A Novel Reciprocal Regulatory Circuit Between Caspase-8 and c-Src

By
Jennifer Lai-Yee Tsang
MD, FRCPC

A thesis submitted in conformity with the requirements

for the degree of Doctor of Philosophy

Graduate Department of the Institute of Medical Science

University of Toronto

© Copyright by Jennifer Lai-Yee Tsang, 2014

TITLE: A NOVEL RECIPROCAL REGULATORY CIRCUIT BETWEEN CASPASE-8 AND C-SRC

DEGREE AND ANTICIPATED YEAR OF CONVOCATION: DOCTOR OF PHILOSOPHY, 2014

NAME: JENNIFER LAI-YEE TSANG

GRADUATE DEPARTMENT AND UNIVERSITY: INSTITUTE OF MEDICAL SCIENCE, UNIVERSITY OF TORONTO

ABSTRACT

Apoptosis and cell survival are two seemingly opposing fate-determining processes that are regulated by distinct and complex signaling pathways. Caspase-8, an apical caspase, plays a pivotal regulatory role in initiating apoptosis. c-Src, a prototypical member of the Src family kinases (SFKs), regulates a myriad of cellular processes including cell mitogenesis, proliferation, growth and migration. Although the regulation of caspase-8 by c-Src has been suggested, the reciprocal regulation of these two seemingly opposing signaling molecules, caspase-8 and c-Src, has never been explored. To study this reciprocal regulation, we asked three questions. (1) Can active caspase-8 negatively regulate c-Src activity to allow the propagation of apoptosis? (2) Can c-Src negatively regulate caspase-8 activity to prevent the propagation of apoptosis? (3) Can caspase-8, when its enzymatic activity is inhibited, further promote c-Src activity to allow the propagation of cell survival? To address these questions, we first investigated the effect of active caspase-8 on the activation and activity of c-Src. We discovered that active caspase-8 inhibited c-Src activation and some of its downstream effectors. Next, we investigated whether c-Src could tyrosine phosphorylate caspase-8. We discovered that c-Src could phosphorylate caspase-8 at multiple tyrosine sites. We then examined whether tyrosine phosphorylated caspase-8 prevents apoptosis. We found that phosphorylation of caspase-8 at Y465 prevented its cleavage, and activity towards

activating caspase-3 and towards causing cell morphological changes associated with apoptosis. Finally, we studied whether tyrosine phosphorylation of caspase-8 could further promote the activation of c-Src. We showed that phosphorylation of caspase-8 at both Y465 and Y397 resulted in the activation of c-Src and extracellular signal-regulated kinase 1/2 (Erk1/2). In conclusion, this work demonstrated the reciprocal regulation of two opposing signaling molecules, caspase-8 and c-Src. These results also suggest an elegant mechanism for a cell to commit efficiently and rapidly to a fate-determining process, either apoptosis or survival, by further suppression of the opposing signaling pathway.

ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr. John Marshall, for giving me this wonderful opportunity to work in his lab as a graduate student. I am thankful for his unending moral support even in times of desperation. He stood by me and continued to encourage me to pursue my dream. More importantly, he gave me complete freedom in choosing my project, in formulating my own scientific questions and in performing experiments that I designed. He allowed me to develop my research skills and he molded me into an independent scientist. I am also grateful for his vision in science. He has taught me to think outside the box and to ask scientific questions that are at a higher philosophical level.

I would like to express my sincere gratitude towards my co-supervisor, Dr. Andras Kapus, for adopting me in his lab family. This thesis would not have been possible without him. His wisdom in science is second to none. He was able to pick out interesting and significant results when I failed to even notice them. He pulled me through the toughest time of my PhD career by providing scientific guidance and psychotherapy. He gave me confidence when I felt I failed miserably in my graduate studies. He played a pivotal role in the inception till the completion of this project. He spent endless hours discussing science with me, pushing me to think critically, to question and to formulate explanations for every bit of my scientific results. I cherished every minute of our discussions because the best thing for a graduate student is to spend time with her supervisor trying to extract as much wisdom as possible from him. I will miss our scientific discussions terribly when I leave the lab. I will always be grateful and thankful for his endless scientific and psychological support.

I could never forget the unconditional support that Jean Parodo gave me. She is the technician and practically my lab mom, who took care of me throughout my PhD career in the lab. She welcomed me to the Marshall lab with open arms. She taught me neutrophil isolation, flow cytometry, Western blot and many other techniques. She took care of all the mundane tasks in the lab, including purchasing, making buffers and Western gel, taking care of cell culture, isolating neutrophils, etc, to allow me to focus on my experiments. I remember the time when she would literally chase down sales representatives to make sure I got my antibodies in time for my Western blots. She even made purchase orders when she was on vacation! More importantly, she gave me endless words of encouragement throughout the last five years. There was never an occasion when she would say no to my requests. She is someone I could truly depend on in the lab.

Dr. Songhui Jia is the research associate in the lab. He is a guru in cloning genes. I would like to extend my sincere appreciation for all the genes that he cloned for my PhD project. I would also like to thank him for all the words of encouragement that he gave me.

I would like to extend my sincere gratitude towards Dr. Monika Lodyga, Dr. Kapus' former post-doc. She has been fundamental in my survival in the lab. Her knowledge in molecular biology, especially in Src kinase biology, was instrumental in the design of my project. She provided me with the constitutively active c-Src plasmid, which had revolutionized my PhD project. She provided endless scientific and psychological support. Those late night dinners together after a long day of lab work

washed away the frustration and fatigue from tedious and fruitless experiments. I would not have survived the wrath of PhD studies without Monika.

Dr. Caterina Di Ciano-Oliveira, a research associate in Dr. Rotstein's lab, is one of the smartest, funniest and most efficient persons I have worked with in a lab. Her laughter is infectious! She has painstakingly reviewed and revised my CIHR fellowship application and my PhD thesis. She has also helped me with the 3D protein structures. More importantly, she has also provided a great deal of scientific advices. Although sometimes, she ran away from me when I tried to show her my Western blot results. I would like to thank Caterina for being a great friend and a science mentor.

I would like to thank Dr. Emmanuel Charbonney, a former graduate student of Dr. Kapus, for all the patient samples he had provided me. I would also like to extend my appreciation for all the collaborative opportunities he had given me. His words of encouragement throughout my PhD helped me through my toughest time.

I would also like to thank Dr. Kati Szaszi for giving me endless moral support throughout the last five years, especially when I thought I would never finish my PhD. She also gave me many scientific advices and she was always there for me when I needed someone to talk to.

I would like to thank Elaine Caon, Dr. Marshall's administrative assistant, for helping me with grant application, manuscript preparation, PAC meeting arrangement and many other things. She has always made herself available whenever I needed her.

I would like to thank all my PAC members (Drs. Haibo Zhang, Conrad Liles, James Booth and Mingyao Liu) and the PIs on the sixth floor (Drs. Jane Batt, Wolfgang Kuebler, Warren Lee, Ori Rotstein and Claudia dos Santos) for all their scientific advices.

I would like to thank the lab gangs including Dr. Zeenat Malam, Dr. Yasamen Amoozadeh, Dr. Matthew Zuly, Matthew Rozycki, Faiza Waheed, Pam Speight, Julie Kang, Sandra Naccarato, Dr. Matteo Parotto, Dr. Peter Speith, Dr. Vito Fanelli, Alice Luo, Dr. Jack Haisma, Dr. Tatiana Maron, Judy Correa, Dr. Yuexin Shan, Dr. Pamela Plant, Chris Spring, Xiao Feng Lu for all the fun, laughter and scientific support they gave me. Moreover, I would like to thank the many healthy volunteers in the lab who had donated their precious blood for my studies.

There are also many people outside of the lab who had played a pivotal role during my PhD years and more. They are my friends and mentors both in the scientific and clinical world, including Drs. Robert Fowler, Margaret Herridge, Neill Adhikari and Sharmistha Mishra. I would like to thank them for all the support they gave me, including editorial, moral, scientific, and clinical support.

Last but not least, I would like to thank my family, especially Blanche, Johnny, Jessica, Vincent and Rica for being there for nutritional and moral support.

DATA ATTRIBUTION

I would like to acknowledge Dr. Songhui Jia for his contribution to this work, as he was responsible for the cloning of caspase-8 and the generation of various caspase-8 mutants.

I would like to acknowledge Dr. Flynn from West Virginia for providing me with the constitutively active c-Src construct.

TABLE OF CONTENTS

ABSTRACT ii
ACKNOWLEDGMENTS iv
DATA ATTRIBUTION viii
TABLE OF CONTENTS ix
LIST OF FIGURES xii
LIST OF ABBREVIATIONS xiv
OVERALL PHD WORK xvi
PUBLICATION LIST xviii
Published Refereed Papers xviiiii
Published Book Chapters xviiiii
Published Abstracts xix
Book Chapter in Press xix
Paper Under Editorial Review xx
Chapter 1. INTRODUCTION 1
1.1 CASPASES 1
1.2 CASPASE-8 4
1.2.1 Caspase-8 Isoforms 4
1.2.2 Cleavage of Caspase-8 8
1.2.3 Three-Dimensional Structure of Caspase-8 12
1.2.4 Substrate Recognition and Specificities 17
1.2.5 Activation by Induced Proximity Model 17
1.2.6 Localization of Caspase-8 19
1.3 INHIBITORS OF CASPASE-8 20
1.3.1 Viral-Encoded Caspase-8 Inhibitors 20
1.3.2 Mammalian Caspase-8 Inhibitors 22
1.4 CASPASE-8 KNOCKOUT 24
1.5 CELLULAR FUNCTIONS OF CASPASE-8 25
1.5.1 Extrinsic (Death Receptor-Induced) Apoptosis 28
1.5.2 Intrinsic Apoptosis 28
1.5.3 Integrin-Mediated Death (IMD) 29
1.5.4 Necroptosis 29
1.5.5 Autophagy 30
1.5.6 Inflammation 30
1.5.7 Immune Cells Homeostasis, Proliferation, Differentiation and Activation 31
1.5.8 Cell Adhesion, Migration and Motility 33
1.5.9 Endosome Trafficking 34
1.5.10 Cell Signaling – NF-κB Activation 34
1.5.11 Cell Signaling – Erk1/2 Activation 36
1.5.12 Cell Signaling – Src Kinase 36
1.5.13 Tumorigenesis 37

1.5.14 Gene Transcription ... 38
1.6 POSTTRANSLATIONAL MODIFICATIONS OF CASPASE-8 ... 39
1.6.1 Ubiquitination of Caspase-8 ... 39
1.6.2 Cleavage of Caspase-8 and its Functions ... 39
1.6.3 Phosphorylation of Caspase-8 ... 40
1.7 APOPTOSIS ... 42
1.7.1 Introduction ... 42
1.7.2 Intrinsic Apoptosis ... 45
1.7.3 Extrinsic Apoptosis ... 46
1.7.4 The Consequences of Effector Caspases Activation ... 49
1.8 SRC FAMILY PROTEIN TYROSINE KINASE ... 50
1.8.1 Overview ... 50
1.8.2 Structure of Src Family Protein Tyrosine Kinase ... 52
1.9 ACTIVATION OF C-SRC ... 55
1.10 REGULATION OF C-SRC ... 58
1.10.1 Intramolecular Regulation of c-Src ... 58
1.10.2 Receptor-Mediated Activation of c-Src ... 59
1.10.3 Other Mechanisms of c-Src Regulation ... 60
1.11 CELLULAR FUNCTIONS OF C-SRC ... 61
1.11.1 Regulation of Mitogenesis and Cell Growth ... 61
1.11.2 Regulation of Cell Survival and Apoptosis ... 63
1.11.3 Regulation of Cell Adhesion, Motility and Invasion ... 64
1.11.4 The Role of c-Src in Tumorigenesis and Tumor Progression ... 66
1.12 INTERACTION BETWEEN CASPASE-8 AND SFKS ... 68
1.13 BCL-2 FAMILY OF PROTEINS ... 69
1.13.1 Three Families of Mammalian Bcl-2 Proteins and Their Domain Structures ... 69
1.13.2 The Regulation of Apoptosis and Survival by Bcl-2 Family Proteins ... 71
1.14 SUMMARY ... 74

Chapter 2. RATIONALE, AIMS AND FUNDAMENTAL RESEARCH QUESTIONS ... 75
2.1 RATIONALE ... 75
2.2 AIMS ... 75
2.3 FUNDAMENTAL RESEARCH QUESTIONS ... 76

Chapter 3. MATERIALS AND METHODS ... 78
3.1 Cell Lines ... 78
3.2 Antibodies, Inhibitors and Dyes ... 78
3.3 DNA Construct ... 79
3.4 Plasmid Construction ... 79
3.5 Transfection of Cell Line ... 81
3.6 Protein Studies ... 81
3.7 Mass Spectrometry ... 82
3.8 Microscopy and Immunofluorescence Analysis ... 82
3.9 Statistical Analysis ... 83

Chapter 4. THE RECIPROCAL NEGATIVE REGULATION OF CASPASE-8 AND C-SRC ... 84
4.1 SUMMARY ... 84
4.2 INTRODUCTION ... 85
4.3 RESULTS ... 87

4.3.1 Caspase-8 Enzymatic Activity Regulates the Capacity of c-Src to Tyrosine Phosphorylate Caspase-8 87
4.3.2 Caspase-8's Tyrosine Phosphorylation Sites by Src Kinases 91
4.3.3 Caspase-8 Inhibits c-Src Kinase Activity and its Specific Downstream Signaling Pathway 95
4.3.4 The Inhibition of c-Src by Caspase-8 is Caspase-8 Enzymatic Activity Dependent 101
4.4 DISCUSSION 111

Chapter 5. MULTIPLE TYROSINE PHOSPHORYLATION SITES SWITCH CASPASE-8 FROM AN APOPTOTIC TO A PRO-SURVIVAL MOLECULE 117
5.1 SUMMARY 117
5.2 INTRODUCTION 118
5.3 RESULTS 120
5.3.1 Generation of Phosphomimetic and Non-Phosphorylatable Mutants 120
5.3.2 Phosphomimetic Modification at Y465 but not Y397 of Caspase-8a Prevents its Cleavage 121
5.3.3 Phosphomimetic Modification of Y465 but not Y397 of Caspase-8 Abolishes its Capacity to Activate Caspase-3 and to Induce Apoptosis 128
5.3.4 Phosphomimetic Modification at Y465 of Caspase-8 Enhances Activation of c-Src 133
5.3.5 Phosphomimetic Modification at Y465 of Caspase-8 Enhances the Activation of Erk1/2 but not Akt 142
5.3.6 The Activation of c-Src by Y465E Phosphomimetic Caspase-8 Mutant is Dependent of Phosphorylation of Y397 147
5.4 DISCUSSION 153

Chapter 6. OVERALL DISCUSSION AND FUTURE DIRECTION 159
6.1 Summary of Major Findings 159
6.2 What is the Mechanism of Inhibition of Phosphorylation of c-Src at Y416 by Active Caspase-8? 162
6.2.1 c-Src Cleavage by Caspase-8 or its Downstream Effectors 162
6.2.2 Active Caspase-8-Induced Downregulation of a c-Src Adaptor Molecule Resulting in Inhibition of c-Src Phosphorylation at Y416 163
6.2.3 Active Caspase-8 Activates A Protein Tyrosine Phosphatase Resulting in Dephosphorylation of c-Src at Y416 164
6.3 Can Tyrosine 465 of Caspase-8 be Phosphorylated by c-Src? 167
6.4 What Could be the Mechanism of Phosphorylation of Tyrosine 465 of Caspase-8 in Preventing its Cleavage? 168
6.5 What is the Mechanism whereby Phosphorylation of Tyrosine 397 of Caspase-8 Promote c-Src Activation? 170
6.6 What are the Implications of our Findings on Cancer Biology? 175
6.7 Overview and Concluding Remarks 177

REFERENCES 178

LIST OF FIGURES

Figure 1. Structure of Caspases 3
Figure 2. Caspase-8 Isoforms 6
Figure 3. Protein Sequence of Caspase-8a and Caspase-8b Isoforms 7
Figure 4. Cleavage of Caspase-8 9
Figure 5. Three-Dimensional Structure of Caspase-8 Dimer 13
Figure 6. Three-Dimensional Structure of Procaspase-8 and Active Caspase-8 16
Figure 7. Caspase-8's Cellular Functions 26
Figure 8. Intrinsic and Extrinsic Apoptotic Pathway 43
Figure 9. Stress (Intrinsic) and Death-Receptor (Extrinsic) Apoptotic Pathways 47
Figure 10A. The Secondary Structure of c-Src 54
Figure 10B. The Activation of c-Src 56
Figure 11. Bcl-2-Related Proteins and Their Structural Domains 70
Figure 12. The Regulation of Cell Survival and Apoptosis by Bcl-2 Family Proteins 73
Figure 13. Caspase-8 Enzymatic Activity Regulates the Capacity of c-Src to Tyrosine Phosphorylate Caspase-8 89
Figure 14. c-Src Tyrosine Phosphorylates Caspase-8 at Multiple Sites 93
Figure 15. Caspase-8 Inhibits c-Src Activation and its Activity 97
Figure 16. Caspase-8 Inhibits Erk1/2 Activation but not Akt Activation 99
Figure 17. Inactive Caspase-8 (C377S) Results in Enhanced c-Src Activation and Activity 102
Figure 18. Inactive Caspase-8 (C377S) Results in Enhanced Erk1/2 Activation but not Akt Activation 104
Figure 19. Caspase-8 Enzyme Inhibition Results in Enhanced c-Src Activation and Activity 107
Figure 20. Caspase-8 Enzyme Inhibition Results in Enhanced Erk1/2 Activation but not Akt Activation 109
Figure 21. The Balance of Death and Survival 115
Figure 22. Generation of Caspase-8 Mutants 120
Figure 23. Wild Type, Y397F, Y397E, Y465F Caspase-8 Mutants were Cleaved after D233 and D391 whereas C377S and Y465E Caspase-8 Mutants were only Cleaved after D233 123
Figure 24. C377S and Y465E Caspase-8 Fail to Activate Caspase-3 and Fail to Induce Cell Morphological Changes 130
Figure 25. C377S and Y465E Caspase-8 Mutants Augment whereas WT, Y397F, Y397E and Y465F Suppresses the Activation and Activity of c-Src 136
Figure 26. C377S and Y465E Caspase-8 Mutants Augment the Activation of Erk1/2 but not Akt 143
Figure 27. The Pro-Survival Function of Y465E Caspase-8 Mutant is Dependent on the Phosphomimetic Modification at Y397 149
Figure 28. Phosphorylation of Y397 and Y465 of Caspase -8 Converts it into a Pro-Survival Protein 156
Figure 29. Apoptosis and Survival Signaling Involve Reciprocal Regulatory Mechanisms 160

