Systems) and 1 μg/ml CHX. Cells were harvested directly into heated 1x SDS sample buffer at 0, 6 and 12 hour time points. Cell lysates were sonicated and western blotted for apoptotic markers as described above.

As seen in Fig. A-5 above, PACS-2 -/- MEFs show a marked reduction in apoptotic response to TNFα/CHX as determined by immunoblot for the active p17 form of Caspase-3, Caspase-9 cleavage and Bid cleavage. These results demonstrate that PACS-2 plays an essential role in the apoptotic response of MEF cells to TNFα/CHX to activate Caspase-3 to drive apoptotic cell death.

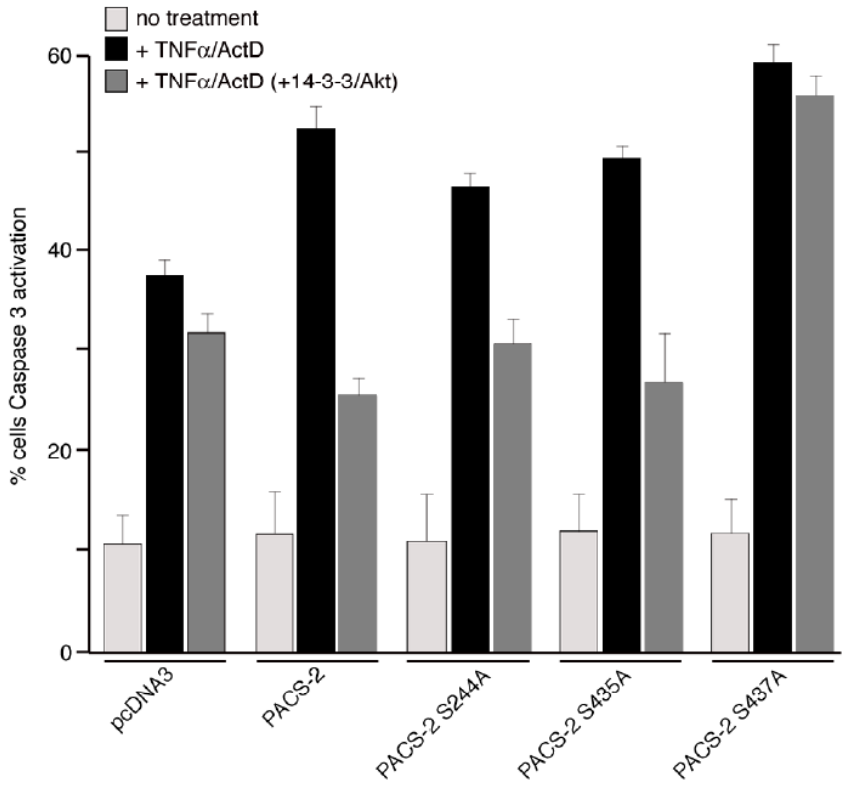


Figure A- 6. 14-3-3 does not reduce apoptosis in HEK293 cells expressing PACS-2HA-Ser437A.

HEK293 cells were seeded into 12-well cell culture plates on glass coverslips (Fisher) one day prior to Lipofectamine 2000 transfection with pSensor and pcDNA3 or pcDNA3-PACS-2 constructs with or without 14-3-3ζ- pcDNA3 and Akt- pcDNA3. 24 hours transfection, cells were treated with 5 ng/ml human TNFα / 0.5 μg/ml ActD for 16 hours, washed in PBS, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and mounted onto glass microscope slides (Fisher). 10 fields of each sample were visualized with the 20x objective on a Zeiss microscope using Openlab software (Improvision) and scored for eYFP localization in triplicate.

I found that TNFα/ActD increased the eYFP nucleus-positive cells from less than 10.5% to 38%. Co-expression of PACS-2 or the PACS-2 mutants all increased caspase-3 activity in response to TNFα/ActD. I also found that 14-3-3/Akt markedly repressed the ability of PACS-2, and the $S_{244}$A, and $S_{435}$A mutants to increase caspase-3 activity. By contrast, 14-3-3/Akt failed to repress the $S_{437}$A-mediated increase in caspase-3 activity. These results suggest that the binding of 14-3-3 proteins to phosphorylated $Ser_{437}$ regulates the apoptotic activity of PACS-2.

# Appendix B. Sustained phosphorylation of Bid is a marker for resistance to Fas-induced apoptosis during chronic liver diseases

Arndt Vogel[1,2], Joseph E. Aslan[3], Holger Willenbring[1], Christian Klein[2], Milton Finegold[4], Howard Mount[5], Gary Thomas[3] and Markus Grompe[1]

From the [1]Department of Molecular and Medical Genetics, Oregon Health & Science University, Portland, Oregon, [2]Department of Hepatology, Gastroenterology and Endocrinology, Medical School Hannover, Hannover, [3]Vollum Institute, Oregon Health and Science University. Portland, Oregon, [4]Department of Pathology, Texas Children's Hospital, Houston, Texas, and [5]Centre for Research in Neurodegenerative Diseases, Department of Medicine, University of Toronto, Ontario, Canada

[2]Address correspondence to : Dr. Arndt Vogel, Department of Hepatology, Gastroenterology and Endocrinology, Medical School Hannover, Carl-Neubergstr. 1, 30625 Hannover, tel. +49 511 5326324, fax +49 511 5324092, E-mail: avogel@gmx.de

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In this appendix I performed the experiments in Figures 3B, 4A, 4B, 4C, 4D, and 5B. A. Vogel performed the experiments in Figures 1, 2, 3, 4E, 4F, 4G, 5A, 5C, 5D, 5E, 5F and Table 1 with the assistance of H. Willenbring and C. Klein. Histochemistry was performed by M. Finegold. H. Mount supplied $ATM^{-/-}$ mice. The manuscript was authored by J.E. Aslan, A. Vogel and M. Grompe.

**Summary**

Increased rates of apoptosis have been reported to play a role in the pathophysiology of many disorders, including liver diseases. Conversely, genetic mutations which result in impairment of programmed cell death have been associated with cancer development. However, apoptosis resistance can also be the result of non-genetic stress adaptation as seen in the cancer-prone metabolic liver disease hereditary tyrosinemia (HT1). To clarify whether stress induced apoptosis resistance is a general feature of cancer-prone chronic liver diseases, an animal model of chronic cholestasis was examined. Studies were performed with mice before and two weeks following bile duct ligation and with $Fah^{-/-}$ and $Fah/p21^{-/-}$ mice before and after NTBC withdrawal. Here we show that bile-duct ligation induced a profound resistance against Fas mAb-mediated hepatocyte death. The apoptosis-signaling pathway was blocked downstream of caspase 8 activation and proximal to mitochondrial cytochrome C release. In controls, activation of the Fas receptor resulted in rapid dephosphorylation of Bid and its subsequent cleavage, whereas Bid remained phosphorylated and uncleaved in chronic cholestasis and other models of hepatic apoptosis resistance. We propose a model in which the phosphorylation status of Bid determines the apoptotic threshold of hepatocytes in vivo. In addition, resistance to apoptosis in chronic cholestasis may contribute to the long-term risk of cancer in this setting.

## B.1 INTRODUCTION

Fas (CD95/Apo-1) belongs to the subfamily of death receptors and plays an important role in liver homeostasis. Hepatocytes are exquisitely sensitive to apoptosis mediated by the Fas receptor. Hence cell death induced by this mechanism has been implicated in acute liver injury and liver failure (Galle and Krammer, 1998). However, chronic liver disease is in some cases associated with apoptosis resistance. For example, we have recently shown that the chronic injury found in the metabolic liver disease hereditary tyrosinemia (HT-1) is associated with profound cell death resistance (Vogel et al., 2004). This observation raised the question whether death resistance is a specific adaptation seen only in the rare genetic disease HT-1 or whether it represents a more general response in chronic liver disorders. Here, an animal model of chronic cholestasis was used to address this question. Cholestasis is defined as an impairment of bile flow and is seen frequently in human liver disease, manifesting itself as jaundice. Many conditions associated with chronic cholestasis have not only an elevated risk of cholangiocellular carcinoma but also of hepatocellular carcinoma (Caballeria et al., 2001; Findor et al., 2002; Gores, 2003; Keeffe et al., 1993). During cholestasis toxic bile acids accumulate within hepatocytes, leading to structural changes, inflammatory responses and, ultimately, to hepatotoxicity. The mechanisms leading to hepatocyte death in cholestasis are not well defined and two distinct mechanisms have been reported. Several studies have proposed that Fas-receptor and Bid dependent apoptosis is an important mechanism for cholestatic liver injury (Higuchi et al., 2001; Schoemaker et al., 2003; Yerushalmi et al., 2001). In contrast, more recent studies carefully re-evaluated the mode of cell death *in vivo* and showed that the mechanism of hepatocyte death is almost

exclusively oncotic and not apoptotic following bile duct ligation (Fickert et al., 2005; Gujral et al., 2004). Despite these reports demonstrating liver cell death acutely after accumulation of bile acids, it is increasingly recognized that hepatocytes develop protective adaptations against bile acid induced cell death during chronic cholestatic liver diseases. In HT-1, the cell death resistant state is characterized by protection against multiple triggers including the toxic tyrosine metabolite homogentisic acid, the drug acetaminophen and the monoclonal anti-mouse Fas-activating antibody $Jo_2$ (Fas mAb) (Vogel et al., 2004). In this study, we therefore determined whether hepatocytes in chronic cholestasis also display resistance against classical apoptosis induced by Fas mAb and analyzed the mechanism underlying this adaptive response. We found that resistance to Fas mAb-induced apoptosis is indeed a feature of chronic cholestasis. Furthermore, we show that phosphorylation/dephosphorylation of Bid correlates with this adaptive response in different apoptosis resistance models. Our data suggest a central role for Bid in the regulation of apoptosis sensitivity in the liver.