Figure 30. Potential Mechanisms of Active Caspase-8 Inhibition of c-Src Phosphorylation at Y416 165
Figure 31. Three-Dimensional Structure of Caspase-8 Dimer 169
Figure 32. Phosphomimetic and Non-Phosphorylatable Caspase-8 Mutants and Their Ability to Promote c-Src Autophosphorylation at Y416.......... 172
Figure 33. Three-Dimensional Structure of the Caspase-8 Monomer with the Linker Region.......... 173
Figure 34. Caspase-8 as a Substrate and Activator of c-Src.......... 174
Figure 35. Cell Permeable Peptide as a Potential Therapeutic Option in Colon Cancer 176

LIST OF TABLES

Table 1. Viral-Encoded Caspase-8 Inhibitors 21
Table 2. Src Family Kinase Members and Their Pattern of Expression.......... 51
Table 3. Antibodies, Inhibitors and Dyes 78
Table 4. List of Primers Used for Point Mutations of GFP-Caspase-8 Constructs. 80
Table 5. Predicted Capsaes-8 Tyrosine Phosphorylated Sites.......... 91
Table 6. Major Functional Characteristics and Effects of Various Caspase-8 Mutants 161

LIST OF ABBREVIATIONS

AFAP	Actin filament-associated protein
AIF	Apoptosis-inducing factor
APAF-1	Apoptosis protease activating factor-1
ATG7	Autophagy-related protein 7
ATP	Adenosine triphosphate
Bcl	B cell lymphoma protein
BH	Bcl2-Homology
Bid	BH3-ineracting domain death agonist
CAD	Caspase-activated DNase
CAMKLK	$Ca^{2+/}$calmodulin-dependent protein kinase-like kinase
CARD	Caspase-associated recruitment domain
CARMA1	Caspase-recruitment domain membrane-associated guanylate kinase protein 1
CARP	Caspase-8 and -10 associated RING protein
CAS	Crk-associated substrate
CBM	CARMA1-Bcl10-MALT1
CDK	Cyclin dependent kinase
CED	Cell death abnormal gene
cIAP	Cellular inhibitor of apoptosis protein
CMV	Cytomegalovirus
CPN2	Calpain 2
CrmA	Cowpox virus cytokine response modifier A
CSF-1R	Colony-stimulating factor 1 receptor
Csk	Carboxy-terminal Src kinase
CUL3	Cullin3
DD	Death domain
DED	Death effector domain
DISC	Death-inducing signaling complex
DR	Death receptor
ECF	Extracellular matrix
EGF	Epidermal growth factor
Erk1/2	Extracellular signal regulated kinase 1/2
FADD	Fas-associated protein with death domain
FAK	Focal adhesion kinase
FGFR	Fibroblast growth factor receptor
FLICE	FADD-homologous ICE/CED-3-like protease
FLIP	FLICE-inhibitory protein
GAP	Guanosine triphosphatase activating protein
G-CSF	Granulocyte-colony stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte/monocyte-colony stimulating factor
GTP	Guanosine triphosphate
HGFR	Hepatic growth factor receptor
HPV	Human papillomavirus

IAP	Inhibitor of apoptosis protein
ICE	Interleukin-1β converting enzyme
IKK	IκB kinase
IL-1β	Interleukin-1β
IMD	Integrin-mediated death
LMWPTP	Low molecular weight protein tyrosine phosphatase
M-CSF	Macrophage-colony stimulating factor
MALT1	Mucosa-associated lymphoid tissue translocation protein 1
MAPK	Mitogen activated protein kinase
MEK	Mitogen activated protein kinase kinase
MLCK	Myosin light chain kinase
MOMP	Mitochondrial outer membrane permeabilization
NF-κB	Nuclear factor κB
NLRP3	NOD-like receptor family, pyrin domain containing 3
NMR	Nuclear magnetic resonance
NOD	Nucleotide-binding oligomerization domain
PAK2	p21-activated kinase 2
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PS	Phosphatidylserine
PTP	Protein tyrosine phosphatase
RING	Really interesting new gene
RIP	Receptor interacting protein
ROCK-I	Rho-associated coiled-coil forming kinase I
RRAS	Ras-related protein
RSK2	Ribosomal S6 kinase 2
RPTPa	Receptor protein tyrosine phosphatase α
SFK	Src Family Kinase
SH	Src homology
SHP	SH2-containing phosphatase
SMAC	Second mitochondria-derived activator of caspases
STAT	Signal transducer and activator of transcripiton
TAK1	Transforming growth factor β activated kinase-1
TCR	T cell receptor
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF	TNFR-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRANCE	TNF-related activation induced cytokine
vICA	Viral inhibitor of caspase-8 activation
XIAP	X-linked inhibitor of apoptosis protein

OVERALL PHD WORK

My PhD work focuses on the molecular mechanisms of organ injury in critical illnesses including acute respiratory distress syndrome (ARDS) and sepsis. In particular, I am interested in the mechanism of prolonged neutropil survival. In the first part of my PhD work, I studied the regulation of apoptosis and priming of neutrophil oxidative burst by serine protease inhibitor, diisopropylfluorophosphate. This work has resulted in a first author abstract in *Intensive Care Medicine* and a first author peer-reviewed paper in the *Journal of Inflammation.* I am also a co-author on a peer-reviewed paper (provisionally accepted by *American Journal of Pathology*) titled “Activated neutrophils induce epithelial cell apoptosis through oxidant-dependent tyrosine dephosphorylation of Caspase-8”.

My scholarly work during my PhD also includes one published first author book chapter on the role of neutrophil in inflammation in *Critical Care Nephrology* and another first author book chapter on the treatment of sepsis in the *Encyclopedia of Trauma Care* that is in press.

Moreover, I am also involved in a single centre study focusing on endotoxemia following polytrauma and its correlation with the presence of shock at admission and the subsequent development of organ failure. This work has resulted in a co-author abstract in *Intensive Care Medicine* and we are in the process of preparing a manuscript for submission. I am also a site principal investigator for a multi-centre CIHR-funded translational biology study looking at the correlation of serum free DNA and the mortality of septic shock.

My other scholarly work includes a co-author review paper on the biomarkers in ARDS in the *Journal of Critical Care,* a co-author editorial paper in *Intensive Care Medicine* titled “What’s new: sepsis and the innate-like response”, and a co-author case report on acute refractory hypoxemia after chest trauma reversed by high-frequency oscillatory ventilation published in the *Journal of Medical Case Reports.* I also serve as an invited reviewer for *Critical Care Medicine* and *Journal of Critical Care.* I am also a co-principal investigator of a two-year research grant ($168 000) titled “Cellular and molecular mechanisms of prolonged neutrophil-mediated

inflammation in trauma and sepsis: the role of PBEF/Nampt/Visfatin" from Physicians' Service Incorporated Foundation. Since July 2013, I serve as a part-time clinical assistant professor in the Department of Medicine (Critical Care Medicine) in McMaster University.

The second part of my PhD focuses on the regulation of cell survival and apoptosis through a novel reciprocal regulatory circuit between caspase-8 and c-Src. This work was presented in the American Thoracic Society Annual Meeting in 2012 (abstract published in the *American Journal of Respiratory and Critical Care*) and in the European Society of Intensive Care Medicine Meeting in 2013 (abstract published in *Intensive Care Medicine*). In order to stay focus, I elected to only include this part of my PhD work in my thesis. As part of this thesis, I am in the process of submitting a review paper on the non-apoptotic role of caspase-8. As for the data section of my thesis, my supervisors and I decided to go ahead with defending my thesis before submitting this work because there are a couple additional experiments we would like to perform in order to aim for a higher impact journal.

In summary, during my PhD work, I have published a first author peer-review paper, three first author abstracts, two first author invited book chapters, one co-author peer-reviewed paper, one co-author case report, one co-author review paper, one co-author editorial paper and two co-author abstracts and hold a two-year research grant as a co-principal investigator.

PUBLICATION LIST

Published Refereed Papers

1. Charbonney E, **Tsang JLY**, Wassermann J, Adhikari NKJ. **Acute Refractory Hypoxemia after Chest Trauma Reversed by High-Frequency Oscillatory Ventilation: a Case Report.** *J of Medical Case Reports* 2013; 7:186
   Co-Author

2. **Tsang J**, Parodo J, Marshall J. **Regulation of Apoptosis and Priming of Neutrophil Oxidative Burst by Diisopropylfluorophosphate**. *J of Inflammation* 2010; 7:32-40.
   Principal Author

3. **Tsang JLY**, Mendelsohn A, Tan M et al. **Discordance between Physicians' Estimation of Patient Cardiovascular Risk and Use of Evidence-Based Medical Therapy.** *American Journal of Cardiology* 2008; 102:1142-1145
   Principal Author

4. Hackam DG, Leiter LA, Mendelsohn A, Tan M, **Tsang JL** et al. **Missed Opportunities for Secondary Prevention of Cardiovascular Disease in Canada.** *Canadian Journal of Cardiology* 2007; 23: 1124-30
   Co-Author

5. Yan A, Yan R, Tan M, Hackam D, Leblanc K, Kertland H, **Tsang J**, Jaffer S, Kates M, Leiter L, Fitchett D, Langer A, Goodman S. **Contemporary Management of Dyslipidemia in High-Risk Patients: Targets Still Not Met**. *Am J of Med* 2006; 119:676-83.
   Co-Author

6. Plint A, Perry J, **Tsang J**. **Pediatric Wrist Buckle Fractures: Should We Just Splint and Go?** *CJEM* 2004; 6:397-401.
   Co-Author

Published Book Chapters

1. **Tsang JLY**, Marshall JC. **The Neutrophil and Inflammation**. Chapter 164 in Ronco, Bellomo, Kellum (Eds) *Critical Care Nephrology 2nd Edition; Elsevier Press.* 2009; 777-783
   Principal Author

2. **Tsang J**, Ferguson N. **Liberation from Mechanical Ventilation in Acutely Brain-Injured Patients**. *Year Book of Intensive Care and Emergency Medicine*. 2006; 486-493
   Principal Author

Published Abstracts

1. Tsang JL, Jia S, Parodo J, Lodyga M, Charbonney E, Kapus A, Marshall JC. **Caspase-8 Tyrosine Phosphorylation is a Potential Mechanisms of Increased Neutrophil Survival in Septic Patients.** Intensive Care Medicine 2013;
   Principal Author

2. Duggal A, Ganapathy G, Ratnapalam M, Tsang J, Sinuff T, Burns KE, Meade MO, Adhikari NK. **A Systemic Review of Pharmacotherapies for Acute Repiratory Syndrome (ARDS).** Intensive Care Medicine 2012; 38: (Suppl 1): S165
   Co-Author

3. Tsang JL, Parodo J, Battisti-Charbonney, E, Jia SH, Kapus A, Marshall JC. **Caspase-8-Induced Activation of Lyn – A Potential Mechanism of Sustained PMN Activation Seen in Trauma and Septic Patients.** American Journal of Respiratory and Critical Care Medicine 2012; 185: A5763
   Principal Author

4. Charbonney E, Klein D, Tsang JLY, Romaschin A, Nathens A, Marshall JC. **Endotoxemia following Polytrauma Correlates with the Presence of Shock at Admission and with the Subsequent Development of Organ Failure.** *Intensive Care Medicine* 2011; 37:S112
   Co-Author

5. Tsang JLY, Parodo JC, Marshall JC. **Differential Regulation of Neutrophil Survival and Inflammatory Function by Endogenous Serine Protease Inhibitor**. *Intensive Care Medicine* 2008; 34:S119
   Principal Author

6. Tsang J, Mendelsohn A, Tan M, Hackam D, Leiter L, Fitchett D, Langer A, Goodman S. **The Use of Evidence-Based Cardioprotective Medications in High Vascular Risk Canadian Outpatients is Suboptimal**. *Circulation* 2006; 112:II-445-6
   Principal Author

Book Chapter in Press

1. Tsang JLY. **The Treatment of Sepsis.** *Encyclopedia of Trauma Care*
   Principal Author

Paper Under Editorial Review

1. Jia SH, Parodo J, Charbonney E, **Tsang JLY**, Jia SY, Rotstein O, Kapus A, Marshall J. **Activated Neutrophils Induce Epithelial Cell Apoptosis Through Oxidant-Dependent Tyrosine Dephosphorylation of Caspase-8**. *American Journal of Pathology*
   Co-Author

2. Binnie, A, **Tsang JLY**, Dos Santos, C. **Biomarkers in ARDS.** *Current Opinion in Critical Care*
   Co-Author

3. Josh Douglas, **Tsang JLY**, Walley, K. **What's New: Sepsis and the Innate-Like Response**. *Intensive Care Medicine*
   Co-Author

Chapter 1. INTRODUCTION

1.1 CASPASES

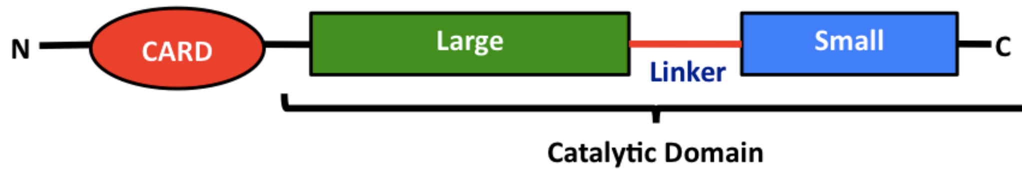
Caspases are a group of proteases known as **c**ysteine dependent **asp**artate-directed prote**ase**s that are homologs of the *Caenorhabditis elegans* "cell death abnormal" gene *CED-3*. They are peptidases that employ a cysteine residue as the catalytic nucleophile and cleave their substrates exclusively after aspartic acid residues (1-6). Caspases are signaling proteases that cleave protein by making a limited number of cuts. They do not destroy protein structure nor degrade protein (7). Interleukin-1β converting enzyme (ICE), also known as caspase-1, is the first identified mammalian homolog of CED-3 (8-10). To date, there are more than 10 known homologs of ICE/CED-3.

In humans, there are a total of 13 caspases, which are grouped into two main families based on their functions. They are involved in the regulation of inflammation and programmed cell death (apoptosis). The caspases that are involved in the regulation of inflammation are caspases-1, -4, -5, -11 and -12. Of these, caspases-1, -4, and -5 are proposed to induce cell death associated with massive activation of inflammatory cells, known as pyroptosis. The caspases that are involved in the regulation of apoptosis are caspases-2, -3, -6, -7, -8, -9 and -10. Caspase-14 has neither a clear inflammatory, nor apoptotic function. It is only expressed in keratinocytes and is involved in keratinocyte differentiation (11, 12). The apoptotic caspases are further divided into apical or initiator caspases (caspases-2, -8, -9 and -10) and effector or executioner caspases (caspases-3, -6 and -7). The structure of caspases is composed of two to three domains as depicted in Figure 1. Apical caspases have a large N-terminal dimerization domain (death effector

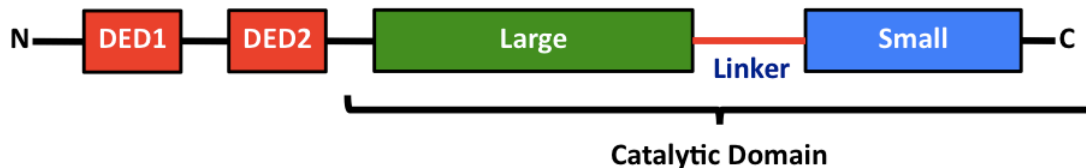
domain – DED for caspase-8 and -10; caspase activation and recruitment domain – CARD for caspases-2 and -9). Effector caspases have short N-terminal domain. Inflammatory caspases (caspases-1, -4, -5, -11, -12) also have a CARD domain (13) (Figure 1). The active (catalytic) domains of apical and effector caspases consist of a large and a small subunit (12).

Figure 1

Initiators (Caspases-2, -9); Inflammatory (Caspases-1, -4, -5, -11, -12)



Initiators (Caspases-8, -10)



Executioners (Caspases-3, -6, -7)

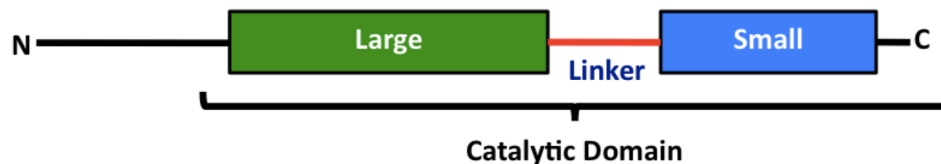


Figure 1. Structure of Caspases

Caspases are classified as inflammatory caspases (Caspase-1, -4, -5, -11, -12), apical or initiator caspases (Caspases-2, -8, -9, -10) and effector or executioner caspases (Caspase-3, -6, -7). Caspases-2, -9 (apical or initiator caspases) and all the inflammatory caspases have a caspase activation and recruitment domain (CARD) in the N-terminus followed by a large and small subunit forming the catalytic domain. Caspase-8 and -10 (apical or initiator caspases) have two tandem death effector domains (DED) in the N-terminus followed by a large and small subunit. Effector or executioner caspases have a short N-terminal fragment followed by the catalytic domain comprised of a large and a small subunit.

1.2 CASPASE-8

Caspase-8, an apical caspase in the extrinsic apoptotic pathway, is also known as FLICE, MACHα1 and MCH5 (14-16). It shares approximately 20% homology with the Ced-3 protein in the nematode *C. elegans* (17). The caspase-8 gene, located in chromosome 2 band q33q34 (16), is comprised of 10 exons. It was initially discovered as a protein that is associated with Fas/FADD complex using nano-electrospray tandem mass spectrometry (nano-ES MS/MS) (14) and it was cloned in the same year (16).