## B.2 MATERIALS AND METHODS

***Mice:*** We used the *Fah*$^{-/-}$ strain mice previously described (Grompe et al., 1993). All *Fah*$^{-/-}$ animals were treated with NTBC containing drinking water at a concentration of 7.5 mg/L unless otherwise indicated. 129S1 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). Common bile duct ligation (BDL) was performed as previously described (Faubion et al., 1999). In brief, mice were anesthetized and the

peritoneal cavity was opened. The common bile duct was double ligated and cut between the ligatures. At the end of each pretreatment, the animals were injected intraperitoneally with Fas antibody ($Jo_2$) (BD Pharmingen, San Diego, CA) and sacrificed at indicated time points. Animal care and experiments were all in accordance with the Guidelines of the Department of Animal Care at Oregon Health Sciences University. At the time of sacrifice, a small fragment of each liver was fixed in buffered formalin. The remaining liver tissues were flash-frozen in liquid nitrogen and stored at -80 °C until analysis.

***Histology and TUNEL assay:*** Liver issues were fixed in 10% phosphate-buffered formalin, pH 7.4, dehydrated in 100% ethanol, and embedded in paraffin wax at 58°C. Five-micron sections were rehydrated and stained with hematoxylin/eosin (H&E). The terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL) assay (ApopTag, Serological Corporation, Norcross, GA) was performed according to the manufacturer's recommendations.

***DNA Fragmentation Analysis:*** Genomic DNA was isolated and purified from mouse livers using a genomic DNA isolation kit (Qiagen, Valencia, CA). DNA samples (1μg each) were electrophoretically separated on 2% agarose gel containing ethidium bromide (0.5 g/L).

***Aminotransferase Determinations:*** For aminotransferase analysis, animal blood was drawn. After spinning at 200*g*, plasma was recovered and stored at 80°C until used for determination of aminotransferase activities.

***Mitochondria Isolation:*** Liver homogenates were prepared and sub-fractionated as described previously. Liver samples were homogenized in 2 ml of Buffer A (225mM mannitol, 75mM sucrose, 0.1 mM EGTA, 10 mM HEPES, pH 7.2) with a dounce glass

homogenizer (type B pestle) and centrifuged at 4 °C for 10 min at 600 x *g*. The supernatants were further centrifuged at 12,000 x *g* for 10 min. The supernatants were collected as the cytosol fraction. The pellet (heavy membranes or mitochondria) was washed once again in Buffer A by centrifugation at 600 x *g* for 10 min and recovered by centrifugation at 12,000 x *g* for 10 min. The isolated mitochondria were resuspended in Buffer B (395mM sucrose, 10 mM HEPES-NaOH, pH 7.5).

***Caspase Activity:*** Liver lysates were prepared by homogenization in hypotonic buffer (25 mM HEPES, pH 7.5, 5 mM MgCl2, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF). Homogenates were centrifuged at 15,000 r.p.m. for 15 min, and extracted proteins (50 μg) were tested in triplicate experiments by measuring the proteolytic cleavage of specific fluorogenic substrate for caspase-3 and caspase-8 (CaspACE Assay System; Promega, Madison, WI).

***Antibodies***: Antibodies against Fas-receptor, caspase-9, Flip, and Bid were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against Bak, Bax, and cytochrome c were purchased from Santa Cruz, and antibodies against caspase-8, c-Iap-2, Bcl-2, Bcl-x, phospho-Bad from BD Biosciences. The antibody, which recognizes S61 phosphorylated Bid, was generously provided by JC Martinou.

***Western Blot Analysis:*** Protein extracts were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). Coomassie staining was used to demonstrate equal protein loading. Western Blotting was performed as recently described [2]. Detection of immunolabeled proteins was done using the chemiluminescence kit (BioRad, Hercules, CA) and Hyperfilm enhanced chemiluminescence film (Amersham Biosciences, Piscataway, NJ).

***Hepatocyte Preparation and Flow Cytometry*.** Livers were perfused as previously described. For cell surface Fas, cell suspensions were incubated with Fas-specific fluorescein isothiocyanate-conjugated antibody (PharMingen, San Diego, California). Data were acquired using an Elite Epics flow cytometer (Coulter, Hialeah, Florida) and analyzed with CellQuest software (Becton Dickinson, San Jose, California). Analysis gates were set to exclude contaminating immune cells (lymphocytes and macrophages).

***$NF_KB$ p65 Immune-fluorescence:*** Cryosections of livers were performed. Sections were fixed in methanol/acetone, dried and rinsed in TBST. After incubation with a specific antibody directed against the mouse NF-$_K$B p65 subunit (Santa Cruz Biotechnology, USA), positive cells were detected by incubation with *Alexa Fluor$_{(594nm)}$* donkey anti-rabbit secondary antibody (Molecular Probes, USA). Staining of liver nuclei was performed by incubation with DAPI containing mounting Medium (Vector Laboratories, USA).

***In Vitro Cleavage of Hepatic Caspase-3 and -9 by Recombinant Caspase-8.*** Liver cytosols (2 μg/μl) of BDL and control mice were incubated with activated recombinant caspase-8 (1 ng/μl, BD PharMingen) at 30 °C for 120 min. Extracts were then separated on a 10% SDS-PAGE followed by Western blotting with anti-caspase-3 and –9 antibodies.

***In Vitro Induction of Mitochondrial Cytochrome c Release.*** Recombinant tBid at various concentrations (1, 5, 10 and 25 nM) were mixed with 100 μg of hepatocyte mitochondria (2 mg/ml) in 75 μl buffer B. Mitochondria of 4 mice in each group were analyzed. After incubation at 30 °C for 30 min, the supernatants were separated from the

mitochondria by centrifugation at 16,000 × g at 4 °C for 10 min (Biofuge Fresco, Heraeus Instruments, Inc.). An aliquot of 50 µl was removed from the supernatant, transferred to a new tube and 5 µl if 5% Triton-X was added. Two control samples containing only mitochondria and no tBid were run for each assay to determine the total amount of cytochrome c that can be released from mitochondria and the amount of spontaneously released cytochrome c from untreated mitochondria. The cytosolic fractions were then subjected to a 12% SDS-PAGE followed by Western blotting with the anti-cytochrome c antibody.

***In Vitro Phosphorylation of Bid:*** Recombinant murine Bid-$His_6$ (5 µg) was in vitro phosphorylated by 3 µg hepatocyte lysate. Control phosphorylation reactions were carried out using 100ng recombinant CK1. Reactions were incubated for 30 minutes at 30°C in a total volume of 25 µL CK1 reaction buffer (20 mM MOPS pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT) containing 200 µM cold ATP and 2.5 µCi [$\gamma$-$^{32}$P]-ATP (3000 Ci / mmol; ICN). CK1 activity was inhibited by 100 µM CKI-7 (US Biological). Phosphorylation reactions were terminated by the addition of SDS-PAGE loading buffer. Bid protein was separated by 15% SDS-PAGE, transferred to nitrocellulose and analyzed by autoradiography. Additionally membranes were probed with the phospho-specific Bid antibody.

***CK1 Substrate Peptide Assay:*** 3 µg hepatocyte lysate was used to phosphorylate the CK1 Substrate Peptide RRKDLHDDEEDEAMSITA (Upstate). A reaction containing 250 µM substrate peptide in a total volume of 25 µL CK1 assay buffer using ATP concentrations mentioned above was incubated for 10 minutes at 30°C. 100 µM CKI-7 was used to inhibit CK1 activity. CK1 substrate reactions were stopped by the addition of

trichloroacetic acid and spotted onto P81 grade Whatman filter paper. Filters were washed 3 times in 0.75% phosphoric acid and one time in acetone. Filters were counted with a Packard 1900 TR Liquid Scintillation Analyzer to measure $^{32}P$ incorporation into peptide bound to filter.

***Microarray analysis:*** Total RNA was extracted with the RNeasy kit (Qiagen). Microarray assays were performed in the Affymetrix Microarray Core of the Oregon Health and Science University Gene Microarray Shared Resource, which uses Affymetrix Expression Analysis software (www.ohsu.edu/gmsr/amc/). The screen was performed in duplicate on GeneChip® Mouse Genome 430 2.0 Arrays.

## B.3 RESULTS

Cholestasis was induced in a cohort of experimental mice by surgical bile duct ligation, which was well tolerated. Two weeks after the procedure, no signs of hepatic apoptosis were present despite Tunel positive bile infarcts (data not shown) as reported by others (Fickert et al., 2005; Gujral et al., 2004; Schoemaker et al., 2003). Apoptosis was determined by DNA laddering (Fig. 1b), caspase activity assays (data not shown) and analysis of the mitochondrial apoptosis pathway (Fig. B-2b). At this time point, BDL mice still maintained the same weight and overall health as controls, despite clearly abnormal liver functions including marked hyperbilirubinemia, mild elevation of transaminases (Fig. B-2a) caused by oncotic liver cell injury. Because of the absence of spontaneous apoptosis, it was determined next whether BDL mice were protected against Fas-induced apoptosis. Remarkably, all BDL mice survived an otherwise 100% lethal

dose of the Fas mAb (Fig B-1a). Histology, DNA laddering (Fig. B-1b) and TUNEL staining (Fig. B-1c) showed massive apoptosis in control mice, whereas BDL mice were completely protected. Additionally, transaminase and bilirubin levels did not increase further (Fig. B-2a). Together these data show that hepatocytes in chronic cholestasis acquired resistance to Fas-induced apoptosis.

In our previous work, heat shock protein (70, 32 and 27) up-regulation and stress kinase down-regulation were implicated in the resistance to Fas mAb induced apoptosis in the *Fah*$^{-/-}$ model. In bile duct ligated and in comparisons of Fah$^{-/-}$ and *Fah/p21*$^{-/-}$ mice, however, none of these changes was consistently seen. Hsps were also up-regulated in *Fah/p21*$^{-/-}$ mice, which are no longer resistant against apoptosis (Willenbring et al manuscript submitted), and the stress kinase pathways, which were blocked in apoptosis resistant *Fah*$^{-/-}$ mice, were activated in BDL mice (data not shown).

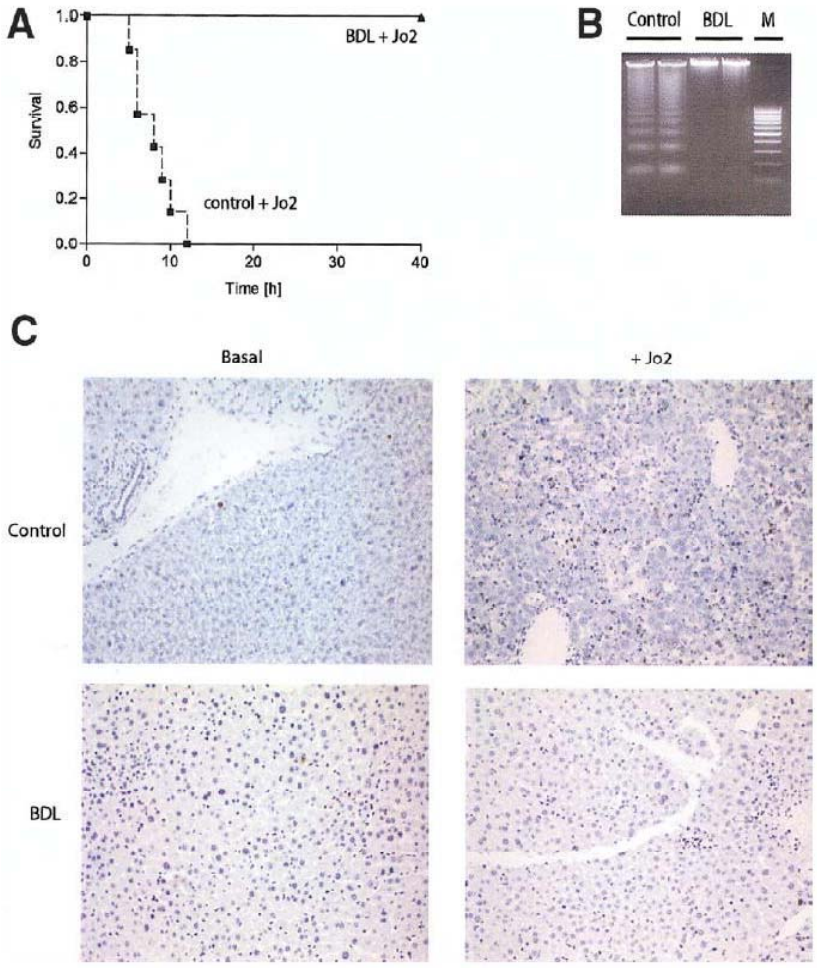


Figure B- 1. Bile duct–ligated mice develop a resistance against Fas-induced apoptosis.

(a) WT mice underwent bile duct ligation and were challenged with the Fas mAb Jo2 (0.35 µg/g) 14 days later. Survival of control (squares) and BDL (triangles) mice are shown. All control mice (n=10) died from acute liver failure within 12 hours following injection of Jo2, whereas all BDL mice survived (n=10). (b) DNA laddering following challenge with Jo2 was only seen in control mice. Results are shown for duplicate animals under each condition. A standard DNA ladder marker (M) is shown. (c-f) TUNEL staining (brown color/ black arrows) of liver sections is shown (original magnification x200) in control and BDL mice before and after Jo2 injection. Panels c, d: control mice before (c), and 4 hrs (d) after Jo2 challenge. Panels e, f: mice 2 weeks after bile duct ligation, before (e) and 4 hr (f) after Jo2 challenge. Tunel positive cells were only visible in control mice following Jo2 injection.

The transcription factor NFκB has been implicated in both hepatocyte proliferation and apoptosis and recent studies have suggested that NFκB can be activated in some models of cholestasis (Bird et al., 2003; Pikarsky et al., 2004; Schoemaker et al., 2003). We therefore considered whether NFκB signaling could account for the resistance against Fas-induced apoptosis in BDL mice. p65 translocation into the nucleus was used as marker of NFκB activation. A mild, transient activation of NFκB occurred only within the first 48h following BDL, there was no nuclear staining of p65 at later time points. Interestingly, no consistent activation of NFκB was evident within the liver even at the earlier time points, only scattered hepatocytes showed nuclear accumulation of p65 (data not shown). In agreement with these observations, we were unable to observe a significant decrease of IκBα and β two weeks following BDL. Additionally, we performed a microarray analysis to ascertain the levels of known NFκB target genes such as Fas receptor (Kuhnel et al., 2000), A20 (Arvelo et al., 2002), inos (Hatano et al., 2001), ciap1 (Pikarsky et al., 2004), ciap2 (Hatano et al., 2001), xiap (Cavin et al., 2004), bfl1 (Pikarsky et al., 2004), bclxl, Gadd45β (Pikarsky et al., 2004), Afp (Cavin et al., 2004), $IP_3R$-1 (Camandola et al., 2005) or mSOD (Table B-1), which have been implicated in the regulation of apoptosis. There was no overlap of genes differentially regulated in the two apoptosis resistant models and there was no consistent up-regulation in apoptosis resistant mice (BDL and *Fah*$^{-/-}$ off NTBC) in comparison to apoptosis sensitive mice (*Fah*$^{-/-}$/*p21*$^{-/-}$ off NTBC).

**Table 1.** Comparison of Gene Expression in Apoptosis Resistant Versus Sensitive Livers

| | BDL/wild-type | $Fah^{-/-}$ off/ $Fah^{-/-}$ on | $Fah/p21^{-/-}$ off/ $Fah/p21^{-/-}$ on | $Fah^{-/-}$ off/ $Fah/p21^{-/-}$ off |
|---|---|---|---|---|
| *Fas receptor*[a] | 1.09 | 0.89 | 1.46 | 0.61 |
| *Fadd* | 1.27 | 0.69 | 0.87 | 0.79 |
| *Tnf-R* | 1.30 | 1.13 | 0.88 | 1.28 |
| *Caspase-9* | 1.05 | 0.77 | 0.79 | 0.97 |
| *Caspase-8* | 1.10 | 1.68 | 1.60 | 1.05 |
| *Caspase-7* | 0.90 | 0.69 | 0.64 | 1.08 |
| *Caspase-6* | 0.87 | 0.64 | 0.69 | 0.93 |
| *Caspase-3* | 1.22 | 1.14 | 0.75 | 1.52 |
| *Smac/Diablo* | 1.02 | 1.04 | 0.89 | 1.17 |
| *Cytochrome c* | 0.66 | 1.53 | 1.90 | 0.81 |
| *Apaf-1* | 1.67 | 1.45 | 1.74 | 0.83 |
| *Bak* | 1.59 | 0.85 | 0.70 | 1.21 |
| *Bax* | 1.37 | 2.53 | 2.35 | 1.08 |
| *Bok* | 1.13 | 0.90 | 0.56 | 1.61 |
| *Bad* | 1.01 | 0.57 | 0.74 | 0.77 |
| *Bid* | 1.77 | 3.06 | 1.82 | 1.68 |
| *Bim* | 1.16 | 2.12 | 2.56 | 0.83 |
| *Bag-1* | 1.16 | 1.39 | 1.02 | 1.36 |
| *Bcl-2* | 1.49 | 0.72 | 2.39 | 0.0 |
| *Bcl-xl*[a] | 1.01 | 1.67 | 1.38 | 1.21 |
| *Bcl-w* | 2.04 | 0.90 | 1.14 | 0.79 |
| *Bfl1/A1*[a] | 3.16 | 1.91 | 1.77 | 1.08 |
| *Mcl-1* | 1.43 | 0.82 | 0.75 | 1.09 |
| *Bis* | 1.03 | 1.79 | 1.26 | 1.42 |
| *clap-1*[a] | 1.64 | 0.99 | 1.22 | 0.81 |
| *clap-2*[a] | 1.31 | 0.57 | 0.82 | 0.70 |
| *xlap*[a] | 1.03 | 1.00 | 1.30 | 0.72 |
| *Flip* | 0.68 | 0.69 | 0.96 | 0.72 |
| *Survivin* | ND | ND | 37.41 | — |
| *Nos-2*[a] | ND | ND | ND | — |
| *Afp*[a] | 1.66 | 50.09 | 52.48 | 0.95 |
| *A20*[a] | ND | ND | ND | — |
| *Cox-2*[a] | 1.16 | 0.85 | 1.37 | 0.62 |
| *Sod-2*[a] | 0.93 | 0.75 | 0.71 | 1.05 |
| *$IP_3R$-1*[a] | 1.13 | 0.79 | 0.65 | 1.21 |

NOTE. Values are expressed as fold change. Transcriptional regulation of several pro-and anti-apoptotic genes were analyzed for (column 1) apoptosis-resistant BDL mice in comparison to apoptosis-sensitive WT mice, (column 2) apoptosis-sensitive $Fah^{-/-}$ on NTBC in comparison to apoptosis-resistant $Fah^{-/-}$ off NTBC, (column 3) apoptosis-sensitive $Fah^{-/-}/p21^{-/-}$ on NTBC in comparison to apoptosis-sensitive $Fah^{-/-}/p21^{-/-}$ off NTBC withdrawal and (column 4) apoptosis-resistant $Fah^{-/-}$ off NTBC in comparison to apoptosis-sensitive $Fah^{-/-}/p21^{-/-}$ off NTBC. ND, not detectable.
[a]NFκB target genes.