1.2.1 Caspase-8 Isoforms

Caspase-8 exists in multiple isoforms reflected by different mRNA transcript sizes (15). Six of the caspase-8 isoforms (**caspase-8a, 8b, 8d, 8f, 8g** and **-8h**) shared a common 182 amino acid N-terminal region (which encompasses the DED domains), but have different C-termini (15, 18) (Figure 2). The ninth caspase-8 isoform is known as **caspase-8L**, which is generated by the alternative splicing process of intron 8 of the human caspase-8 gene in peripheral blood lymphocytes (19), where a 136 bp insertion is introduced between exon 8 and exon 9 of the full-length caspase-8 mRNA (20). Since this alternative spliced product generates a premature stop codon, a truncated protein comprising only of the two N-terminal DED domains without the C-terminal protease domain is generated. It is proteolytically inactive and acts as a dominant negative form of caspase-8 (21). The tenth caspase-8 isoform, known as **caspase-8s** was found in a patient with acute leukemia and in bone marrow mononuclear cells. It only encodes the first DED and part of the second DED (22) (Figure 2). Only caspase-8a and -8b isoforms are ubiquitously expressed in many cell lines (18). Both of these isoforms possess two tandem DED domains as well as the catalytic subunits p18 and p12 (Figure 2).

Moreover, only caspase-8a and -8b isoforms are recruited to the Fas receptor in an activation-dependent manner (18). Caspase-8a contains an additional 2-kDa fragment that is located between the second DED and the large catalytic subunit resulting in different length of the caspases-8a and -8b, p55 and p53, respectively (23). With the alignment of caspase-8a and caspase-8b protein sequences, it is apparent that there is a 32-amino acid insertion in caspase-8a and a 15-amino acid insertion in caspase-8b, both in the N-terminal pro-domain, contributing to a total of 17-amino acid difference between caspase-8a and -8b, with caspase-8a being the longer one (Figure 3). Caspase-8a and -8b are ubiquitously expressed in cells. Therefore, they will be the focus of discussion in the remaining of this chapter.

Figure 2

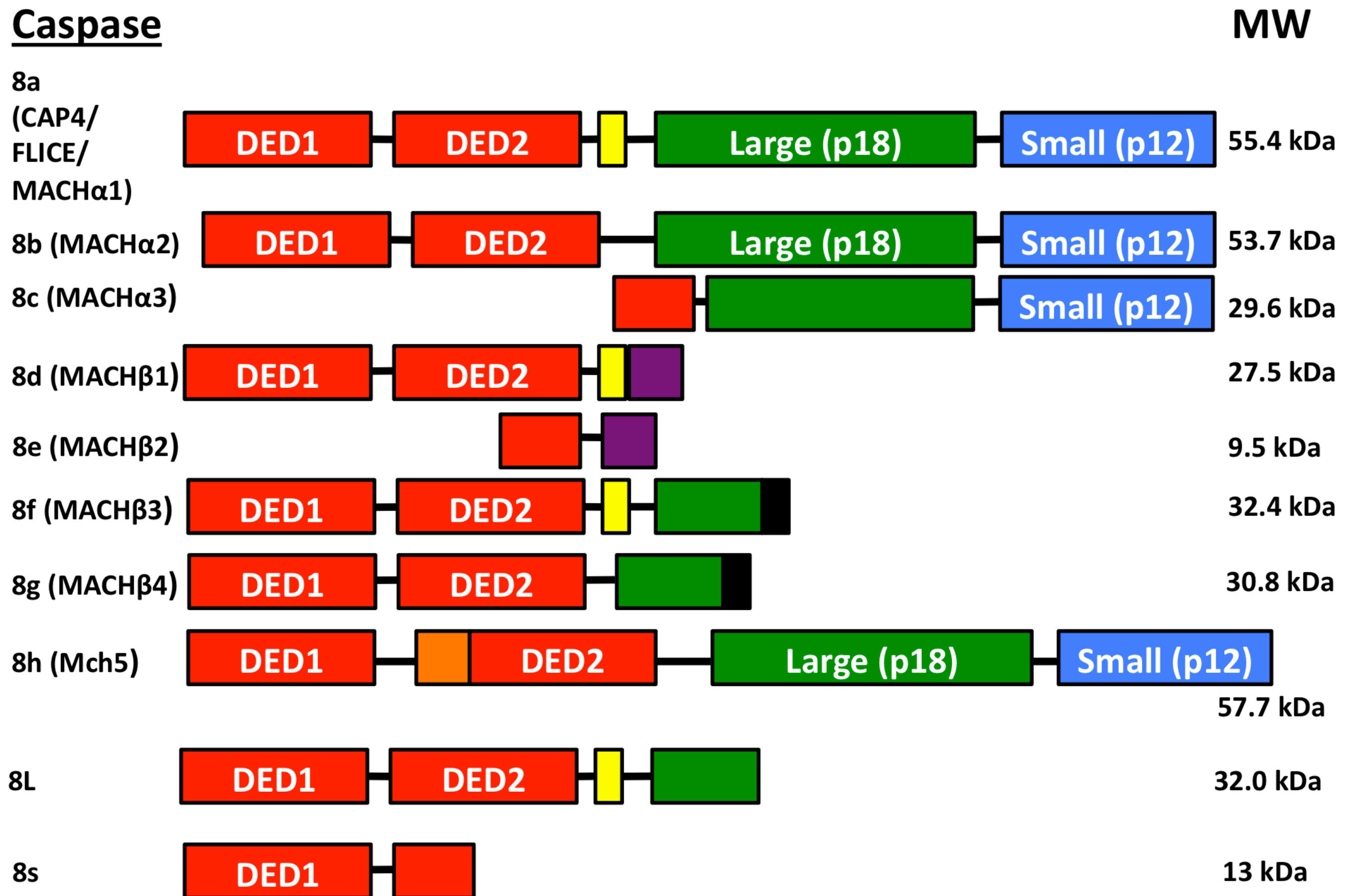


Figure 2. Caspase-8 Isoforms

Caspase-8 exists in 10 different isoforms: caspase-8a, -8b, -8c, -8d, -8e, -8f, -8g, -8h, -8L and -8s.

The domains of all isoforms and their respective molecular weights are illustrated.

Figure 3

```
1   MDFSRNLYDIGEQLDSEDLASLKFLSLDYIPQRKQEPIKDALMLFQRLQEKRMLEESNLS 60
1   MDFSRNLYDIGEQLDSEDLASLKFLSLDYIPQRKQEPIKDALMLFQRLQEKRMLEESNLS 60

61  FLKELLFRINRLDLLITYLNTRKEEMERELQTPGRAQISAYRFHFCRMSWAEANSQCQTQ 120
61  FLKELLFRINRLDLLITYLNTRKEEMERELQTPGRAQISAY------------------- 101

121 SVPFWRRVDHLLIRVMLYQISEEVSRSELRSFKFLLQEEISKCKLDDDMNLLDIFIEMEK 180
102 -------------RVMLYQISEEVSRSELRSFKFLLQEEISKCKLDDDMNLLDIFIEMEK 148

181 RVILGEGKLDILKRVCAQINKSLIKIINDYEEFSK---------------GEELCGVMTI 225
149 RVILGEGKLDILKRVCAQINKSLIKIINDYEEFSKERSSSLEGSPDEFSNGEELCGVMTI 208

226 SDSPREQDSESQTLDKVYQMKSKPRGYCLIINNHNFAKAREKVPKLHSIRDRNGTHLDAG 285
209 SDSPREQDSESQTLDKVYQMKSKPRGYCLIINNHNFAKAREKVPKLHSIRDRNGTHLDAG 268

286 ALTTTFEELHFEIKPHDDCTVEQIYEILKIYQLMDHSNMDCFICCILSHGDKGIIYGTDG 345
269 ALTTTFEELHFEIKPHDDCTVEQIYEILKIYQLMDHSNMDCFICCILSHGDKGIIYGTDG 328

346 QEAPIYELTSQFTGLKCPSLAGKPKVFFIQACQGDNYQKGIPVETDSEEQPYLEMDLSSP 405
329 QEAPIYELTSQFTGLKCPSLAGKPKVFFIQACQGDNYQKGIPVETDSEEQPYLEMDLSSP 388

406 QTRYIPDEADFLLGMATVNNCVSYRNPAEGTWYIQSLCQSLRERCPRGDDILTILTEVNY 465
389 QTRYIPDEADFLLGMATVNNCVSYRNPAEGTWYIQSLCQSLRERCPRGDDILTILTEVNY 448

467 EVSNKDDKKNMGKQMPQPTFTLRKKLVFPSD 496   Caspase-8a
466 EVSNKDDKKNMGKQMPQPTFTLRKKLVFPSD 479   Caspase-8b
```

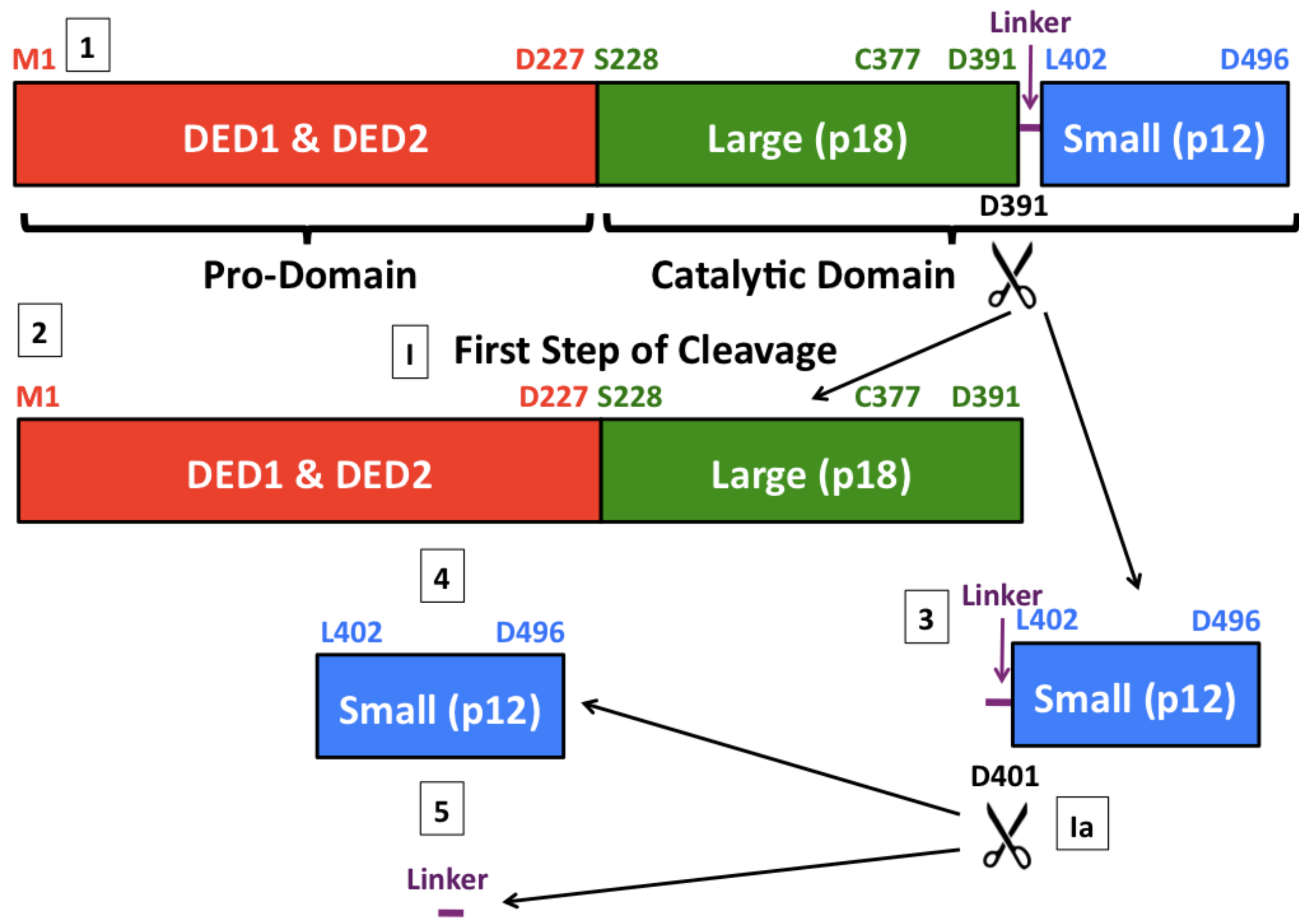
Figure 3. Protein Sequence of Caspase-8a and Caspase-8b Isoforms

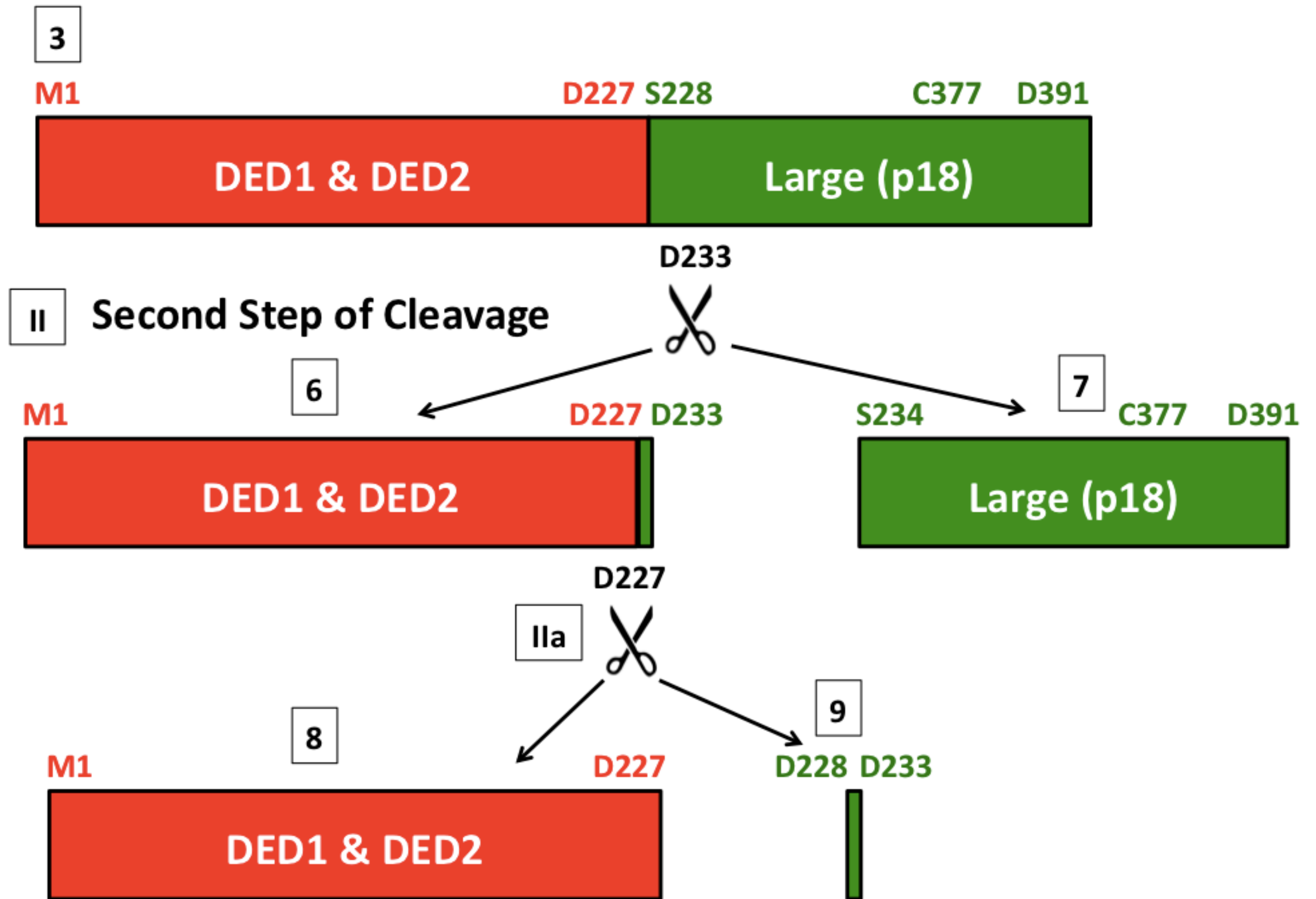
The alignment of protein sequence of caspase-8a and -8b isoforms is illustrated. The total number of amino acid in caspase-8a and caspase-8b are 496 and 479 respectively. The difference in protein sequence occurs in the pro-domain. The caspase-8 catalytic domains are identical in both isoforms.

1.2.2 Cleavage of Caspase-8

Caspase-8 is comprised of a N-terminal pro-domain (two DEDs) (14-16, 24, 25), followed by a large (p18) and a small (p12) subunit separated by a linker region (Figure 4). It is cleaved in a multi-step process to generate a mature enzyme composed of two p18 and two p12 subunits. The first cleavage step occurs between the large and small subunit after aspartic acid D391 (using Caspase-8a isoform numbering) (23, 25, 26), which occurs as early as 30 seconds upon death receptor stimulation (25, 27). The small subunit is further cleaved after D401. Cleavage after both D391 and D401 are important for the activity of active caspase-8 as failure of cleavage in either site results in enzyme that is about 40- and 10-fold less active, respectively (25). The second cleavage step occurs after D233 then after D227. These two cleavage sites result in the removal of the 28-kDa pro-domain. The second cleavage step is not crucial in the activation of caspase-8 (25) (Figure 4).