Table B- 1. Comparison of Gene Expression in Apoptosis Resistant Versus Sensitive Livers.

Comparison of gene expression in apoptosis resistant vs. sensitive livers.
Transcriptional regulation of several pro-and anti-apoptotic genes were analyzed for (row 1) apoptosis-resistant BDL mice in comparison to apoptosis-sensitive WT mice, (row 2) apoptosis-sensitive Fah-/- on NTBC in comparison to apoptosis-resistant Fah-/- off NTBC, (row 3) apoptosis-sensitive Fah-/-/p21-/- on NTBC in comparison to apoptosis-sensitive Fah-/-/p21-/- off NTBC withdrawal and (row 4) apoptosis-resistant Fah-/- off NTBC in comparison to apoptosis-sensitive Fah-/-/p21-/- off NTBC. n.d. not detectable; * NFkB target genes.

Finally, several publications have shown that activation or inactivation of NFκB does not significantly modulate Fas-induced apoptosis in the liver in contrast to the established anti-apoptotic activity of NFκB in TNF- and Trail-induced liver injury (DeAngelis et al., 2001; Finotto et al., 2004; Nagaki et al., 2000; Van Antwerp et al., 1996; Zender et al., 2005).We therefore sought to determine specifically at which step the Fas-induced apoptosis pathway is blocked and which other factors could contribute to observed resistance against apoptosis.

### 5.9 The apoptosis-signaling pathway is blocked above and/or at the mitochondria

Upon ligand binding, activated death receptors engage the Fas Associated Death Domain adaptor protein (FADD) (Siegel et al., 2000). FADD in turn recruits caspase-8 via a homophilic death effector domain (DED) interaction forming the death-inducing signaling complex (DISC). Disc formation activates caspase-8 through a proximity induced dimerization mechanism requiring no autoproteolysis (Boatright et al., 2003; Salvesen and Dixit, 1999). Here, a clear increase of caspase-8 activity was detectable in both control and BDL mice two hours following Fas mAb injection, indicating that the early steps of the pathway were unaltered following BDL (Fig. B-2a). There was no difference in baseline activation of caspase-8 and -3 two weeks following bile duct ligation. All values were therefore normalized to the same WT controls.

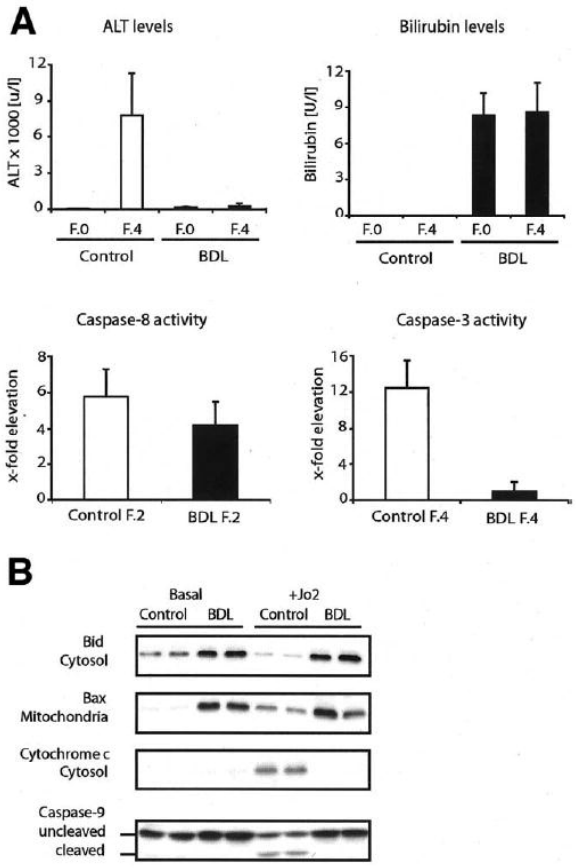


Figure B- 2. The mitochondrial pathway is blocked in bile duct–ligated mice.

(a) Liver injury after Jo2 challenge in control (WT; open columns) (n=4) and BDL mice (filled columns) (n=4). ALT and bilirubin levels were measured in plasma before (F.0) and after (F.4) challenge with Jo2. ALT levels increased only in control mice following Jo2 injection. Bilirubin was significantly elevated in BDL mice. Caspase-8 and –3 activities were determined in liver homogenates 2 hours (F.2; caspase 8) or 4 hours (F.4; caspase 3) following Jo2 injection (n=4 each). In both groups activation of caspase-8 was observed, whereas caspase-3 activity was only detectable in control mice (b) Western blots of Bid, Bax, cytochrome C and caspase-9 before (Basal) and 4h after (Fas) induction of apoptosis with Jo2 in control and BDL mice (n=4 each; 2/4 samples shown here) to determine activation of the mitochondrial pathway of apoptosis. Cleavage of Bid (decrease of full length Bid), translocation of Bax, release of cytochrome C and cleavage of caspase-9 following Jo2 occurred only in control mice. F.0: before Fas mAb injection, F.2/F.4 : 2/4 hours following Fas mAb injection.

In type-II cells such as hepatocytes, activation of caspase-8 at the DISC alone is insufficient to efficiently trigger cell death and an amplification loop via the mitochondria is required. Previous studies have shown that this link between the Fas receptor and the mitochondria is dependent on cleavage and translocation to the mitochondria of the proapoptotic Bcl-2 family member Bid by caspase 8 (Li et al., 1998; Luo et al., 1998). Caspase-8 cleaved tBid was not detectable in *vivo* experiments with various commercially available antibodies, possibly because of the small pool of Bid molecules involved, or because of its rapid turnover (Werner et al., 2002).Therefore, to examine this step of the apoptotic machinery, protein levels of full length (uncleaved) Bid were therefore compared after Fas mAb injection in BDL mice and controls. A marked reduction of full-length Bid was visible in the Fas mAb -treated control group (Fig. B-2b) but full length Bid remained unchanged in BDL mice.

During the apoptotic process in normal type-II cells, tBid activates Bax or Bak to initiate mitochondrial dysfunction and cell death and therefore the role of these proteins was also examined (Desagher et al., 1999; Eskes et al., 2000). As expected, analysis of the intracellular distribution revealed translocation of Bax from the cytosol to the mitochondrial fraction in Fas mAb injected control mice (Fig. B-2b). Surprisingly, Bax was constitutively localized to the mitochondrial fraction even before Fas mAb injection in BDL mice and these high levels of Bax remained unchanged after Fas mAb administration (Fig. B-2b). Normally, activation and oligomerization of Bax and Bak induce structural changes at the mitochondrial membrane resulting cytochrome C release (Shimizu et al., 1999; Wei et al., 2000). Here, cytochrome C was found only in the

cytosol of control animals following Fas mAb injection, but not in BDL mice despite the translocation of Bax to the mitochondria (Fig. B-2b). Following release of cytochrome c from mitochondria, APAF-1 is known to recruit caspase-9 and the resulting assemblage of proteins activates effector caspases such as caspase-3 (Acehan et al., 2002). As expected from the observed release of cytochrome C, the cleaved, activated fragment of caspase-9 and activity of caspase-3 were only detectable in controls, and no caspase activation was found in BDL mice (Fig. B-2a and Fig. B-2b).

### B.3.1 Resistance against apoptosis is not mediated by direct inhibition of caspases

Apoptosis is a tightly regulated process, which involves many pro- and anti-apoptotic proteins capable of altering the balance between life and death. We first wondered whether direct inhibition of caspases contributed to the observed failure of mitochondrial cytochrome C release in BDL mice. Specifically, the expression of death receptor, Flice-like inhibitory proteins (c-Flip) and inhibitors of apoptosis proteins (Iap) were analyzed. In agreement with the activation of caspase-8 seen in BDL mice (Fig. B-2a), mRNA levels for components of the DISC complex such as the Fas receptor, Fadd, and caspase-8 were unaltered (Table B-1). This was confirmed at the protein level for the Fas receptor and caspase-8 (Fig. B-2b and B-3a). Furthermore, Fas receptor at the cell surface was not downregulated assessed by flow cytometric cell cycle analysis, indicating that the adaptive response did not result from lack of available Fas receptors (data not shown). Additionally, levels of c-Flip$_{s/l}$, inhibitors of caspase-8 (Krueger et al., 2001), remained unchanged in BDL mice (Table1 and Fig. 3a). Another group of proteins that function as

intrinsic regulators of caspase activation are the Iaps. Iaps are known to regulate the activity of both initiator (caspase-9) and effector caspases (caspase-3 and -7). However, there were no significant changes for several Iaps including cIap-1 (Fig. 3a), cIap-2 and xIap (Table B-1). These findings indicated that the expressions of proteins known to have direct effects on caspase activities were not changed in BDL. This was further verified by showing that purified active recombinant caspase-8 was able to normally induce cleavage of caspase-9 and –3 in cytosolic extracts of BDL mice in vitro (Fig. B-3b).

### B.3.2 Detailed analysis of Bcl-2 like family members

During apoptosis mitochondria suffer specific damage, which ultimately results in loss of their function. Mitochondrial changes include the release of several pro-apoptotic factors which trigger and amplify the cell death signal leading to activation of caspases and DNA fragmentation (Wang, 2001). The family of Bcl-2 like proteins plays a pivotal role in protection against such irreversible mitochondrial damage and the interactions between the pro- and anti-apoptotic family members determine the overall susceptibility to a cell death signal (Cory et al., 2003). Anti-apoptotic members include Bcl-2, Bcl-x, Bcl-w, A1, and Mcl-1. Pro-apoptotic members can be subdivided into more fully conserved members possessing Bcl-2 homology domains 1-3 such as Bax, Bak and Bok and the BH3-only members such as Bid, Bad, Bim, Noxa, Puma, and Bik. Activation of Bcl-2 like family members is regulated in multiple ways including transcriptional up-regulation, proteolytic cleavage, dimerization, phosphorylation and translocation (Gross et al., 1999a).

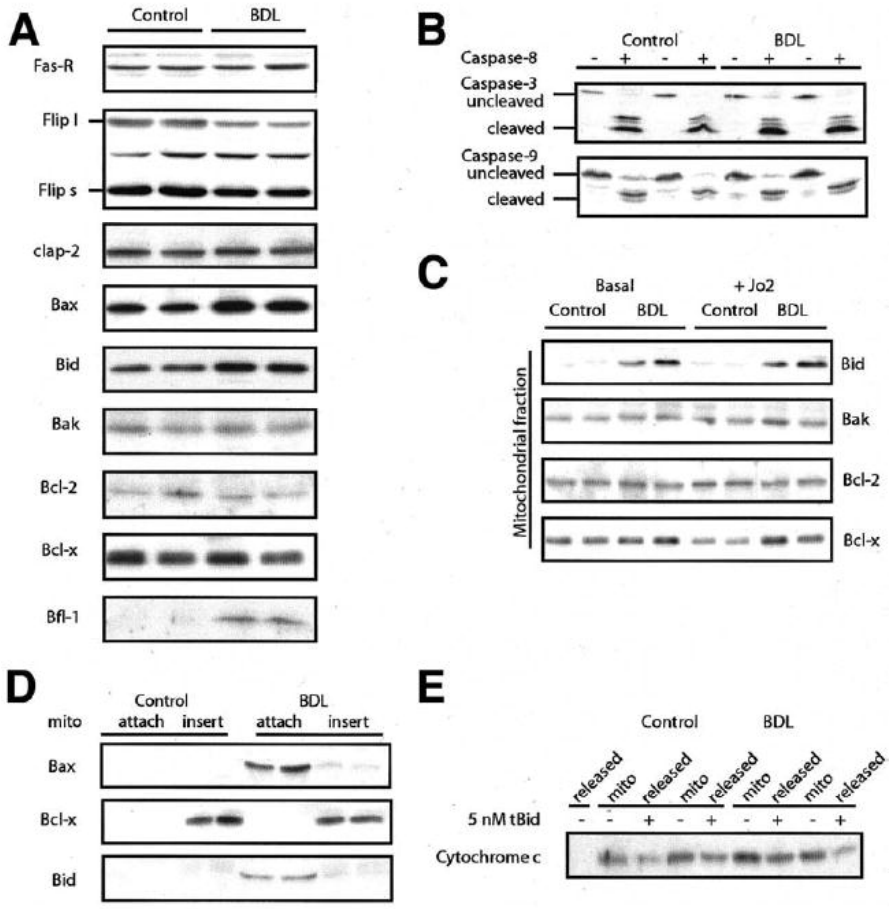


Figure B- 3. Analysis of the mitochondrial pathway in wild-type and bile duct–ligated mice.

Analysis of the mitochondrial pathway in WT and bile duct ligated mice.
(a) Western blot analysis of liver tissues in control and BDL mice in the basal state ithout Fas mAb injection. Shown are data from 2 out of 4 mice in each group. Liver tissues were analyzed for total cellular levels of Fas-R, Flips/l, cIap-2, Bax, Bid, Bak, Bcl-2, Bcl-x and A1/Bfl-1. Of all the proteins analyzed, only A1/Bfl-1 was significantly induced 2 weeks following bile duct ligation. (b) Liver extracts of control and BDL mice were incubated with in vitro activated recombinant caspase-8. Western blots for in vitro cleavage of caspase-3 and –9 revealed no difference in both groups. (c) The mitochondrial fraction of control and BDL mice was analyzed for BH-3 like proteins. Levels of Bak, Bcl-2 and Bcl-x were not significantly changed before and after challenge with Jo2, whereas Bid is significantly increased in this fraction 2 weeks following bile duct ligation even before Jo2 injection. (d) Alkaline extraction of mitochondria from BDL mice and controls. Bid and Bax were only loosely attached and not inserted into the mitochondrial membrane of BDL mice in contrast to BCL-x. (e) Isolated liver mitochondria (n=4 each; 2/4 samples shown here) were incubated with different concentration of in vitro cleaved tBid and translocation of cytochrome c from the mitochondrial (mito) fraction to the cytosol (released) was monitored by immunoblotting with a cytochrome c antibody. cytochrome C release following incubation with 5 nM tBid is shown. First row (-): spontaneous cytochrome C release without tBid incubation. 2nd-9th row: The overall mitochondrial cytochrome C content of the respective sample (n=2 per group) is shown followed by the released cytosolic cytochrome C fraction following incubation with tBid. There was no difference in cytochrome C release between BDL mice and controls.

First, it was determined whether any of the Bcl-2 like genes were up-regulated in response to bile duct ligation. Interestingly, only *Bfl-1/A1* was more than twofold induced at the RNA level.mRNA levels of all other members of the bcl-2 like family were only slightly modulated following bile duct ligation (Table B-1). In agreement with these microarray data, protein levels of Bfl-1/A1 were mildly increased in the cytosol of BDL mice (Fig. B-3a), whereas Bak, Bcl-2 and Bcl-x remained unchanged.

Figure B-4. Bid is not dephosphorylated following Fas injection in BDL and in Fah-/-/p21-/- mice. (a) Phosphorylation of Bid at S 61 was measured in cytosolic liver extracts from control and BDL mice by Western blot. Increased phosphorylation of Bid in BDL mice (n=4 each; 2/4 samples shown here) before challenge with Jo2 and more importantly, no dephosphorylation following Jo2 injection were seen. In contrast, Bid was almost completely dephosphorylated following induction of apoptosis in control mice (b) Western blot analysis of CK1 epsilon and PP2A levels in cell extracts from control and BDL mice. The cytosolic CK1 epsilon levels were higher in BDL mice. Levels of PP2A were not changed in cytosolic extracts (data not shown). (c) CK1 activity was measured in cell extracts of control and BDL mice with a CK1 specific peptide (n=4). Note: Activity assay is not specific for CK1 epsilon, but measures overall CK1 activity. CK1 activity was higher in BDL mice at all time points. Interestingly, CK1 activity was reduced in control mice following challenge with Jo2, but this reduction was not seen in BDL mice. (d) Cellular extracts of control and BDL mice following Jo2 injection were analyzed for in vitro phosphorylation activity of His6-Bid (representative blot, n=4 each); negative control (-) contains no cell extract, positive control (CK1) contains recombinant CK1. Shown is a Western blot of the membranes probed with the phosphor-specific Bid (S61) antibody. Phosphorylation of Bid was only detectable in BDL mice and was almost absent in controls. CK1 inhibitor (CKI-7) significantly inhibited phosphorylation of His6-Bid in BDL mice. To-tal His6-Bid levels of non-CKI-7 treated reactions are shown (e) Fah-/- and Fah-/-/p21-/- mice were chal-lenged with Jo2 two weeks following NTBC withdrawal. Dephosphorylation and subsequent cleavage of Bid and cytochrome C release occurred only in the double-knockout mice. Total CK1 epsilon levels were unchanged, whereas cytosolic levels were lower in Fah-/-/p21-/- mice following Jo2 injection. (f) WT mice were injected with Jo2 alone or concomitantly with suramin. Suramin prevents dephosphorylation of Bid and subsequent Bid cleavage and cytochrome C release. Cytosolic CK1 epsilon levels are higher in Suramin treated mice. (g) In suramin treated mice only a few scattered Tunel positive hepatocytes were visible following Jo2 injection.