Figure 4





3
M1
D227
S228
C377
D391
DED1 & DED2
Large (p18)
D233
II
Second Step of Cleavage
6
M1
D227
D233
DED1 & DED2
7
S234
C377
D391
Large (p18)
D227
IIa
8
M1
D227
DED1 & DED2
9
D228
D233

Figure 4. Cleavage of Caspase-8

A schematic diagram of caspase-8 (caspase-8a isoform) structure is illustrated. It comprises of a 28-kDa pro-domain (consists of two DEDs), followed by a large (p18) and a small (p12) subunit, separated by a linker region. The p18 subunit, p12 subunit and the linker region together form the catalytic domain of the enzyme. The active site cysteine residue (C377) located within p18 subunit is illustrated. The amino acids corresponding to the beginning and the end of each region are illustrated at the top of the figure.

The cleavage process of caspase-8 (caspase-8a isoform) is illustrated here. Caspase-8 (1) is first cleaved after D391 (I), generating a N-terminal fragment comprised of the pro-domain and the large (p18) subunit (2), and a C-terminal fragment comprised of the linker region and the small (p12) subunit (3). The C-terminal fragment is further cleaved after D401 (Ia) to generate a small (p12) subunit (4) and a small linker fragment (5). A second cleavage step (II) occurs within the N-terminal fragment (3) after D233 generating a N-terminal fragment comprised of the pro-domain (6), and the large (p18) subunit (7). The N-terminal fragment is then quickly processed after D227 (IIa) to generate a shorter N-terminal fragment (8) and a very small 6 amino acid fragment (9).

1.2.3 Three-Dimensional Structure of Caspase-8

In order to understand how caspase-8 becomes activated, its three-dimensional structure must be discussed. The three-dimensional structure of caspase-8 asymmetric (or antiparallel) unit contains the p18-p12 heterodimer. The small subunits are at the dimer interface. To be biologically active, the caspase-8 molecule must consist of two heterodimers that form a tetramer. The dimer has a typical α/β folding motif with a central six-stranded β sheet made up of five parallel strands and one antiparallel strand. There are three α helices on one side and two on the other side of the main β sheet. A two-stranded, anti-parallel β sheet that is part of the binding pocket is located at the top of the main β sheet. Four loops and the linker regions extend from the top of the main β sheet (28-31) (Figure 5).

The three-dimensional structures of inactive caspase-8 and active caspase-8 are similar in that the β strands and α helices of caspases-8 superimpose well. The main differences lie in the binding pocket that is composed of the loops and the linker region of the molecule (31) (Figure 6).

Figure 5

A

```
001MDFSRNLYDI GEQLDSEDLA SLKFLSLDYI PQRKQEPIKD ALMLFQRLQE050
051KRMLEESNLS FLKELLFRIN RLDLLITYLN TRKEEMEREL QTPGRAQISA100
101YRFHFCRMSW AEANSQCQTQ SVPFWRRVDH LLIRVMLYQI SEEVSRSELR150
151SFKFLLQEEI SKCKLDDDMN LLDIFIEMEK RVILGEGKLD ILKRVCAQIN200

                                                    <--
201KSLLKIINDY EEFSKGEELC GVMTISDSPR EQDSESQTLD KVYQMKSKPR251

 -β1---->-(--α1'-)-----L1---->-------α1------>   <--β2
251GYCLIINNHN FAKAREKVPK LHSIRDRNGT HLDAGALTTT FEELHFEIKP300

 -> L2<-------α2----->     <---β3--><-------L3-------><-
301HDDCTVEQIY EILKIYQLMD HSNMDCFICC ILSHGDKGII YGTDGQEAPI350

 --α3-->             <--β4--><----end of p18----><-Linker-
351YELTSQFTGL KCPSLAGKPK VFFIQACQGD NYQKGIPVET DSEEQPYLEM400

 ><Start of p11><--β5--><------L4-----><---------α4-----
401DLSSPQTRYI PDEADFLLGM ATVNNCVSYR NPAEGTWYIQ SLCQSLRERC450

 -->  <-----α5-----><-----L5-----><-β6->
451PRGDDILTIL TEVNYEVSNK DDKKNMGKQM PQPTFTLRKK LVFPSD496
```

Adapted from Watt *et al* 1999

B

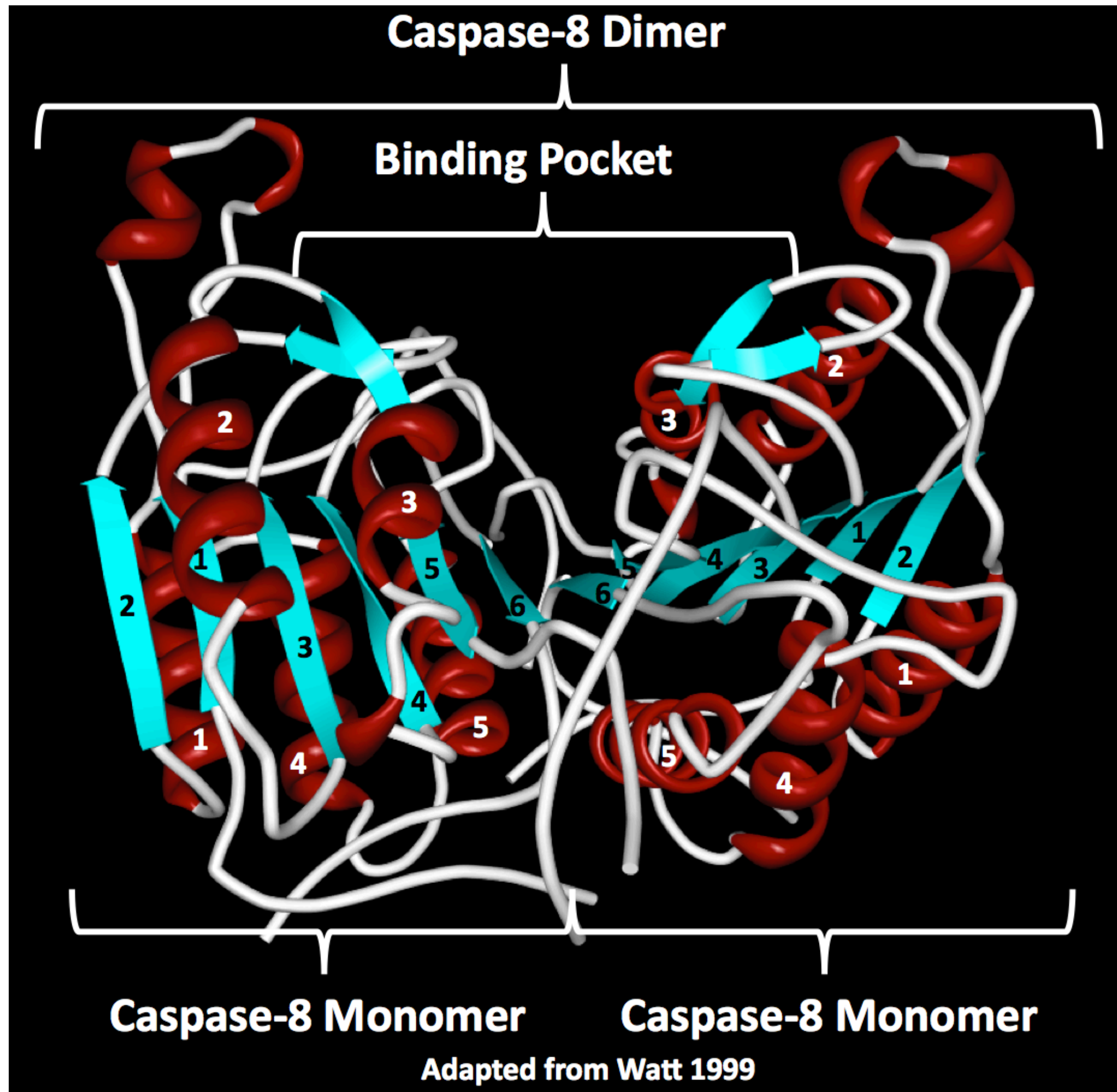


Figure 5. Three-Dimensional Structure of Caspase-8 Dimer

A) Caspase-8 protein sequence with corresponding α helix and β sheet secondary structure elements are depicted. B) The three-dimensional structure of caspase-8 dimer is depicted here. Caspase-8 structure has a typical α/β folding motif with a central six-stranded β sheet made up of five parallel strands (strands 1 to 5) and one antiparallel strand (strand 6). There are five α helices in the core, three on one side (1, 4 and 5) and two on the other side (2 and 3) of the main β sheet. A two-stranded, anti-parallel β sheet that is part of the binding pocket is located at the top of the main β sheet.

Figure 6

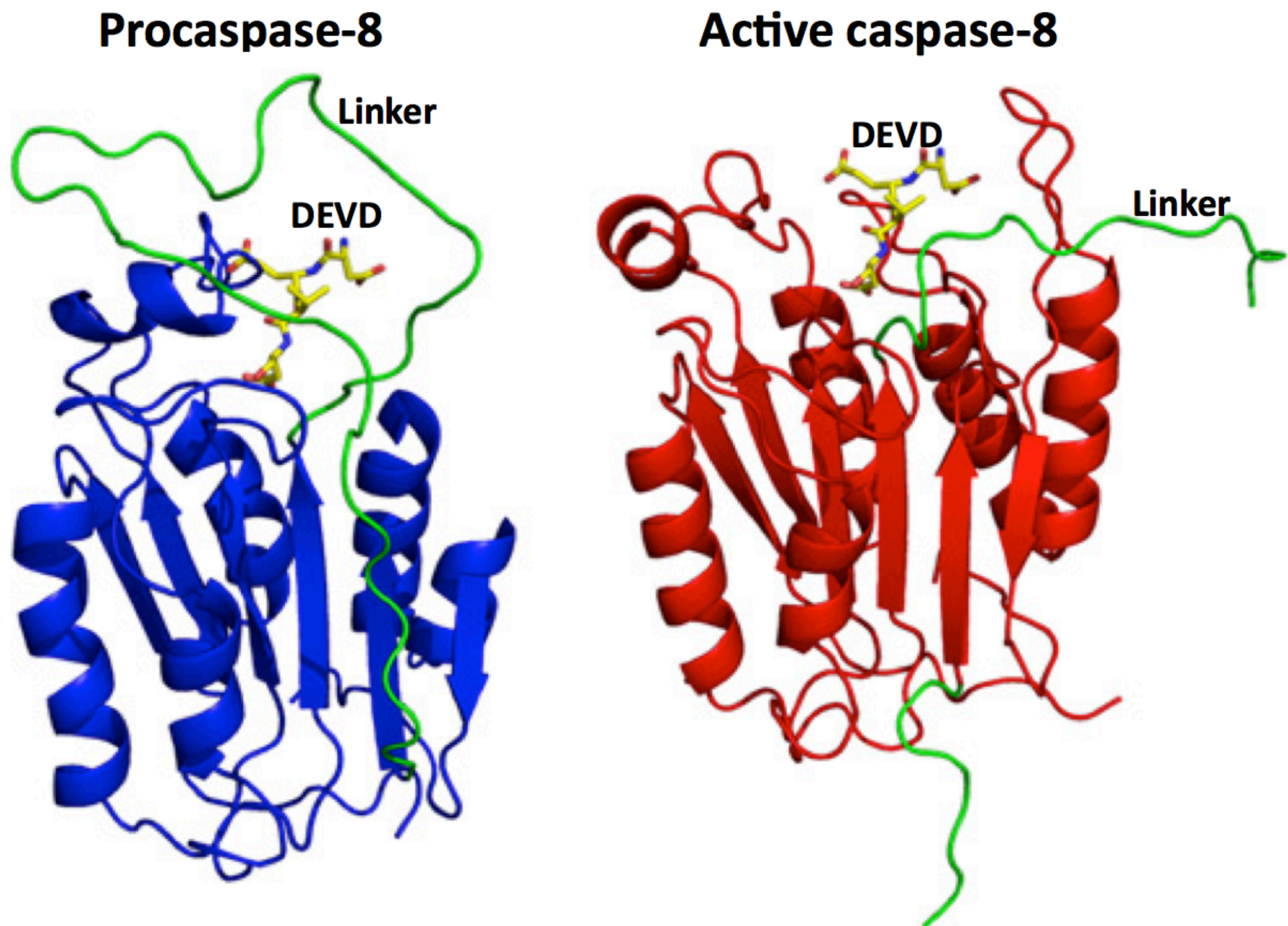


Figure 6. Three-Dimensional Structure of Procaspase-8 and Active Caspase-8

The three-dimensional structures of procaspase-8 and active caspase-8 are depicted along with DEVD tetrapeptide binding to active site pocket of caspase-8. The three-dimensional structures of procaspase-8 and active caspase-8 are similar in that the β strands and α helices of caspases-8 superimpose well. The main differences lie in the binding pocket that is composed of the loops and the linker region of the molecule.

1.2.4 Substrate Recognition and Specificities

Caspases are one of the most specific proteases, with an absolute requirement for cleavage after an aspartic acid (D) (2). The recognition of at least four contiguous amino acids N-terminal to the cleavage site is another necessary requirement for efficient catalysis (32). The nomenclature for caspase substrate specificity denotes P1 as the aspartic acid, P2, P3 and P4 as one, two and three amino acids N-terminal to P1, respectively. Aside from their stringent requirement for D in P1 position, P4 is the most critical determinant of specificity while P2 is the most liberal position. The preference for P3 position is glutamic acid. The consensus sequence for caspase-8 cleavage is (I/L/V)E*X*D. I is isoleucine, L is leucine, V is valine, E is glutamic acid, *X* is any amino acid and D is aspartic acid (32). In addition to sequence specificities, the tertiary structure of the substrate is also crucial in specificity (2). Caspase-8 can cleave procaspases-3, -4, -7 and -9 *in vitro* (25, 33-35). Caspase-8 can also cleave Bid, allow it to propagate intrinsic apoptotic pathway (36). Other caspase-8 substrates include receptor interacting protein (RIP) (37, 38), p21-activated kinase 2 (PAK2) (39), Ca^{2+}/calmodulin-dependent protein kinase-like kinase (CaMKLK) (39) and CLYD (40).

1.2.5 Activation by Induced Proximity Model

In contrast to the executioners of apoptosis, which are expressed as dimers; the initiators, such as caspase-8, are expressed as inactive monomeric zymogens. The dissociation constant (K_D) for homodimerization of the casapse-8 catalytic domains was proposed to be in the low micromolar range, which is well above its nanomolar *in vivo* concentration (31, 41, 42). Therefore, in order for caspase-8 to dimerize, it has to be brought into close proximity by the binding of its DED domains to the DED domains of

adaptor molecules in macromolecular complexes such as the death inducing signaling complex (DISC) (15). This is known as the **Induced-Proximity Model** that serves as the basis for the operation of DISC, an assembly that forces a locally high concentration of caspase-8 in a process mediated by recruited FADD. The dimerization of caspase-8 (43-45) is sufficient to induce some basal proteolytic activity, which is approximately 1% of the fully processed enzymes (44, 46).

After dimerization, caspase-8 is first cleaved at the linker region by intramolecular processing (i.e. *cis* processing). After intramolecular processing, cleaved caspase-8 can then process another caspase-8 molecule in *trans* (intermolecular processing) (45) after D391 and D401 (using Caspase-8a isoform numbering) (23, 25, 26). The autoproteolytic cleavage of the zymogens results in separation of the catalytic unit into two units (see section on Cleavage of Caspase-8). An additional cleavage between the prodomain and the catalytic domain after D233 (using Caspase-8a isoform numbering) (25) allows the release of the active enzyme from the activation complex (31). This phenomenon is called the sequential accessibility mechanism (47). Despite some groups having shown that cleavage of linker region occurs intramolecularly, other groups have demonstrated that both cleavage sites within linker region can be processed intermolecularly by a proximal protease, which could either be another caspase-8 dimer or a different protease such as granzyme B (31, 44, 47, 48).

Whether cleavage of caspase-8 is necessary for activation remains controversial. By using uncleavable caspase-8 mutant, it has been shown that dimerization is necessary, sufficient and reversible in its activation (41, 42, 47). The cleavage of caspase-8 mainly serves to stabilize caspase-8 dimer and is not required for activation (31, 41, 42, 48).

Furthermore, the cleaved linker region serves to stabilize the dimer and allows the formation of active caspase-8 upon substrate binding via an induced-fit mechanism (49). However, others have demonstrated that both inducible dimerization and cleavage are required for caspase-8 activation (50). The dimerization of caspase-8 requires threonine 467 and phenylalanine 468 (caspase-8b isoform numbering) (31).

1.2.6 Localization of Caspase-8

Caspase-8 has been observed in different cellular locations (51). It can localize to the cytosolic compartment (26), actin-rich ruffles (52, 53), endosomes (54), including those at the front of migrating cells (55), focal adhesions (56) and stable microtubules structures, such as centrosomes (57). The different domains of caspase-8 appear to favor localization to different cellular compartments. It is possible that these different preferred locations may ultimately influence caspase-8 functions (51).

1.3 INHIBITORS OF CASPASE-8

1.3.1 Viral-Encoded Caspase-8 Inhibitors

Various pathogen-encoded molecules can inhibit the activity of caspase-8. They can inhibit caspase-8's activation (binding to DISC, dimerization and/or cleavage) or inhibit its activity without affecting its activation by interacting with its active site (See Table 1). For example, **human papillomavirus (HPV) 16 E6 protein** inhibits caspase-8 dimerization. **Viral FLICE-inhibitory proteins (v-FLIPs)** including molluscum contagiosum virus MC159 protein, Kaposi's sarcoma-associated herpesvirus K13 protein (58), gammaherpesvirus ORF71 (93) and equine herpesvirus 1 E8 can inhibit caspase-8 directly by either inhibiting its binding to DISC, dimerization and cleavage (59). Other caspase-8 inhibitors primarily prevent caspase-8's activation. They include **cytomegalovirus (CMV) viral inhibitor of caspase-8 activation (vICA) protein** (by forming a complex with caspase-8 preventing its binding to DISC) (60-62), bovine herpesvirus 4 BORFE2 protein, **adenovirus E3 14.7 kDa protein** (63) and herpes simplex virus UL39 protein. Another class of caspase-8 inhibitor is serpin, including **cowpox virus cytokine response modifier A (CrmA)**, vaccinia virus B13R protein and myxoma virus Serp2 protein. CrmA inhibits caspase-8 activity (64, 65), not its activation (26, 47), by interacting with active form of caspase-8 (33). The **baculovirus p35 protein** also inhibits caspase-8 activity directly by binding to its catalytic cysteine 360 (C360) residue (13, 60). The C360 residue of caspase-8 (Caspase-8b isoform numbering) is covalently linked to the aspartic acid 87 of p35 through a thioester bond (66). The thioester bond is protected from hydrolysis by the N-terminus of p35 (13).