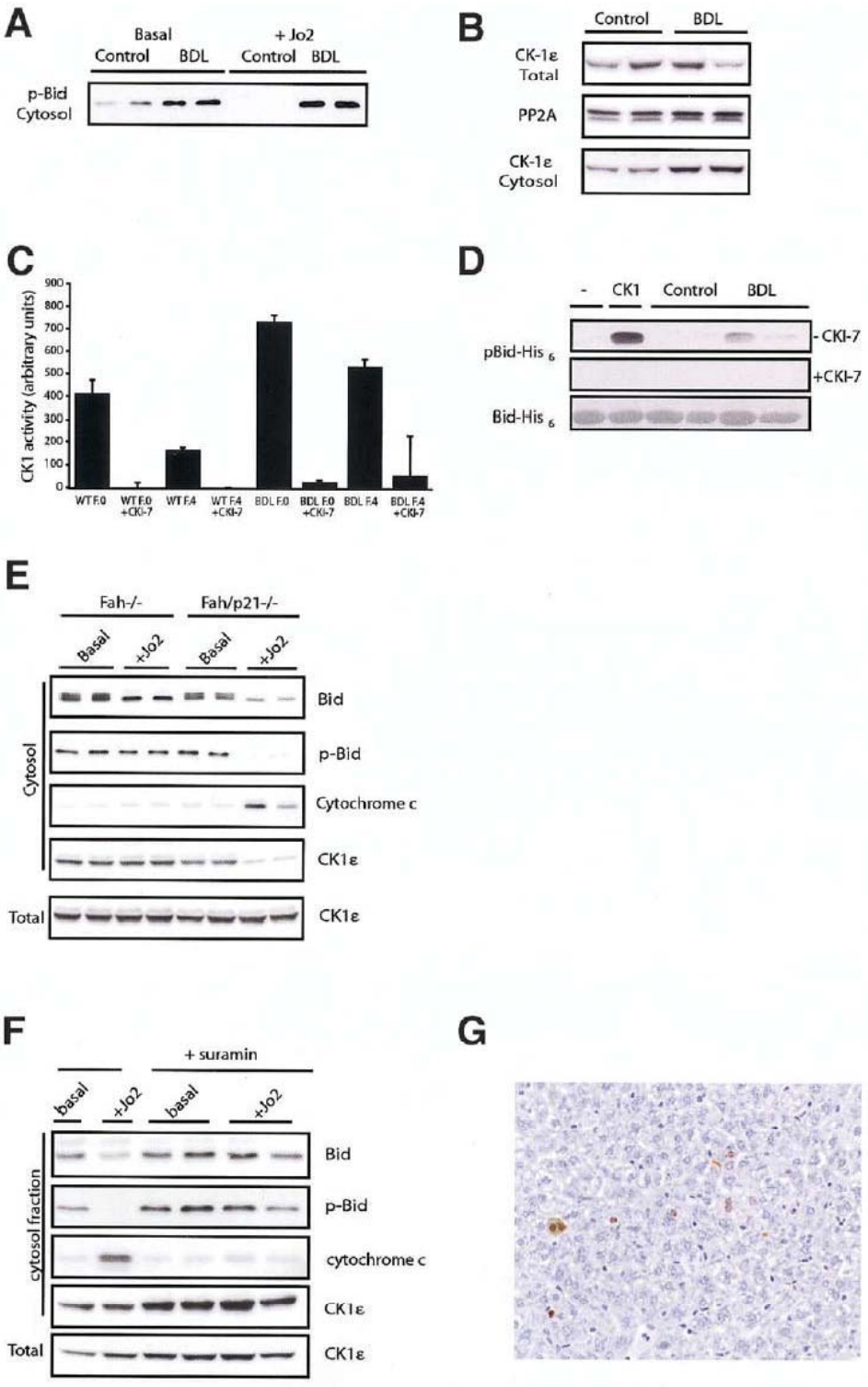


Figure B- 4. Bid is not dephosphorylated following Fas injection in bile-duct ligated *Fah$^{-/-}$/p21$^{-/-}$ mice*

Next, the subcellular location of Bcl-2 like proteins was studied. Bcl-x is found both in the cytosol and attached to mitochondrial membranes of most healthy cells, whereas Bcl-2 and Bak are constitutively associated with mitochondrial membranes (Hsu et al., 1997). The proapoptotic Bcl-2 like proteins Bax and Bid have been reported to be predominantly cytosolic in non-apoptotic cells. In the mitochondrial fraction of BDL mice, an increase of not only Bax but also of full length Bid was seen (Fig. B-2b and Fig. B-3c). On the other hand, levels of Bak, Bcl-x and Bcl-2 in the mitochondrial fraction of BDL mice were not significantly changed before injection of Fas mAb (Fig. B-3c). It has previously been shown that Bax and Bid need to be integrated into the mitochondrial membrane to be active (Wei et al., 2001). Alkaline extraction of mitochondria was therefore used to assess membrane insertion. In contrast to Bcl-x, both Bax and Bid were only loosely attached but not integrated into the mitochondrial membrane. This suggested that these proteins were not activated in BDL mice prior to Fas mAb injection (Fig. B-3d). Mitochondrial integrity was further tested by using different amounts (1, 5, 10 and 25 nM) of caspase-8-cleaved recombinant mouse tBid to induce cytochrome C release from isolated liver mitochondria of apoptosis resistant BDL mice and controls *in vitro*. Importantly, both groups discharged cytochrome c equally well at all tested tBid concentrations (Fig. B-3e). This demonstrates that alterations of the mitochondria themselves did not contribute to the observed resistance against apoptosis in BDL.

### B.3.3 Bid is not dephosphorylated during apoptosis in BDL mice.

The results described above show that the apoptotic block in BDL primarily occurred distal to caspase-8 activation and proximal to mitochondrial damage. In addition, failure of Bid-cleavage despite caspase 8 activation was observed and we therefore sought to understand the mechanism underlying this finding. Phosphorylation of Bid has been shown to prevent its cleavage by caspase-8 (Degli Esposti et al., 2003a; Desagher et al., 2001). In BDL mice slightly elevated levels of Bid mRNA and protein were detectable indicating a transcriptional upregulation of this BH3-only protein (Table B-1, Fig. B-3a). Accordingly, an increase of phosphorylated Bid (S61) was detectable in these mice two weeks following bile duct ligation (Fig. B-4a). During Fas-induced apoptosis, Bid needs to be dephosphorylated before it can be cleaved by caspase-8 (Degli Esposti et al., 2003a; Desagher et al., 2001). The required net dephosphorylation of Bid could be mediated either by the inactivation of a Bid kinase or by activation of a Bid phosphatase. As expected from this model, Bid was almost completely dephosphorylated following induction of apoptosis in control mice (Fig. B-4a). Importantly, dephosphorylation of Bid did not occur at all in apoptosis resistant BDL mice (Fig. B-4a). To date, no phosphatase which dephosphorylates Bid during apoptosis has been identified, but PP2A is a candidate because it is cleaved and activated during apoptosis (Cory et al., 2003). However, here PP2A protein levels were not significantly changed before or following $Jo_2$ injection in control and BDL mice (Fig. B-4b). In the case of murine Bid, CK1 epsilon is known to phosphorylate Bid on Ser 61 in vitro (Degli Esposti et al., 2003a). Several CK1 isoforms with different functions have been identified and are found in the cytosol, associated with membranes, and in the nucleus (Ha et al., 2004; Knippschild et

al., 1997; Sakaguchi et al., 2000). Interestingly, CK1 epsilon was increased in the cytosol of BDL mice (Fig. B-4b) and CK1 activity was slightly increased in cell extracts of BDL mice (Fig. B-4c). More importantly, CK1 activity remained significantly higher in apoptosis-resistant BDL mice compared to control mice following $Jo_2$ injection (Fig. B-4c). To further confirm this finding, Bid phosphorylation activity was measured in vitro with extracts form BDL and control mice following Fas mAb injection. In agreement with the in vivo data, recombinant Bid was only phosphorylated in the cytosol of BDL mice (Fig. B-4d). The phosphorylation on S61 was almost completely blocked by the CK1 inhibitor CKI-7.

### B.3.4 Bid is not dephosphorylated in cell death resistant *Fah*$^{-/-}$ mice during apoptosis in contrast to apoptosis-sensitive *Fah*$^{-/-}$/*p21*$^{-/-}$ mice.

To determine the significance of the alterations of Bid phosphorylation seen here in BDL mice, we wondered whether a similar pattern was present in other apoptosis-resistant liver models. Chronic injury found in the cancer-prone metabolic liver disease hereditary tyrosinemia (HT1) induces profound resistance against hepatocyte death (Vogel et al., 2004). However, this adaptation does not occur in mice which are doubly mutant in the gene responsible for HT1, *Fah,* and the cell cycle regulatory protein *p21*/*Cip*/*Waf*, which develop a similar liver damage as WT mice following $Jo_2$ injection.