Table 1

Viral-Encoded Caspase-8 Inhibitor	Inhibition of:			
	DISC Binding	Dimerization	Cleavage	Active Site
HPV 16 E6		√		
v-FLIP	√	√	√	
CMV vICA	√			
Adenovirus E3	√			√
CrmA				√
p35				√

Table 1. Viral-Encoded Caspase-8 Inhibitors

Six viral-encoded caspase-8 inhibitors and their mechanism of inhibition are depicted. HPV 16 E6 protein inhibits caspase-8 by preventing its dimerization. v-FLIP protein prevents caspase-8 from binding to DISC, from dimerization and from cleavage. CMV vICA protein prevents caspase-8 from binding to DISC. Adenovirus E3 protein prevents caspase-8 from binding to DISC and also binds to caspase-8 active site to prevent its activity. Both CrmA and p38 bind to caspase-8 active site to prevent its activity.

1.3.2 Mammalian Caspase-8 Inhibitors

A classic viral inhibitor of the Fas-induced apoptotic pathway is v-FLIP. It is one of the many ways virus evade cell death of infected cells. v-FLIPs interfere with caspase-8 recruitment to the DED of FADD (59, 67-69). After the discovery of v-FLIPs, multiple groups have discovered its mammalian homologue, cellular FLIP (c-FLIP, also known as CASPER, I-FLICE, Flame, CASH, CLARP, MRIT or usurpin) and it is expressed in several splice variants (70-76). c-FLIP$_L$ contains two DEDs and a caspase-like domain with significant homology to caspase-8 but it lacks the critical cysteine and histidine residue that are important for catalytic activity. Instead, they are replaced by tyrosine and arginine, respectively. c-FLIPα also contains two DEDs and a caspase-like domain. c-FLIPβ and c-FLIPγ has two DEDs and lack a part of the caspase-like domain. The shortest variant is c-FLIP$_S$ or c-FLIPδ, which is similar to v-FLIPs in length and only has two DEDs (67). All c-FLIP variants bind to the DED of FADD and caspase-8/-10 via DED-DED homophilic interaction. c-FLIP$_L$'s caspase domain can also bind to the caspase domain of caspase-8 to prevent its activation. The end result is to prevent the propagation of apoptotic signals through the Fas receptor. However, some have shown that activation of caspase-8 can be enhanced by c-FLIP$_L$ upon their heterodimerization (77, 78). The theory that would resolve the above conflict is the following: at high levels of expression, c-FLIP$_L$ competes with caspase-8 for recruitment to the DISC by saturating FADD molecules during DISC formation, thereby inhibiting caspase-8 activation. However, at low levels of expression, c-FLIP$_L$ promotes caspase-8 activation by forming heterodimer with caspase-8 through its protease-like domain, which strongly

induces enzymatic activity in caspase-8 via direct protein-protein interactions (47, 77, 79, 80).

Another family of apoptotic inhibitors, known as Caspase-8 and -10 associated RING protein (CARPs), has been shown to interact specifically with caspases-8 and -10. A C-terminal CARP RING domain with high homology to the inhibitor of apoptosis protein (IAP) family contains E3 ubiquitin ligase activity and contributes to ubiquitin-mediated proteolysis of caspases-8 and -10 but not -9. Thus, overexpression of CARPs results in ubiquitin-mediated proteolysis of caspase-8 and -10 (81). Toso, a member of the immunoglobulin gene superfamily, is another inhibitor of caspase-8. It inhibits caspase-8 processing, and hence its activation (82).

1.4 CASPASE-8 KNOCKOUT

In order to facilitate our understanding of caspase-8 functions, the phenotype of *Caspase-8* knockout must be discussed. Varfolomeev *et* al generated the first *caspase-8* knockout mice in 1998 (83). *Caspase 8* +/- heterozygous mice appeared phenotypically normal, whereas, *caspaes-8* -/- mice all died at day 11.5 *in utero*. The most salient feature of the abnormal phenotype of *caspase-8* -/- mutant was marked impaired heart muscle development and congested accumulation of erythrocytes indicating circulatory failure. These results were reproduced by another group in 2002 (84). *Caspase-8* -/- fibroblasts were resistant to death induction triggered by death receptors such as Fas and TNF receptors. However, they responded normally to noncytocidal effects of death receptors in the activation of NF-κB (83). In addition, conditional knockout of *caspase-8* gene in mice (bone marrow cells) also led to hematopoietic progenitor deficiency (85). The phenotype observed in *caspase-8* deficient mice suggests that in addition to its classic apoptotic function, caspase-8 also harbors non-apoptotic functions. I will now focus on the discussion of both apoptotic and non-apoptotic functions of caspase-8.

1.5 CELLULAR FUNCTIONS OF CASPASE-8

Caspase-8 is involved in many cellular functions, both apoptotic and non-apoptotic. It is involved in many cell-signaling pathways, some of which require caspase-8 proteolytic activity, some of which do not. Caspase-8 has been known as an apoptotic protein. When it is activated, its main function is to set off an apoptotic program and to cause cell death. Caspase-8 also regulates other forms of cell death, including necroptosis, integrin mediated death (IMD) and autophagy. In addition to regulating cell death, caspase-8 can also regulate immune cell function, inflammation, cell adhesion, migration and motility, endosomal trafficking, gene transcription and cell signaling including NF-κB and Erk1/2 signaling. Here, I will discuss the major functions of caspase-8 (Figure 7).

Figure 7

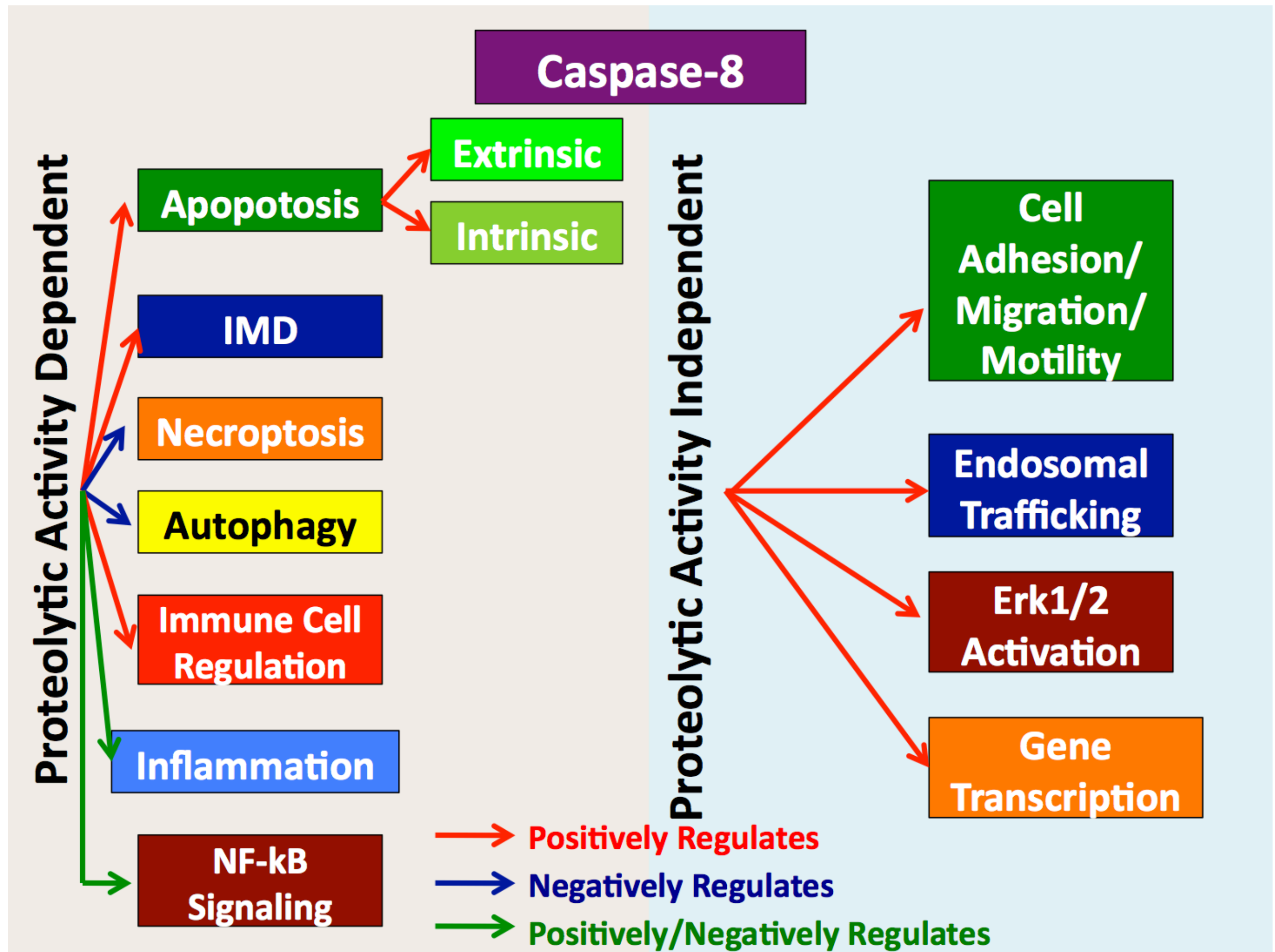


Figure 7. Caspase-8's Cellular Functions

Caspase-8 has both proteolytic activity dependent and independent functions. With its proteolytic activity, it can promote (red arrow) apoptosis, both extrinsic and intrinsic pathways; integrin mediated death (IMD) and immune cell regulation. It can also inhibit (blue arrow) necroptosis and autophagy. Moreover, the proteolytic activity of caspase-8 can either positively or negatively regulate (green arrow) inflammation and NF-κB signaling. Caspase-8 can also regulate other cellular functions and pathways independent of its proteolytic activity. They include the regulation of cell adhesion, migration and motility, endosomal trafficking, gene transcription and Erk1/2 activation.

1.5.1 Extrinsic (Death Receptor-Induced) Apoptosis

Caspase-8 is known as an apical caspase in the cascade of apoptosis. It is critical for the maintenance of peripheral immune tolerance and the elimination of cells infected by viruses (86-88). It has been shown that caspase-8 is essential for Fas-mediated apoptotic signaling (83, 85, 89, 90). As described in the section of extrinsic apoptosis, when death ligand such as FasL binds to death receptor such as Fas, caspase-8 is recruited to the DISC within seconds and caspase-8 then becomes activated in an induced-proximity model and subsequently activates executioner caspases and apoptosis ensues (18, 91, 92). Fas-induced apoptosis is defective in mouse (83, 85, 93) and human deficient of caspase-8 (68), which further supports the crucial function of caspase-8 in Fas-induced apoptosis. Caspase-8 is also involved in apoptotic signaling via other death receptors, such as tumor necrosis factor receptor 1 (TNFR1) (83, 91), death receptor 3 (DR3) (83), DR4, DR5 (90, 94) and TNF-related apoptosis-inducing ligand (TRAIL) receptors (94-96).

1.5.2 Intrinsic Apoptosis

Caspase-8 is not only involved in extrinsic apoptosis. The activation of caspase-8 can propagate intrinsic apoptosis pathway by the cleavage of Bcl2 protein Bid into truncated Bid (tBid) in certain cells (36) (See Intrinsic Apoptosis section). tBid then triggers cytochrome *c* release from the mitochondria (97), allowing the formation of apoptosome and hence intrinsic apoptosis ensues.

1.5.3 Integrin-Mediated Death (IMD)

Caspase-8 is also involved in a process known as integrin-mediated death (IMD). Cells that are non-adherent to the extracellular matrix via integrin binding leads to the recruitment of caspase-8 to the β integrin tails. It results in the activation of caspase-8 in a death receptor-independent manner and cell death ensues (98, 99). Upon integrin ligation to the extracellular matrix, however, the integrin-caspase-8 containing complex is disrupted, therefore the activation of caspase-8 is inhibited and cell survival increases (99).

1.5.4 Necroptosis

Programmed necrosis induced by death receptor, also known as necroptosis, is mediated by receptor interacting protein 1 (RIP1) (100) and RIP3 (101). Caspase-8 can form complex with both FADD and RIP1 upon death receptor activation. In the absence of c-$FLIP_L$, it promotes apoptosis (102). However, in the presence of c-$FLIP_L$, RIP3 is recruited to complex II converting it into a necrosome and promotes necroptosis (103, 104). Upon cellular inhibitor of apoptosis protein (cIAP) depletion, RIP3 can also form complex with FADD and RIP1 independent of death receptor signaling, forming ripoptosome (105, 106). In the presence of c-FLIPs, ripoptosome promotes necroptosis (106).

The role of caspase-8 in the inhibition of necrosis became more apparent when the inhibition of caspase-8 with CrmA resulted in the sensitization of death-receptor-mediated necrosis (47, 107-109). With the generation of caspase-8 and RIP3 double knockout mice, which, unlike caspase-8 knockout, are not embryonic lethal, it became apparent that caspase-8 regulates RIP-dependent necroptosis (110, 111). Moreover,

caspase-8 has been shown to cleave both RIP1 and RIP3, inhibiting their role in caspase-independent cell death (37, 112) and it has been shown to regulate intestinal homeostasis and protect intestinal epithelial cells from TNF-α-induced necroptotic cell death (113). Caspase-8 can also regulate RIP1 and RIP3 dependent necroptosis by cleavage of CLYD, an enzyme essential in necroptosis (40). In summary, caspase-8 serves to inhibit the propagation of necroptosis mediated by RIP1 and RIP3.

1.5.5 Autophagy

Autophagy promotes a cell survival response to nutritional starvation involving the formation of membrane bound vacuoles that target organelles and proteins to the lysosome for degradation. Autophagy requires the activation of *ATG7* and *beclin* genes. These genes are induced by caspase-8 inhibition. Therefore, caspase-8 negatively regulates autophagy (109, 114-116).

1.5.6 Inflammation

Conditional deficiency of caspase-8 results in chronic inflammation in the liver (117) and in the skin (118) suggesting that caspase-8 can restrict the signaling of some inflammatory mechanisms. Moreover, Kang *et al* showed that dendritic cells deficient in caspase-8 are hyperresponsive to induction of assembly of NLRP3 inflammasome (119), a signaling complex that, through the activation of caspase-1, leads to the processing of proinflammatory cytokines such as IL-1β (120). The enhanced activation of the inflammasome as a result of caspase-8 deficiency is dependent on the functions of RIP1 and RIP3 (119).

On the other hand, caspase-8 has been shown to induce IL-1β maturation through the stimulation of TLR3 and 4 (121) and dectin-1 (an extracellular pathogen sensor) via a

non-canonical caspase-8 inflammasome (MALT-1-caspase-8-ASC complex) (122). Together, these suggest a dual role of caspase-8 in the process of inflammation depending on the cellular circumstances.

1.5.7 Immune Cells Homeostasis, Proliferation, Differentiation and Activation

Caspase-8 has been shown to play a role in Fas-induced apoptosis of self-reactive lymphocytes which is important to achieve homeostasis, to maintain peripheral immune tolerance and to prevent autoimmunity (86, 123, 124). However, caspase-8 has also been implicated in naïve T cell proliferation (68, 125) and IL-2 production (93, 126) induced by CD3, effects that are independent of the classical Fas/FasL interaction (127). Rather, in caspase-8 deficient T cells, impaired proliferation is due to defects in cell cycle progression with diminished entry into S phase (128, 129), decrease phosphorylation of ribosomal S6 protein (128) and lack of proper regulation of cell cycle-associated proteins (126). Similar mechanisms of B-cell proliferation impairment were also observed in conditional caspase-8 deficient mice (130).

Moreover, upon antigen stimulation of T cells, caspase-8 forms a heterodimer with a paracaspase called MALT1. This complex promotes caspase-8 to undergo limited autoproteolytic cleavage resulting in an active form that exhibits diminished activity towards caspase-8 but maintains its activity toward c-FLIP$_L$ (131). c-FLIP$_L$ has been shown to be involved in lymphocyte proliferation (132, 133). Intriguingly, caspase-8 can migrate with c-FLIP$_L$ to lipid rafts upon T cell receptor (TCR) ligation. There, the active caspase-8- c-FLIP$_L$ complex associates with NF-κB signaling molecules including RIP1, TNFR associated factor 2 (TRAF2), and TRAF6 as well as upstream NF-κB regulators PKCθ, CARMA1, Bcl-10 and MALT1, which connect to the TCR. When caspase-8

activity is inhibited, NF-κB activation is suppressed (134) suggesting that caspase-8 is involved in T cell proliferation upon antigenic stimulation. Another mechanism of caspase-8's involvement in T cell proliferation upon antigenic stimulation is through its inhibition of RIP-1-dependent necroptosis (135).

Caspase-8 also plays an important role in immune cell differentiation. *Caspase-8* gene deletion in bone marrow cells prevented hematopoietic progenitors from forming myeloid and B-lymphoid colonies. When caspase-8 gene deletion is restricted to cells of the myelomonocytic lineage, the differentiation of monocytes into macrophages was arrested, whereas their differentiation into dendritic cells or granulocytes remained unaffected (85, 109). Likewise, it is also involved in macrophage differentiation induced by macrophage-colony stimulating factor (M-CSF) and thymocyte generation but not dendritic cell and granulocyte differentiation in the presence of GM-CSF and G-CSF, respectively (85, 136). All together, these data suggest that caspase-8 is involved in lymphocyte and monocyte/macrophage differentiation but not dendritic cell or granulocyte differentiation. The cell differentiation effect of caspase-8 appears to be dependent on its DEDs and it requires the presence of a critical lysine (K156 in caspase-8b and K188 in caspase-8a) in the microtubule binding motif within the second DED (DED2) (51).