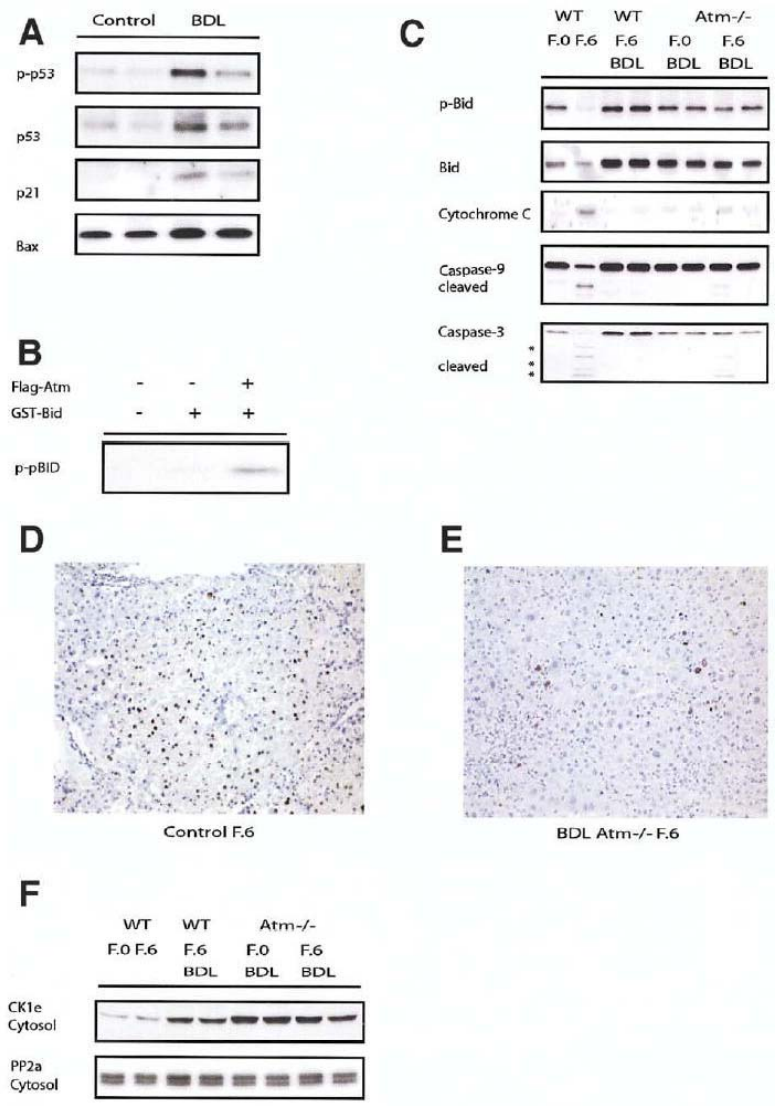


**Figure B- 5.** Atm phosphorylates Bid in vitro, but is not necessary to prevent Fas-induced apoptosis during chronic liver diseases.

(a) The ATM-p53 DNA-damage pathway is activated in BDL mice measured by increased phosphorylation of p53 and increased overall levels of p53 and it effector proteins p21 and Bax. (b) Recombinant Atm phosphorylated Bid-GST in vitro. (C) BDL $Atm^{-/-}$ mice are protected against Fas mAb induced apoptosis similar to WT mice. Dephosphorylation of Bid and subsequent cleavage of Bid, release of cytochrome C and cleavage of Caspase-9 and –3 occurs only in WT mice. (d,e) TUNEL staining (brown color) of liver sections is shown (original magnification x200). Tunel positive cells are only visible in control mice 6 hours after $Jo_2$ injection (d) but not in $Atm^{-/-}$ mice (e). Cytosolic CK1 epsilon levels are again higher in BDL mice, whereas PP2A levels remain unchanged.

Interestingly, *Fah*$^{-/-}$/*p21*$^{-/-}$ mice were highly sensitive against Fas-induced apoptosis despite a marked increase of proliferating hepatocytes two weeks following NTBC withdrawal indicating that proliferation alone does not prevent Fas-induced apoptosis in the liver (Willenbring et al., manuscript submitted) (Desbarats and Newell, 2000). Our observation provides the opportunity to directly compare Bid phosphorylation in apoptosis resistant and apoptosis sensitive livers. First, we compared the transcriptional changes of apoptosis-related genes including pro- and anti-apoptotic BH3-like proteins and inhibitors of caspases between *Fah*$^{-/-}$/*p21*$^{-/-}$ and *Fah*$^{-/-}$ mice on NTBC and two weeks following NTBC withdrawal (Table B-1). We did not observe significant changes of theses genes similar to our results in the BDL mice. Particular *Bfl1/A1* was less than two-fold induced in apoptosis-resistant *Fah*$^{-/-}$ mice and there was no significant difference to apoptosis-sensitive *Fah*$^{-/-}$/*p21*$^{-/-}$. Next, we wondered whether the phosphorylation pattern of Bid would correlate the observed apoptosis sensitivity. *Fah*$^{-/-}$/*p21*$^{-/-}$ and *Fah*$^{-/-}$ mice were taken off the liver protective drug NTBC for 14 days and then injected with $Jo_2$. Phosphorylation levels of Bid were determined in mice of both groups, which at this time point still had the same weight and overall fitness, despite their hepatic dysfunction (Grompe et al., 1995). Phosphorylation of Bid was not different in *Fah*$^{-/-}$/*p21*$^{-/-}$ and *Fah*$^{-/-}$ mice before Fas mAb injection. Importantly however, Bid was not dephosphorylated in the apoptosis resistant *Fah*$^{-/-}$ mutants but almost completely dephosphorylated in the apoptosis competent *Fah*$^{-/-}$/*p21*$^{-/-}$ double mutant mice following $Jo_2$ injection (Fig. B-4e). Similarly, cleavage of Bid measured by decreasing levels of full length Bid and release of cytochrome C occurred only in *Fah*$^{-/-}$/*p21*$^{-/-}$ mice (Fig. B-4e). Dephosphorylation of Bid was associated with decreased cytosolic CK1 epsilon levels in

the apoptosis sensitive mice following $Jo_2$ injection, PP2A levels remained unchanged in both groups (Fig. B-4e).

### B.3.5 Suramin inhibits Fas-induced hepatocellular apoptosis and prevents dephosphorylation of Bid.

Recently, it has been shown that suramin inhibits death receptor-induced apoptosis in vitro and fulminant apoptotic liver damage in mice. Suramin is a polysulfonated napthylurea compound that has been widely used for the treatment of trypanosomiasis and onchocerciasis (Hawking, 1978). Initial observation suggested that suramin acts primarily by interfering with the binding of growth factors, such as platelet-derived growth factor, transforming growth factor and tumor necrosis factor alpha to their corresponding receptors. Subsequent investigation however revealed a marked increase in phosphorylation of several cellular proteins in suramin treated cancer cell lines and identified surmamin as a reversible modulator of protein phosphatases and kinases (Sartor et al., 1992). Previous studies have shown that Surmain does have proapoptotic effects in specific tumors such as prostate carcinoma, whereas it has antiapoptotic affects in lymphoid and hepatic cell lines. Remarkably, Suramin does not prevent apoptosis in type I cells such as SKW6.4 cells whereas it inhibited apoptosis in type II cells such as hepatocytes. Subsequent analysis revealed that the block of apoptosis occurred upstream of the mitochondria and involves inhibition of the Disc. Interestingly however, the general composition of the Disc was not changed by suramin treatment, CD95, FADD,

pro-caspase-8 (p55/53) and their initial cleavage products (p43/44) were present in almost equal amounts. The observations that suramin was only effective in type II cells and acts upstream of the mitochondria suggest that modulation of Bid could also contribute to the observed phenotype. We therefore wondered whether resistance against cell death induced by suramin was also associated with inhibition of Bid dephosphorylation and injected 5 mg of Suramin per mouse concomitantly with $Jo_2$ in WT mice. Co-administration of suramin significantly protected mice from $Jo_2$ induced death. In agreement with our hypothesis, Bid was not dephosphorylated and subsequently not cleaved in suramin treated cells. Cytosolic CK1 epsilon levels were again higher in the apoptosis resistant cells, whereas PP2alpha levels remained unchanged (Fig. B-4f).

### B.3.6 ATM phosphorylates Bid in vitro, but is not necessary for BDL induced resistance against apoptosis.

Our data indicate that phosphorylation/dephosphorylation of Bid strictly correlates with the apoptosis sensitivity in different apoptosis resistance models. Furthermore, apoptosis resistant mice revealed higher cytosolic CK1 epsilon levels in contrast to apoptosis sensitive mice either before or following $Jo_2$ injection implying CK1 epsilon in this adaptive response. To test whether other kinases also phosphorylate Bid, the Scansite of the Massachusetts Institute of Technology was used (Obenauer et al., 2003). The current release of Scansite, version 2.0, includes 63 motifs characterizing the binding and/or substrate specificities of many families of Ser/Thr- or Tyr-kinases, SH2, SH3, PDZ, 14-3-3 and PTB domains, together with signature motifs for PtdIns(3,4,5)P(3)-specific PH

domains. Out of these 63 kinases only Atm (ataxia telangiectasia-mutated) was identified to phosphorylate Bid in silico. Loss of Atm function causes Ataxia telangiectasia (A-T) a rare autosomal recessive disorder characterized by clinical manifestations that include progressive cerebellar ataxia, neuronal degeneration, hypersensitivity to ionizing radiation (IR), and an increased risk for cancer (Savitsky et al., 1995). *Atm* encodes a 370-kD protein that is a member of a family of proteins related to phosphatidylinositol 3-kinase (PI-3-K), which transduce signals through the phosphorylation of proteins on serine or threonine residues (Abraham, 2001). Interestingly, the ATM-p53-p21 DNA-damage pathway is activated in both, $Fah^{-/-}$ mice off NTBC (Willenbring et al. Manuscript submitted) and in BDL mice (Fig. B-5a). Furthermore, recombinant Atm phosphorylates Bid in vitro (Fig. B-5b). To test whether Atm significantly contributes to the observed resistance against apoptosis, $Atm^{-/-}$ mice were bile duct ligated. Two weeks following this procedure, $Atm^{-/-}$ and control mice were injected with a lethal dose of $Jo_2$. However, BDL $Atm^{-/-}$ mice showed the same resistance against apoptosis as the control BDL mice and Bid was not dephosphorylated following $Jo_2$ injection (Fig. B-5c-e). Subsequently, cleavage of Bid and release of cytochrome C occurred only in the non-BDL controls (Fig. B-5c). Cytosolic CK1 epsilon levels were slightly higher in the BDL $Atm^{-/-}$ than in the BDL control mice (Fig. B-5f). PP2alpha levels were similar to the previous experiments unchanged (Fig. B-5f).

## B.4 DISCUSSION

We have recently shown, that mice modeling the genetic disease hereditary tyrosinemia develop resistance against cell death during chronic liver injury (Vogel et al., 2004), an adaptation, which does not occur in mice which are doubly mutant in the gene responsible for HT1, *Fah,* and the cell cycle regulatory protein *p21*/*Cip*/*Waf* (Willenbring et al, manuscript submitted). In the current study, we therefore asked whether apoptosis resistance could also be a feature of other, more common liver disorders. Jaundice and obstruction of bile flow are found in many hepatic diseases; therefore an animal model of chronic cholestasis was used to address this question. Here, we show that BDL mice exhibit a profound resistance against apoptosis. Our observations are not contradictory to previous reports showing that bile acids acutely induced hepatocyte cell death. In HT1, several studies have similarly shown that HGA acutely induces apoptosis in hepatocytes of mice which are doubly mutant in *Hpd* and $Fah^{-/-}$ (Jorquera and Tanguay, 1999; Kubo et al., 1998). In both Fah deficiency and chronic cholestasis, already injured hepatocytes developed resistance to additional cell death triggers. We found in both models that the apoptotic block was primarily related to an inhibition of the mitochondrial amplification loop of Fas-induced apoptosis. We therefore wondered which factors contribute to the observed resistance against Fas-induced apoptosis.

Analysis of pro- and antiapoptotic proteins identified several changes to Bcl-2 like proteins. These alterations included not only quantitative changes, but also posttranslational modifications such as phosphorylation and subcellular localization. First, the anti-apoptotic protein Bfl-1/A1 was transcriptionally unregulated in BDL mice

in comparison to controls. However, we did not observe a significant difference between apoptosis-sensitive *Fah*$^{-/-}$/*p21*$^{-/-}$ and apoptosis-resistant *Fah*$^{-/-}$ mice. Additionally, previous studies have shown that Bfl-1/A1 did not prevent processing of Bid or tBid translocation to the mitochondria, but prevented subsequent collaboration between tBid and Bax/ Bak in the mitochondrial membrane (Werner et al., 2002). It is therefore unlikely that the mild elevation of Bfl-1/A1 can completely explain the lack of Bid cleavage seen in all apoptosis resistant models. Furthermore, we did not observe any additional consistent changes of pro- and antiapoptotic Bcl-2 like proteins in BDL, *Fah*$^{-/-}$ and *Fah*$^{-/-}$/*p21*$^{-/-}$ mice indicating that the resistance against Fas-induced apoptosis is not mediated by transcriptional regulation of these proteins. Another change was the increase of Bax and Bid in the mitochondrial fraction of BDL mice even before injection of Fas. Translocation of Bax to the mitochondria is generally associated with cytochrome C release and/ or mitochondrial dysfunction and apoptosis. For its pro-apoptotic functions, Bax needs to be activated and inserted into the mitochondrial membrane similar to Bid to induce release of cytochrome C. In BDL mice, however, both proteins were only loosely attached to the membrane. To clarify the potential role of the high levels of mitochrondrially located Bax, in vitro cytochrome C release assays were performed. Mitochondrial cytochrome C release was completely normal in BDL mice and therefore these changes are also unlikely to be the main cause of hepatocyte survival.

Instead, failure of Bid cleavage 8 was observed consistently in both models of hepatic cell death resistance, BDL and Fah deficiency. Previously, it has been shown that phosphorylation of Bid regulates its cleavage by caspase-8 (Degli Esposti et al., 2003a;

Desagher et al., 2001). In type II cells, the mitochondrial amplification loop necessary for Fas induced apoptosis is triggered through cleavage of Bid at Asp 59 by caspase-8. In the case of murine Bid, CK1 epsilon phosphorylates Bid in vitro on Ser 61 located exactly next to the cleavage site of caspase-8. Phosphorylation of Bid by CK1 renders Bid resistant to cleavage by caspase-8. Moreover, inhibition of CK1 accelerated Fas induced apoptosis, whereas hyperactivity of the kinase inhibited apoptosis [46]. More recent data additionally indicate that phosphorylation of Bid does not only prevent cleavage by caspase-8, but also the translocation of Bid to the mitochondria (Simmen et al., 2005). The mechanisms underlying the trafficking of Bid to the mitochondria are poorly defined at this time; the current model suggests that cleavage and myristoylation of Bid induces a molecular switch targeting tBid to the mitochondria (Gross et al., 1999b; Zha et al., 2000). However, a recent study suggests that the sorting protein PACS-2 is required for the apoptosis-induced targeting of Bid. The PACS-2 dependent translocation of Bid to the mitochondria appears to occur prior to caspase cleavage and requires the dephosphorylation of Bid (Simmen et al., 2005). Phosphorylated Bid (S61) was readily detectable in WT, BDL, *Fah*$^{-/-}$ and *Fah*$^{-/-}$/*p21*$^{-/-}$ mice. The constitutive phosphorylation of Bid in hepatocytes implies that it must be dephosphorylated during Fas induced apoptosis to transduce and amplify the death signal. Here however, Bid was not dephosphorylated following Fas mAb injection and CK1 activity remained higher in apoptosis resistant BDL mice. This phosphorylation/dephosphorylation pattern of Bid was not only specific for bile duct ligation, but was also seen in the adaptive response in the cancer prone metabolic liver disease HT1. Bid was only dephosphorylated in the apoptosis sensitive *Fah*$^{-/-}$/*p21*$^{-/-}$ double knockout mice following induction of apoptosis, but not in the

resistant *Fah*$^{-/-}$ mice. Finally, the effect of suramin was analyzed, which has been shown to inhibit Fas-induced hepatocellular apoptosis [52]. Interestingly, Suramin does not alter the composition of the DISC in the liver following challenge with APO-1 antibody but prevents release of cytochrome C. Additionally, the anti-apoptotic effect of suramin are specific for type II cell suggesting that a role for the mitochondrial pathway. Here, we show that Bid is not dephosphorylated and subsequently not cleaved in suramin treated mice following $Jo_2$ injection.

Interestingly, lack of dephosphorylation of Bid following Fas mAb injection correlates with increased cytosolic levels of CK1 epsilon in all models analyzed in this study. However, at this point we cannot exclude that additional kinases and/or phosphatases also contribute to the increased Bid phosphorylation level in vivo. Atm phosphorylates Bid in vitro, but was not necessary to sustain phosphorylation levels of Bid in BDL mice following induction of apoptosis with Fas mAb implicating CK 1 epsilon as the candidate kinase. Accumulating data suggest that CK1 is involved in negative regulation of apoptosis. Phosphorylation of different proteins by CK1 prevents their cleavage by caspase (Choi et al., 2003; Desagher et al., 2001). Additionally, CK1 acts as a molecular switch for certain proteins such as Galectin-3 (Takenaka et al., 2004). Phosphorylation of Galectin-3 regulates its cellular translocation from the nucleus to the cytoplasm and, as a result, its anti-apoptotic function. Finally, CK1 phosphorylates several membrane receptors and it has been shown that the p75 TNF receptor is phosphorylated and associated with CK-1, which negatively regulates p75-mediated TNF signaling to apoptosis (Beyaert et al., 1995). The data shown here establish a strong correlation

between the status of Bid-phosphorylation and hepatic apoptosis resistance and suggest a coherent model explaining this phenomenon in distinct models. However, future experiments using phosphorylation resistant Bid-mutants and mutant mimicking constitutive phosphorylation will be needed to determine conclusively whether this is the central mechanism.

In summary, our data show that resistance against apoptosis is a common feature of chronic liver diseases such as HT-1 and chronic cholestasis. In terms of evolution, this can be viewed as an adaptive response, allowing survival during chronic liver injury. However, survival might come at a price, because hepatocytes can retain DNA damage and become malignantly transformed. Our data show that the Fas apoptosis-signaling pathway is blocked at the level of Bid cleavage. We did not observe consistent transcriptional changes to pro- and anti-apoptotic genes including BH3-like genes and inhibitors of apoptosis and propose therefore a model in which phosphorylation/dephosphorylation of Bid determines the apoptotic threshold of hepatocytes during chronic liver diseases. Importantly, $Bid^{-/-}$ mice are not only resistant against Fas mAb-induced hepatocellular apoptosis, but also spontaneously develop a myeloproliferative disorder proving that loss function of a single BH3-only molecule can predispose to cancer (Zinkel et al., 2003). Therefore, understanding the changes in apoptosis sensitivity during chronic diseases might help to reduce the long-term risk of cancer development.

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