Finally, caspase-8 has also been shown to be involved in the activation of lymphocytes including T cells, B cells and natural killer cells (68, 125, 137). Condition knockout of caspase-8 in hematopoietic cells results in embryonic lethality (85). Contrast to *caspase-8* deficient mice, which die *in utero* and have abnormal development, human deficient of *caspase-8* develop normally but they exhibit defects in their activation of T

and B lymphocytes and natural killer cells. All together, these lead to immunodeficiency (68, 93). *Caspase-8*-deficient T cells also had a defect in the production of IL-2 (68). Moreover, caspase-8 plays an essential role in B-cell activation and expansion in response to TLR2, TLR3 and TLR4, but not TLR9 (138, 139).

1.5.8 Cell Adhesion, Migration and Motility

Caspase-8 has also been shown to regulate cell adhesion, migration and motility. Cells that lack caspase-8 have reduced cell motility. Moreover, caspase-8, independent of its proteolytic activity, is capable of restoring cell migration/adhesion to caspase-8 null cells (140), suggesting that caspase-8's proteolytic activity is not required for cell adhesion (141), migration and motility.

The role of caspase-8 in cell adhesion is also illustrated by the fact that cells that are deficient of caspase-8 have reduced calpain activity, resulting in the reduction of Rac activation, lamellipodial assembly and fidelity of cytokinesis (142). From the mechanistic point of view, it has been shown that phosphorylation of tyrosine 380 (Caspase-8b numbering), induced either by epidermal growth factor (EGF) or integrin-mediated adhesion (52), is essential in the restoration of cell adhesion, migration and motility in caspase-8-null cells. Tyrosine 380 phosphorylation of caspase-8 is required for its localization to the cell periphery and its interaction with c-Src (52, 141) and Src homology 2 (SH2) domain of p85α subunit of phosphatidylinositol 3-kinase (PI3K) (140). It has been shown that the interaction of caspase-8 with p85α subunit of PI3K results in Rab5 dependent Rac activation (140, 143).

Moreover, caspase-8 has been shown to interact with a multiprotein complex that include focal adhesion kinase (FAK) and calpain 2 (CPN2) and enhance cleavage of focal

adhesion substrates and cell migration. Caspase-8 association with CPN2/calpastatin disrupts calpastatin-mediated inhibition of CPN2 (56). These together serve to explain the potential mechanisms of caspase-8 dependent cell adhesion, migration and motility.

1.5.9 Endosome Trafficking

Interestingly, tyrosine 380 phosphorylation of caspase-8b isoform also promotes endosomal trafficking. For example, phosphorylation of tyrosine 380 is essential for the interaction with the SH2 domain of p85α, a multifunctional adaptor for PI3K that possesses Rab-GAP activity. The interaction between caspase-8 and p85α promotes Rab5 GTP loading, alters endosomal trafficking, and results in the accumulation of Rab-positive endosomes at the leading edge of the migrating cells (55, 143). This may be another mechanism by which caspase-8 contributes to cell migration.

1.5.10 Cell Signaling – NF-κB Activation

Nuclear factor κB (NF-κB) is a transcription factor that regulates the expression of a myriad of genes that contain the κB DNA elements. NF-κB signaling can occur through the canonical or the non-canonical pathways. The canonical pathway involves the recruitment of TRAF6 and RIP to TNFR upon its ligation by TNF. It results in the phosphorylation and activation of IκB kinase (IKK) complex by TAK1. The non-canonical pathway involves the recruitment of TRAF2 to TNFR. In this case, IKK complex is phosphorylated and activated by NF-κB inducing kinase (NIK). IKK then phosphorylates IκB proteins, which leads to ubiquitination and subsequent proteasomal degradation. The degradation of IκB proteins release NF-κB, allowing it to translocate to the nucleus to activate gene transcription (144).

The NF-κB activation in T cells is slightly different from the canonical and the non-canonical pathways. Upon T cell receptor (TCR) stimulation, protein kinase Cθ (PKCθ) is phosphorylated and activated and resulted in the recruitment of the CARMA1-Bcl10-MALT1 (CBM) complex to the immunological synapse. The CBM complex then activates IKK complex and therefore activates NF-κB (145).

Caspase-8's involvement in NF-κB signaling is a complex one. In human or murine T, B and natural killer cells that are deficient of caspase-8, it has been shown that the activation of NF-κB through the stimulation of antigen receptors, Fc receptors or TLR4 is abolished (126, 146), suggesting a role for caspase-8 in NF-κB signaling. Moreover, the pro-domain of caspase-8 can bind to NIK, RIP, TRAFs and IKK and the enzyme domain of caspase-8 can also interact with NIK, RIP and TRAFs. These data imply that caspase-8 could be important in the NF-κB signaling pathway (147). Furthermore, upon Fas stimulation, caspase-8 (independent on its enzymatic activity) can recruit FADD, c-FLIPs and RIP1 to the receptor complex and thus promotes NF-κB activation (147-150). Caspase-8 is also involved in IL-8 production upon Fas-induced ASC-mediated NF-κB activation (151, 152).

Indeed, the roles of caspase-8 in NF-κB signaling are contradictory depending on its activation state. When caspase-8 is fully activated, it cleaves NIK and renders it inactive in phosphorylating IκB protein, therefore, preventing the activation of NF-κB (153). However, upon TCR stimulation, when caspase-8 is only weakly activated, the full-length caspase-8 binds to CBM complex and recruits IKK complex. This leads to the activation of IKK complex and phosphorylation of serine 536 of p65 subunit of NF-κB and hence the activation of NF-κB (138, 146).

1.5.11 Cell Signaling – Erk1/2 Activation

Extracellular signal-regulated kinase1/2 (Erk1/2), a protein serine/threonine kinase, is a mitogen activated protein kinase (MAPK) that is involved in the regulation of cell cycle progression, cell adhesion, migration, survival, differentiation, metabolism, proliferation and transcription (154). It is activated by a myriad of mitogens and growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and TNFα, etc.

It has been shown that in caspase-8-deficient neuroblastoma cells, the induction of Erk1/2 activation by fibronectin adhesion is absent (141). Likewise, Erk1/2 activation is also absent in these cells upon EGF, PDGF or TNFα stimulation (53), while overexpression of caspase-8 leads to enhanced TNF induced Erk1/2 activation (155). These together suggest a crucial role of caspase-8 in the activation of Erk1/2. The promotion of EGF-induced Erk1/2 activation is independent of the proteolytic activity of caspase-8 and can be recapitulated using only the prodomains of the protein (53).

1.5.12 Cell Signaling – Src Kinase

c-Src is the prototypic member of a family of nine non-receptor tyrosine kinase and was first identified as a proto-oncogene (156). It is involved in cell proliferation and migration (see Src Kinase section). Caspase-8 has been shown to co-localize with c-Src at the cellular periphery via the "RXDLL motif" in its death effector domain (53, 141) and via its linker domain (52). Phosphorylation of Y380 or Y397 (linker region) of caspase-8b and -8a respectively allows it to bind to c-Src, most likely to the SH2 domain of c-Src because caspase-8 has a putative SH2 binding site (SEEQP-pYLEM-DLSS) (52).

1.5.13 Tumorigenesis

Caspase-8 can have either an inhibitory or a promoting effect on tumor development. A characteristic feature of cancers is their inability to undergo apoptosis in response to apoptotic stimuli (157). One potential mechanism of evasion of apoptosis is through the inactivation of caspase-8 by genetic or epigenetic mechanisms, alternative splicing or posttranslational modifications (21). Inactivating mutations of the *caspase-8* gene has been reported in colorectal carcinoma (158), head and neck carcinoma (159), lung carcinoma (160, 161) and gastric carcinoma (162). The *caspase-8* gene can also be deleted through homo- or heterozygous genomic deletion as seen in neuroblastoma cells (163) or through allelic imbalance on chromosome 2q resulting in alterations of *caspase-8* gene (164). Hypermethylation (epigenetic mechanism) of the regulatory sequence of the *caspase-8* gene is another way caspase-8 expression can be altered. It is seen in neuroblastoma (165-170), medulloblastoma (165, 171), glioblastoma, Ewing tumor, retinoblastoma (165), rhabdomyosarcoma (165), lung carcinoma (172, 173) and hepatocellular carcinoma (174). In addition, the protein function of caspase-8 can be impaired by the expression of a dominant negative variant of caspase-8, caspase-8L, as seen in leukemia (175) and neuroblastoma (17, 176). Posttranslational modification, such as phosphorylation, of caspase-8 has been shown to impair its ability to promote apoptosis. Src-dependent phosphorylation of Y380 of caspase-8b or Y397 of caspase-8a has been suggested to suppress its apoptotic function (52, 177, 178) and it has been implicated in colorectal cancer (177).

However, in addition to its apoptotic functions, caspase-8 has also been shown to have non-apoptotic functions, such as cell differentiation, proliferation, activation, adhesion, and migration. These functions can potentially be involved in tumorigenesis. Intriguingly, a significant proportion (10-30%) of aggressive stage IV neuroblastoma maintains expression of caspase-8. Inactivation mutations as described above are surprisingly rare (162, 173, 179, 180). Equally, deletion or silencing of *caspase-8* gene is an extremely rare event in malignancies (181). On the contrary, it has been shown that there is an increased expression of caspase-8 in lung cancers (182). Together, these suggest that the presence of caspase-8 potentially promotes tumorigenesis by promoting cell differentiation, proliferation and migration.

1.5.14 Gene Transcription

Intriguingly, the DED of caspase-8, when cleaved off from the protease domain during the activation of caspase-8, can be chaperoned by Erk1/2 and translocates into the nucleus where it participates in *caspase-8* transcriptional activation by directly binding to TOPORS, a p53 and topoisomerase I binding protein. Binding of DED1 displaces p53 from TOPORS, allowing p53 to activate the expression of caspase-8 (183). This feedback loop allows processed caspase-8 to be continually replenished by newly synthesized caspase-8, potentially allowing the propagation of apoptosis by providing more caspase-8.

1.6 POSTTRANSLATIONAL MODIFICATIONS OF CASPASE-8

Caspase-8 protein can be modified posttranslationally to alter its cellular functions. Ubiquitination, cleavage and phosphorylation are the main posttranslational modifications of caspase-8. I will now discuss these posttranslational modifications of caspase-8 and describe their effects on caspase-8 functions.

1.6.1 Ubiquitination of Caspase-8

The stability of caspase-8 dimer is labile once it is released from the DISC (42, 48). Therefore, in order to sustain its activity, caspase-8 must be modified to increase its stability. This is achieved by K48 and K63 polyubiquitination of caspase-8 by neddylated cullin3 (CUL3)-based ubiquitin E3 ligase, a component of DISC. Upon death receptor ligation, the DISC translocates to lipid-raft platforms (linked to the cytoskeleton) and comes into physical contact with CUL3 and mediates RING box protein (RBX1)-dependent polyubiquitination of caspase-8 on its C-terminal region within the p12 subunit, likely at lysine 461 residue. Polyubiquitination of caspase-8 promotes its aggregation and stability. After polyubiquitination of caspase-8, the Ub-binding protein p62 promotes translocation of the modified caspase-8 from receptor-associated complexes into higher molecular weight (MW) structures and allows aggregation of caspase-8 which enhances its activity and drives the auto-proteolytic release of caspase-8 into the cytosol to activate executioner caspases and trigger apoptosis (184).

1.6.2 Cleavage of Caspase-8 and its Functions

Whether or not cleavage of caspase-8 is necessary for its function remains controversial. It has been shown that cleavage of caspase-8 is required for effective

extrinsic cell death pathway. For example, c-$FLIP_L$, RIP and Bid were ineffectively cleaved by a caspase-8 mutant which cannot itself be cleaved (185). However, the cleavage of caspase-8 is not necessary for endotoxin-stimulated B lymphocytes proliferation, M-CSF-induced macrophage differentiation (85, 139), bone marrow hematopoietic progenitor differentiation (85, 185) and NF-κB activation (145). In general, cleavage of caspase-8 is primarily required for pro-apoptotic function but not required for non-apoptotic functions.

1.6.3 Phosphorylation of Caspase-8

Caspase-8 can be phosphorylated at different sites. Serine 364 (S364) of caspase-8 can be phosphorylated by p38 MAPK in freshly isolated neutrophils (186). Phosphorylation of S364 of caspase-8 has been shown to reduce its enzymatic activity (186). Moreover, serine phosphorylation of caspase-8 has been shown to be sustained in neutrophils that were treated with endotoxin, i.e. a gram negative bacterial toxin that has been shown to prolong the lifespan of neutrophils (178). Together, these data suggest that serine phosphorylation of caspase-8 negatively regulates the pro-apoptotic activity of caspase-8.

Caspase-8 can also be tyrosine phosphorylated by Src family kinases (SFKs) (52, 177, 178), specifically, tyrosine 380 (Y380) of caspase-8b isoform or Y397 of caspase-8a isoform has been shown to be phosphorylated by SFKs (177). Tyrosine phosphorylation of caspase-8 reduces it enzymatic activity (178), suppresses cellular apoptosis (177, 178) and promotes cell migration (52). These findings suggest that tyrosine phosphorylation of caspase-8 might promote its non-apoptotic functions while suppressing its pro-apoptotic functions.

Threonine 263 (T263) of caspase-8b isoform is another site that can be phosphorylated. Ribosomal S6 kinase 2 (RSK2) has been shown to phosphorylate T263, a process that is mediated by EGF (187). Phosphorylation of T263 results in ubiquitination and degradation of caspase-8 (187).

1.7 APOPTOSIS

1.7.1 Introduction

Caspase-8's major function is to initiate apoptosis. I will now focus my discussions on the mechanism of apoptosis. The word "apoptosis" comes from ancient Greek, meaning "falling off of petals from a flower" or "of leaves from a tree in autumn" (188). It was first used in 1972 by Kerr *et al* to designate a cell death process with specific morphological changes (189). Apoptosis is an energy consuming process (190) through which a cell undergoes self-destruction without causing bystander injury. It is a highly regulated process that involves gene transcription, protein translation, activation of protein, and cell signaling. Appropriate control of apoptosis is important for normal functioning (sculpting the embryo, maintaining tissue homeostasis, shaping the immune repertoire, terminating immune responses and restricting the progress of infections). Unregulated apoptosis has been linked to a variety of disease states. Excess apoptosis is thought to contribute to neurodegenerative diseases, whereas insufficient apoptosis can lead to diseases such as autoimmunity and cancer (191, 192). Apoptosis can be activated by a variety of apoptotic cues, including ligand activation of death receptors, growth-factor withdrawal, oncogenes, and anoxia (191). It is well established that caspases are central players of apoptosis. Apical caspases, such as caspase-8, -10 and -9 become activated by apoptotic signals followed by the activation of effector caspases, such as caspase-3, -6 and -7, resulting in a distinct set of biochemical and physical changes involving the cytoplasm, nucleus and plasma membrane (188). In the cytoplasm, vesicles and vacuoles are formed from endoplasmic reticulum. In the nucleus, chromatin condenses and aggregates and becomes fragmented. Then the nucleus becomes

convoluted and buds off into fragments within apoptotic bodies. In the plasma membrane, cell junctions are disintegrated, and the membrane becomes convoluted and blebs (188). Apoptosis can be activated via an intrinsic or an extrinsic pathway depending on the origin of the death stimuli. These pathways will be elaborated below and are illustrated in Figure 8.

Figure 8

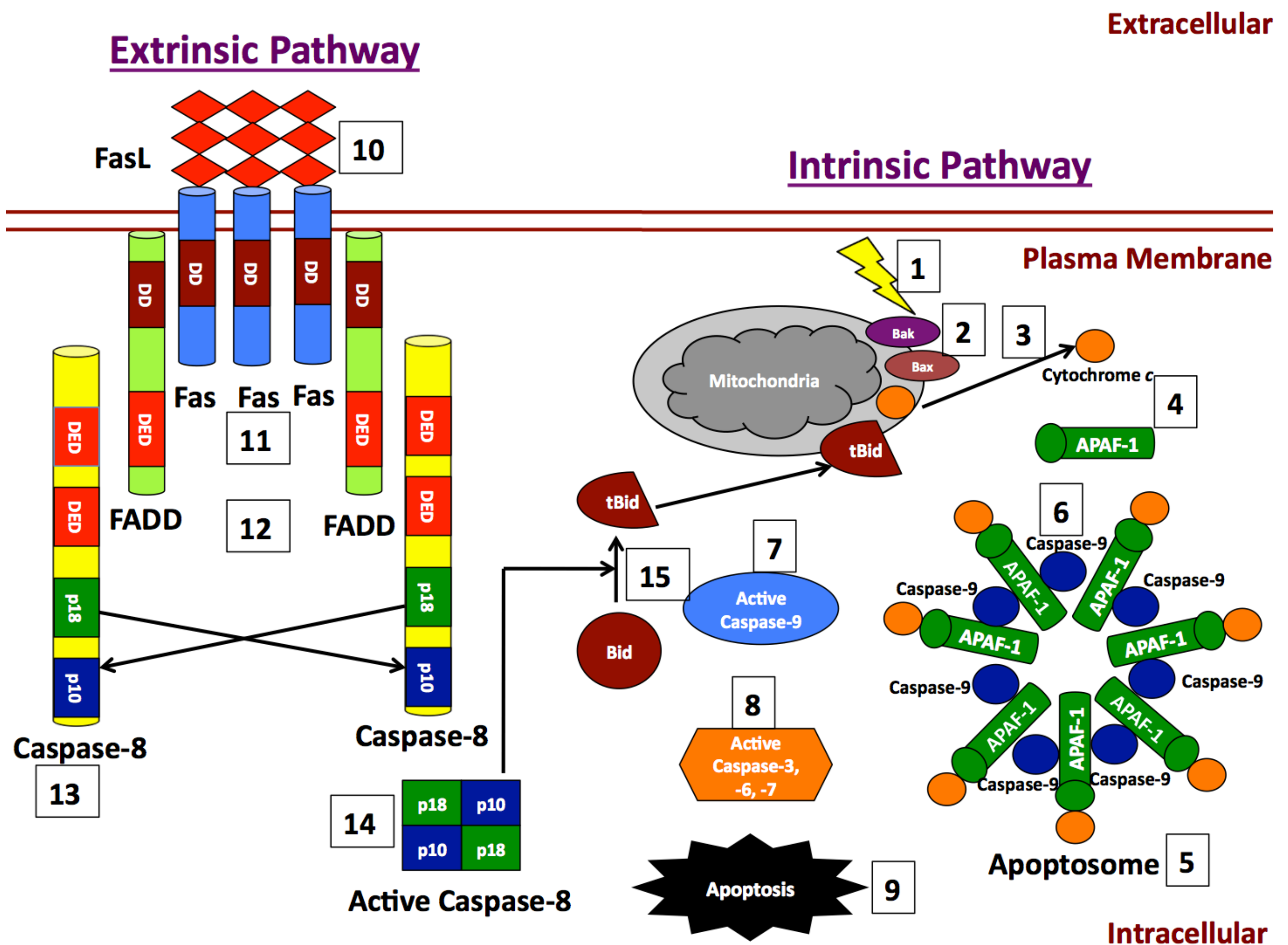


Figure 8. Intrinsic and Extrinsic Apoptotic Pathway

In the intrinsic pathway, an intracellular stress signal (1) such as oxidative stress, oncogene activation or DNA damage, activates BH1, BH2, BH3-containing Bcl-2 proteins, namely Bak and Bax (2). This allows pore formation in the outer membrane of mitochondria, allowing the release of cytochrome *c* into the cytoplasm (3). Cytochrome *c* then recruits APAF-1 in the cytoplasm (4), resulting in the formation of apoptosome (5), which recruits caspase-9 (6). The recruitment of caspase-9 to the apoptosome leads to the activation of caspase-9 (7). Activated caspase-9 in turn activates caspases-3, -6, -7 (8) resulting in the morphological changes seen in apoptosis (9) and cell death.

In the extrinsic pathway, an extracellular death signal, such as FasL (10), binds to death receptor such as Fas and trimerizes it (11). This allows the recruitment of adaptor molecule FADD (Fas Associated Death Domain) via interactions between death domains (DD) (12). Homotypic interactions between the death effector domains (DED) on FADD and caspase-8, lead to caspase-8 recruitment to the complex forming the death-inducing signaling complex (DISC) (13). This leads to activation of caspase-8 by induced proximity (14). Activated caspase-8 then activates caspase-3 (8) and apoptosis ensues (9). Activated caspase-8 can also cleave BH3 containing Bcl-2 protein, Bid, into truncated Bid (tBid) (15). tBid then translocates to mitochondrial outer membrane, allows the release of cytochrome *c* into the cytoplasm (3), activating the intrinsic pathway.

1.7.2 Intrinsic Apoptosis

When a cell senses an intracellular stress signal (such as oxidative stress, oncogene activation and DNA damage), Bcl-2 homology-3 (BH3)-only protein becomes activated, resulting in the oligomerization of the pro-apoptotic Bcl-2 family proteins Bak and/or Bax (BH1, BH2, and BH3 domains containing protein) (192). This allows the formation of pores in the mitochondrial outer membrane resulting in mitochondrial outer membrane permeabilization (MOMP) (193, 194). The permeabilization of mitochondrial outer membrane leads to the release of cytochrome *c* into the cytosol, which then associates with apoptosis protease activating factor-1 (APAF-1), changing its conformation. This then allows APAF-1 to bind to ATP/dATP and oligomerize to form a protein platform named the "apoptosome" (~1 MDa). An apoptosome contains seven APAF-1, seven cytochrome *c*, seven dATP and seven procaspase-9 (195). The apoptosome allows the binding and activation of caspase-9 by dimerization (7, 196-199). Active caspase-9 then goes on to cleave and activate effector caspases-3, -6, and -7 (194) and apoptosis ensues resulting in morphological changes as described above (Figure 8). The permeabilization of mitochondrial outer membrane also releases Smac/Diablo and Omi/HtrA2, which promotes caspase activation by eliminating inhibitor of apoptosis protein (IAP) function; flavoprotein apoptosis-inducing factor (AIF), which is implicated in chromatin condensation and large-scale DNA degradation; and endonuclease G, which might aid the caspase-activated DNase (CAD) nuclease in nucleosomal DNA fragmentation (192). See figure 8 and 9.

1.7.3 Extrinsic Apoptosis

Extrinsic apoptosis is also known as receptor-mediated apoptosis. It begins when a cell senses a death signal from the extracellular space. Extracellular death ligand, such as FasL (200), or tumor necrosis factor (TNF) (188, 201) can bind to its cell-surface death receptor (TNF receptor family) (91, 189, 202) , such as Fas (CD95/APO-1) (92, 190) and TNF receptor (TNFR) (188, 203) respectively, allowing it to form homotrimeric ligand-receptor complex. The trimerization of Fas or TNFR allows the recruitment, via its cytoplasmic death domain (DD) (188, 204), with adaptor proteins such as Fas associated protein with death domain (FADD), also known as MORT1 (15, 192, 205, 206). FADD also contains death effector domain (DED), a domain that interacts with the DED (homophilic interaction) of caspase-8 or -10, in the N-terminus (14, 15, 26, 193, 194, 207). The recruitment of caspase-8 or -10 to Fas/FADD complex forms an oligomeric death-inducing signaling complex (DISC) within seconds of receptor engagement (26, 195, 205, 208). The DISC allows the oligomerization, self-cleavage and activation of apical caspases, caspases-8 or -10 in an induced proximity model (7, 26, 43, 44, 196-199, 209). In cells where the active caspase-8 or -10 directly cleaves and activates effector caspases, caspases-3 and -7, subsequently, caspase-3 activates caspase-6 (194, 210, 211). In cells where the active caspase-8 level is lower, caspase-8 inserts itself after the first cleavage into the mitochondrial membrane where it is anchored by cardiolipin (200, 212). Here caspase-8 further oligomerizes and undergoes cleavage to form a mitochondria-associated caspase-8 active complex. Active caspase-8 then cleaves Bid (a BH3-only member of the Bcl-2 family of proteins), which then triggers the release

of mitochondrial proteins including cytochrome *c* and the XIAP-inhibitor SMAC, leading to exuberant caspase-3/-7 activity (36, 97, 211, 213, 214). See Figure 8 and 9.

Figure 9

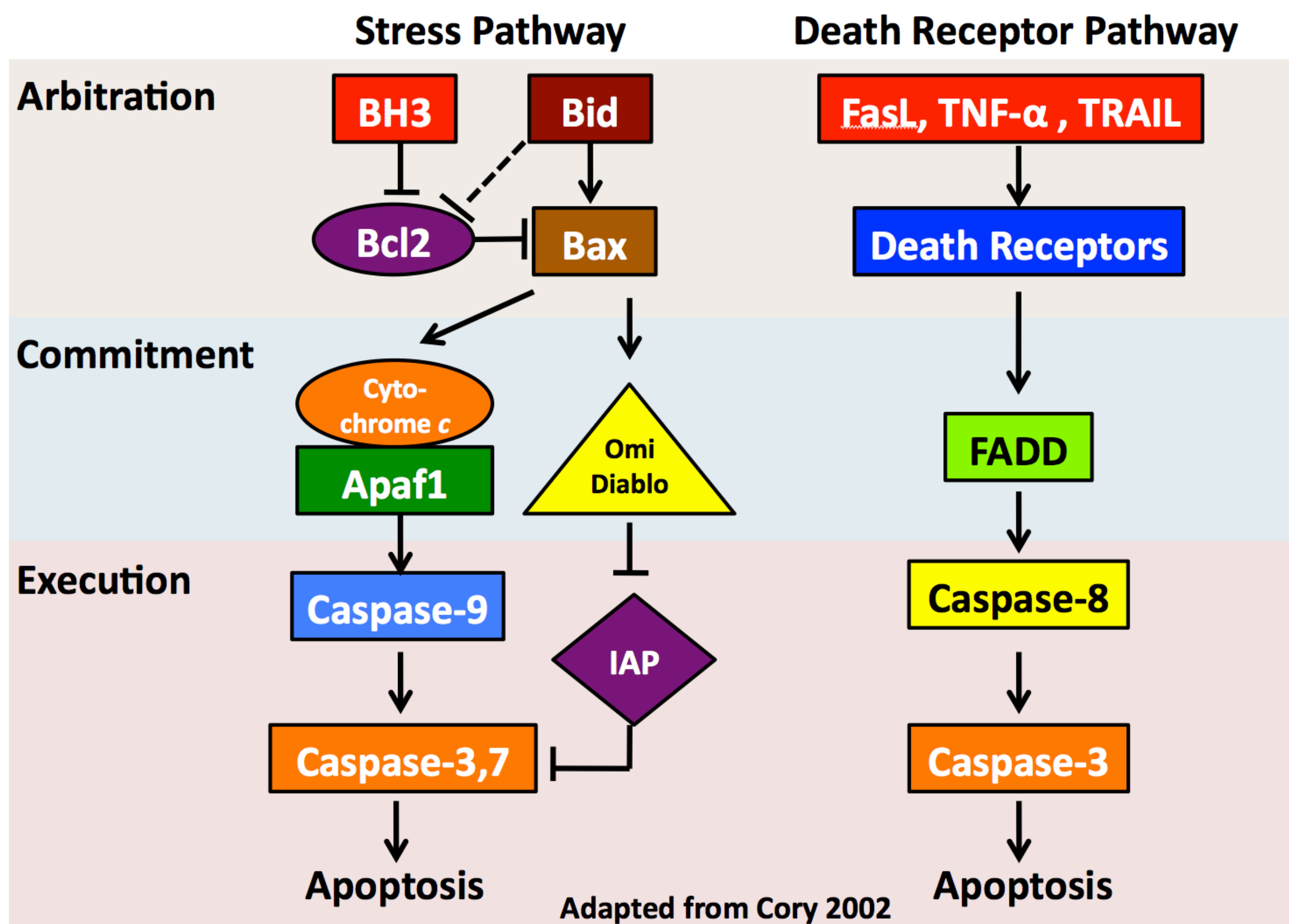


Figure 9. Stress (Intrinsic) and Death-Receptor (Extrinsic) Apoptotic Pathways

Stress (intrinsic) and death-receptor (extrinsic) apoptotic pathways are the two main apoptotic pathways in cells. In the stress pathway, signals are mediated through the Bcl2 family, whereas the death-receptor pathway is activated by signals from other cells. In the stress pathway, when Bax binds to mitochondrial membrane, it allows the release of cytochrome *c* and Omi/Diablo into the cytoplasm. Cytochrome *c* binds to Apaf1 and activates caspase-9, which subsequently activates caspase-3 and -7 and apoptosis ensues. Omi/Diablo antagonizes inhibitors of apoptosis proteins (IAP) therefore preventing it from suppressing caspase-9, -3, and -7 and therefore promoting apoptosis. The death-receptor pathway starts with death ligands (FasL, TNF-α, TRAIL) binding to death receptors, which then recruits FADD and then caspase-8 allowing the activation of caspase-8 and thus activation of caspase-3 and therefore apoptosis. Caspase-8 can also trigger the stress pathway by cleaving Bid and activating it.

1.7.4 The Consequences of Effector Caspases Activation

Both the intrinsic and extrinsic pathways of apoptosis lead to the activation of effector caspases, caspases-3, -6 and -7. The effector caspases are capable of cleaving many important cellular substrates that lead to morphological changes seen in apoptosis and ultimately cell death (188). Caspase-3 can cleave inhibitor of caspase-activated DNase (ICAD) resulting in the release of caspase-activated DNase (CAD), allowing CAD to move into the nucleus and cleave DNA internucleosomally. This results in DNA fragmentation (215-217). Chromatin condensation, nuclear shrinking and budding are due to the cleavage of nuclear lamins (218, 219). The cleavage of cytoskeletal proteins such as actin, fodrin, focal adhesion kinase (FAK) (220) and gelsolin (221) leads to the loss of overall cell shape (221, 222). The active blebbing is orchestrated through caspase-mediated cleavage of PAK2 (223) and Rho-associated coiled-coil forming kinase I (ROCK-I) cleavage renders the kinase constitutively active, leading to constitutive phosphorylation of myosin light chain (224-226). Once cleaved, ROCK-I stimulates actomyosin-based contractility and is responsible for bleb formation in apoptotic cells (224-226). Finally, caspases inactivate flippase, an ATP-dependent enzyme that maintains phosphatidylserine (PS) at the inner layer of the plasma membrane, resulting in the redistribution of PS to the outer leaflet of the plasma membrane, generating an "eat-me" signals that stimulate phagocytosis (via PS receptor) of apoptotic cells by surrounding cells (194, 227, 228). Phagocytosis of apoptotic bodies results in the release of anti-inflammatory cytokines and immune tolerance (229, 230).

1.8 SRC FAMILY PROTEIN TYROSINE KINASE

1.8.1 Overview

c-Src is the prototypic member of a family of non-receptor tyrosine kinases, known as Src family kinases (SFKs), that play a central role in the control of cell proliferation, survival, differentiation, migration, invasion, angiogenesis, adhesion, cytoskeletal rearrangement, vesicle trafficking by cell surface receptors and immune function (231, 232). Including c-Src, there are a total of nine members of SFKs, namely Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes and Yrk (233, 234). Src, Yes and Fyn are described as "ubiquitously" expressed, whereas Lck, Lyn, Blk, Fgr and Hck are uniquely expressed at relatively high levels in various hematopoietic lineages. Myeloid lineage cells primarily express Hck, Fgr and Lyn; B cells primarily express Lyn, Fyn and Blk; whereas T cells primarily express Lck and Fyn-T (a hematopoietic cell-specific isoform which contains an alternatively spliced form of exon 7 that encodes a part of the kinase domain (232, 235)) (234, 236) (Table 2). *c-Src* is a proto-oncogene, a cellular precursor of a retroviral (Rous sarcoma virus) transforming gene, *v-src* (237). However, compared to *v-src, c-src* has low transforming activity in both mammalian cell lines and chicken embryo fibroblasts (237-246). The protein product of *v-src,* like *c-src* (247, 248)*,* is a protein tyrosine kinase (249-256). Interestingly, c-Src has a much lower kinase activity than v-Src (256) potentially explaining the low transforming activity of c-Src.

Table 2

SFK Family Member	**Pattern of Expression**
Blk	B cells
Fgr	Myeloid cells, B cells
Fyn	Ubiquitous
Hck	Myeloid cells
Lck	T cells, NK cells, brain
Lyn	Brain, B cells, myeloid cells
c-Src	Ubiquitous
Yes	Ubiquitous
Yrk	Ubiquitous

Table 2. Src Family Kinase Members and Their Pattern of Expression

There are a total of nine Src family kinase members. Blk is primarily expressed in B cells, Fgr is primarily expressed in myeloid cells and B cells, Hck is primarily expressed in myeloid cells, Lck is primarily expressed in T cells, NK cells and brain cells, Lyn is primarily expressed in B cells, myeloid cells and brain cells. Fyn, c-Src, Yes and Yrk are ubiquitously expressed in all cell types.

1.8.2 Structure of Src Family Protein Tyrosine Kinase

All nine members of the SFKs share a common structure that is comprised of four Src homology (SH) domains. Starting from the N-terminus lies the SH4 domain, followed by SH3, SH2 and SH1 domains (Figure 10A) (257-259). The SH4 domain regulates cellular localization, the SH2 and SH3 domains regulate the interactions with binding partners whereas the SH1 domain regulates enzymatic activity (234, 260). All SFKs are N-terminally myristoylated and seven out of nine family members are also N-terminally palmitoylated (261-264). All SFKs have a conserved glycine residue immediately after the start methionine. The start methionine is removed and myristate is cotranslationally fused to the glycine at position 2 (265, 266). Most SFKs undergo reversible palmitoylation at cysteine 3 and at either cysteine 5 (Lck) or cysteine 6 (Fyn) (262, 267-270). The fatty acid moieties (myristoylation and palmitoylation) are required for localization to cellular membranes (232, 271) and subsequent activation. Removal of glycine 2 (myristoylation) or cysteine (palmitoylation) residues prevents membrane localization of SFKs and subsequent activation by receptor binding stimuli (263, 272). Immediately after the SH4 membrane-targeting region lies a “unique” domain of 50-70 residues, which is divergent among family members (258, 273).

The SH3 domain can interact with proline-rich domains (PXXP) allowing the interaction of SFKs with intracellular substrates (274-276). It also interacts with the short polyproline type II helix between the SH2 and kinase domains in the inactive conformation of the protein (258, 261, 277). The SH2 domains recognizes and interact with phosphotyrosine peptide (278-281). It can also bind to the autoinhibitory tyrosine

residue (tyrosine 527 in chicken c-Src; tyrosine 530 in human c-Src) (261), located in its C-terminal tail, forming a closed inactive conformation (238, 241, 243, 282-285).

The SH1 tyrosine kinase domain contains the autophosphorylation site (tyrosine 416 in chicken c-Src; tyrosine 419 in human c-Src) within a segment of the kinase domain termed the activation loop (258). The autophosphorylation site is important because it has been shown to be required for full c-Src activation (261). Followed by the SH1 domain lies a short C-terminal tail, which is a hallmark of Src kinase, which bears an autoinhibitory phosphorylation site (tyrosine 527 in chicken c-Src; tyrosine 530 in human c-Src) (286). Of note, the C-terminal autoinhibitory phosphorylation site is absent in v-Src (286, 287), contributing to its constitutive activity.

Figure 10A

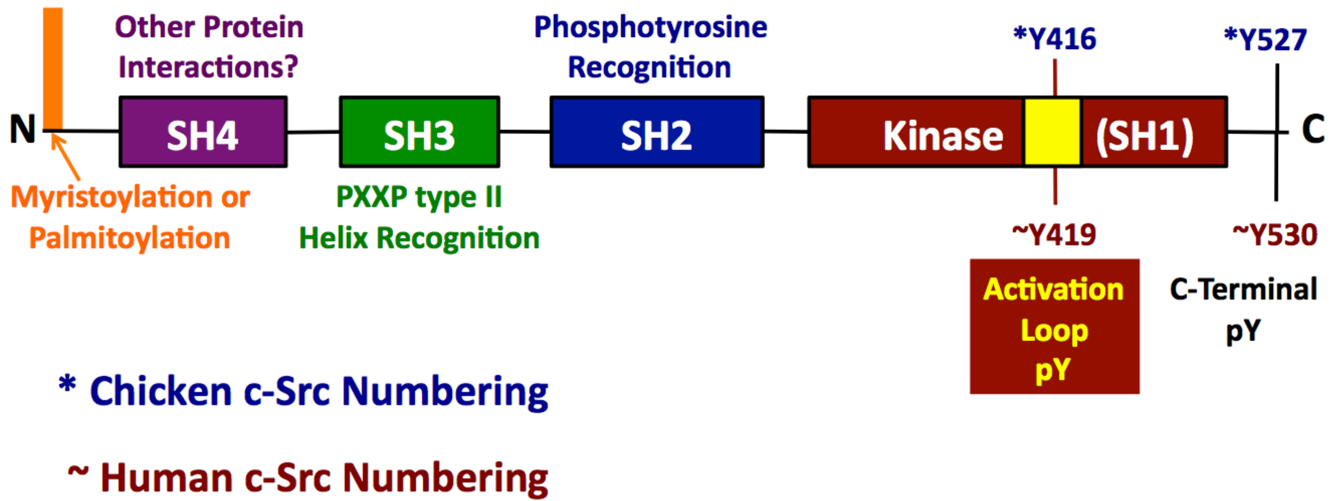


Figure 10A. The Secondary Structure of c-Src

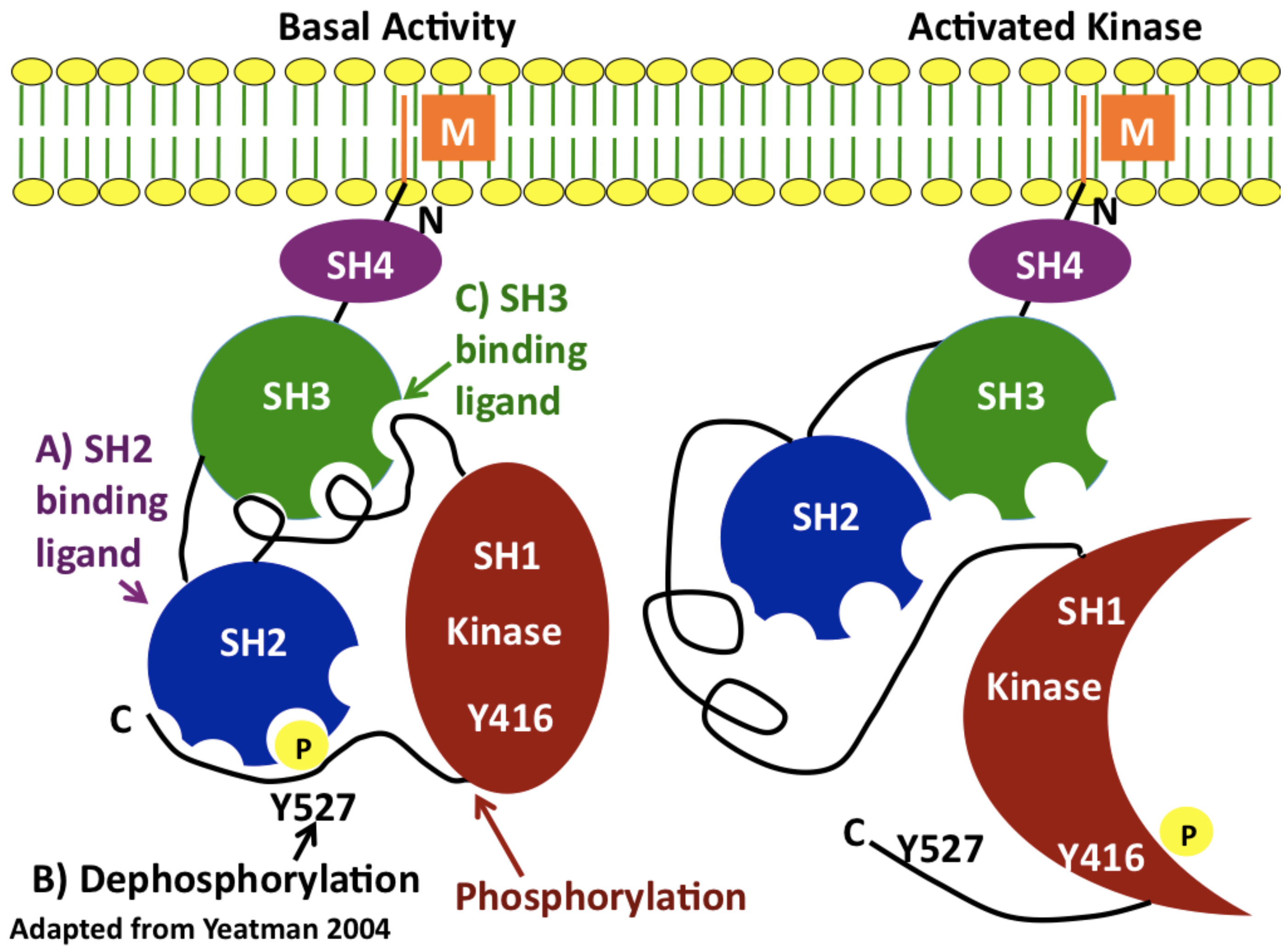
The secondary structure of c-Src is depicted here. The domain structure of Src family kinases (SFKs) consists of four Src homology (SH) domains: the unique region (SH4), followed by the SH3, SH2 and tyrosine kinase (SH1) domains. The SH3 domain binds to proline-rich region (PXXP type II helix recognition). The SH2 domain recognizes and binds to phosphotyrosine peptide, such as the autoinhibitory phosphorylated tyrosine residue in the C-terminal tail (tyrosine 527 in chicken c-Src, tyrosine 530 in human c-Src). The kinase (SH1) domain contains an activation loop tyrosine residue (tyrosine 416 in chicken c-Src, tyrosine 419 in human c-Src). It is involved in autophosphorylation and is required for full activation of SFKs. All SFKs are myristoylated in the N-terminus and seven of the SFKs are also palmitoylated.

1.9 ACTIVATION OF C-SRC

The mechanism of c-Src activation became apparent with the help of the three-dimensional structures of c-Src (243) and Hck (238). In its inactive state, the SH3 and SH2 domains interact with the kinase domain via the short polyproline type II helix between the SH2 and kinase domains in the N lobe of the kinase and the C-terminal tail autoinhibitory phosphotyrosine 527 in the C lobe of the kinase, respectively (278). These protein interaction motifs keep the kinase in an inactive conformation through the intramolecular interactions (259). Intriguingly, the position of the SH3 and SH2 domain does not physically occlude the catalytic cleft. Instead, this inactive conformation prevents the binding of peptide substrates and also protects tyrosine 416 from being phosphorylated (258, 259, 277).

In order for c-Src to be activated, the closed inactive conformation must be opened up. This can be achieved by the binding of SH2 or SH3 ligands (288) or the dephosphorylation of tyrosine 527. These events all lead to the disassembly of the regulatory domains from the catalytic domain, exposing tyrosine 416 to be autophosphorylated (245). The tyrosine 416 residue is located in the catalytic centre. Its phosphorylation is essential in the activation of c-Src in that it allows substrates to bind to the catalytic centre of the kinase domain (246, 258, 277). Tyrosine 416 is phosphorylated through intermolecular autophosphorylation (289) (Figure 10B). The dephosphorylation of tyrosine 527 and phosphorylation of tyrosine 416 are both required in the full activation of c-Src.

Figure 10B



Adapted from Yeatman 2004

Figure 10B. The Activation of c-Src

c-Src is in an inactivated state when its C-terminal tyrosine 527 is phosphorylated and it binds to the SH2 domain. There is also an interaction between the SH3 domain and the back of the kinase domain. These result in a closed molecular structure preventing access of substrates to the kinase domain. A few different mechanisms could result in the opening of the c-Src molecule to allow the access of substrates to the kinase domain and the autophosphorylation of activation loop tyrosine 416 to complete the activation of c-Src. These mechanisms include: A) the binding of phosphotyrosine to the SH2 domain resulting in the release of its binding to C-terminal tyrosine 527; B) dephosphorylation of C-terminal tyrosine 527 resulting in the release of SH2 domain from its binding; and C) the binding of proline rich domain to the SH3 domain resulting in the release its binding from the back of the kinase domain.

1.10 REGULATION OF C-SRC

SFKs are regulated by various inputs, including tyrosine kinase receptors and integrins, G-protein-coupled receptors, such as β-adrenergic receptor (290), antigen and Fc receptors (234), cytokine receptors such as TNF-related activation induced cytokine (TRANCE) (291), protein tyrosine phosphatases such as protein tyrosine phosphatase-α (PTPα) (249) and even by steroid receptors such as progesterone receptor (292).

1.10.1 Intramolecular Regulation of c-Src

c-Src can be regulated by proteins that modify both the intramolecular (inhibitory) interactions and the phosphorylation/dephosphorylation of the inhibitory tyrosine 527 site (259). Firstly, carboxy-terminal Src kinase (Csk) (293-295) or its homolog Chk (51-53) have been shown to phosphorylate tyrosine 527 (286) of c-Src. This leads to inactivation of c-Src (54-56). When the autoinhibitory tyrosine 527 is phosphorylated, it promotes the assembly of the SH2, SH3, and kinase domains into an autoinhibited conformation maintained by intimate interactions among these domains (238, 241, 243, 245, 246).

In order for c-Src to be activated, tyrosine 527 must by dephosphorylated (247). This could be achieved by different protein tyrosine phosphatases, such as PTPα (249, 251), PTP1, SH2-containing phosphatase 1 (SHP1), SHP2 (233) and protein tyrosine phosphatase CD45 (231).

A myriad of cellular stimuli, including polypeptide growth factors, hormones, integrin aggregation, stress, cell cycle progression, can lead to the phosphorylation of signaling proteins. The phosphorylation of signaling proteins then acts as a SH2 binding partner allowing the SH2 domain of c-Src to be released from its own C-terminal tail.

This leads to an active conformation through phosphatase-mediated dephosphorylation of tyrosine 527 then autophosphorylation of tyrosine 416 (232).

In addition, the direct binding of focal-adhesion kinase (FAK) (236) or its molecular partner Crk-associated substrate (CAS) (257) to the SH2 and SH3 (234) domains of c-Src also results in the open, active configuration of c-Src by displacing the C-terminal tail from binding to the SH2 domain of c-Src (261).

1.10.2 Receptor-Mediated Activation of c-Src

Interactions of c-Src with ligand-activated receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR) (265), PDGFR (267, 269, 270), ERBB2 (also known as HER2/Neu)(271), fibroblast growth factor receptor (FGFR) (272), colony-stimulating factor 1 receptor (CSF-1R) (273) and hepatocyte growth factor receptor (HGFR) (274) can result in increased c-Src activation. This is due to the disruption of the intramolecular interactions that hold c-Src in a closed, inactive configuration (261).

The activation of SFKs by RTKs is a sophisticated event, more than just a simple recruitment. First of all, the activation of c-Src by EGFR requires small GTPases, Ras and Ral (296). Secondly, in response to PDGF, EGF and FGF stimulation, protein tyrosine phosphatase SHP2 is recruited to the activated RTKs. There, it dephosphorylates the Csk-binding protein PAG, resulting in the prevention of Csk's access to c-Src (297). This then prevents the phosphorylation of the autoinhibitory tyrosine 527 (298), therefore, resulting in the activation of c-Src.

1.10.3 Other Mechanisms of c-Src Regulation

Another way to regulate SFK function is through their submembrane localization. This is regulated by lipid modification (myristoylation and palmitoylation) and interaction with localized binding partners such as other kinases and phosphatases which are responsible in regulating SFKs' catalytic activity (260). Ubiquitination followed by degradation by the proteasome CBL ubiquitin ligase is another regulatory mechanisms of c-Src activity (299). Intriguingly, in cancer cells, the ubiquitin-proteasome pathway is deregulated, resulting in exuberate c-Src activation (300). Finally, one of the most intriguing potential mechanisms for c-Src activation is through naturally occurring mutational events. It has been shown that in some colon cancer cells (301) and endometrial cancer cells (302), c-Src is truncated at amino acid 531, just C-terminal to the autoinhibitory tyrosine 530 (equivalent to tyrosine 527 in chicken c-Src). This results in increased c-Src activation because the autoinhibitory tyrosine 530 could no longer interact with its own SH2 domain to keep the c-Src molecule in closed, inactive conformation.

1.11 CELLULAR FUNCTIONS OF C-SRC

The functions of c-Src are pleiotropic, ranging from regulation of mitogenesis, cell growth, survival, apoptosis, adhesion and migration. Other functions of c-Src include vesicle trafficking, cytoskeletal rearrangement, B cell and T cell development and activation. Based on the functions of c-Src, it is understandably that c-Src plays an important role in tumorigenesis and tumor progression.

1.11.1 Regulation of Mitogenesis and Cell Growth

v-Src has been shown to suppress the expression of cyclin-dependent kinase (CDK) inhibitor p27. This allows the progression of cell cycle (transition of the G1 phase of the cell cycle and prevention of entry of the quiescent state) despite survival factors are limited (303, 304). Moreover, v-Src in quiescent cells sequentially induces the expression of cyclins D1/E and A and activates cyclin D1, D3/CDK4/6, cyclin E/CDK2 and cyclin A/CDK2 (304). The activities of the phosphatidylinositol 3-kinase (PI3K) and mitogen activated protein kinase kinase (MEK) pathways are crucial in the cell cycle effects stimulated by v-Src. In addition, the activation of MEK/extracellular signal-regulated kinase (ERK) signaling (304, 305), possibly mediated by tyrosine phosphorylation of FAK and coupling to the Grb2/Ras/MEK pathway (306) are required to drive cells through G1 and into S-phase (304).

A stimulation of c-Src tyrosine kinase activity was seen in mitotic cells (307), which suggests that c-Src might play a role in the regulation of mitosis. The mechanism of c-Src activation seen during mitosis is as follow. During mitosis, Cdc2 kinase phosphorylates c-Src at serine and threonine residues (308, 309), resulting in the modification of tyrosine 527-SH2 interaction. This leads to partial activation of c-Src

followed by the dephosphorylation of tyrosine 527 by a protein tyrosine phosphatase which is active during mitosis (310).

By using a kinase-dead c-Src mutant or by using antibodies to inhibit c-Src, studies have shown that c-Src is crucial in the mitogenic activity of PDGF (311-314), EGF (233, 247, 251, 265, 314, 315), FGF-1 (316, 317), CSF-1 (231, 236), stem cell factor (SCF) (318, 319) and insulin growth factor (IGF-1) (320). The mechanism involves the production of c-Myc (313, 321), which is a transcription factor required for mitogenesis in response to growth factors (322). In addition, signal transducer and activator of transcription 3 (STAT3) (269) is implicated in c-Src-induced mitogenesis through the proposed PDGF/PDGFR/c-Src/STAT3/c-Myc pathway (306).

There is also evidence that suggests c-Src cooperates with the EGFR in growth signaling (231, 251). Interestingly, in breast cancer cells, both EGFR and c-Src protein expression are often upregulated. Moreover, in fibroblasts that overexpress c-Src (in the presence of myristolyation and catalytic activity), there is an augmented mitogenic response to EGF stimulation (247). When c-Src (catalytically active) and EyouGFR are co-expressed in CH3H10T1/2 cells, it stimulates EGF-induced anchorage-independent growth and tumor development in nude mice (265, 315). The c-Src/STAT/c-Myc pathway is implicated in the EGF-induced mitogenesis (323) because after the activation of ErbB by EGF, c-Src is required for the activation of STAT transcription factors (324).

Other potential mitogenic targets of SFKs include Vav2, a guanine nucleotide exchange factor (325, 326), Golgi-associated Ras activation (327, 328), and protein kinase C family members (329-333). c-Src is specifically required at points during G1 and for completion of G_2/M transition (233, 307).

1.11.2 Regulation of Cell Survival and Apoptosis

The role of v-Src on apoptosis is complex. It can either prime cells for it or protect cells against it (334). When survival factors are limiting such as in low serum conditions, v-Src promotes cell survival via the activation of PI3K and Akt (334). v-Src transformed cells can overcome the normal adhesion requirement of cell cycle progress and promote anchorage-independent growth. This is due to its ability to promote cell cycle progression in the absence of growth factors (334).

In the process known as “anoikis”, cells undergo programmed cell death upon detachment from extracellular matrix (335). Intriguingly, v-Src can protect epithelial cells from anoikis and also promote cell survival upon UV treatment (336) through the activation of the PI3K/Akt pathway (337) and the MEK/MAPK-dependent induction of the anti-apoptotic Bcl2-family protein Bcl-X_L (338). STAT3, a transcription factor for Bcl-X_L (339), is also thought to be involved (340).

The role of c-Src activity in carcinoma cells is thought to involve the induction of anoikis resistance by the expression of anti-apoptotic Bcl-2 family protein via the activation of the PI3K/Akt pathway (341), resulting in cancer progression (340). This could potentially explain the survival of cancerous cells in the absence of extracellular matrix attachment and in the presence of noxious stimuli (334). Of note, the c-Src expression and activity levels correlate well with resistance to anoikis. Moreover, c-Src has been shown to play a role in TRANCE-receptor/TRAF/Src/PI3K/Akt activation that mediates cell survival (291). Moreover, Src, PI3K/Akt-1 and MEK/Erk pathways are responsible for the survival of human epithelial cells (342) and human neuronal cells (343).

1.11.3 Regulation of Cell Adhesion, Motility and Invasion

c-Src has been shown to regulate cell adhesion, motility and invasion. In c-Src transfected fibroblasts and cancer cells, there are less intercellular adhesion, increased motility and augmented ability to invade matrix substrates. In addition, there is a decreased number of bundled actin filaments, cellular contacts (focal adhesion of mesenchymal cells, cell-cell adhesions of epithelial cells) and gap junction communications (340). Indeed, not only is c-Src expression increased in the early course of colon cancer development (such as in colonic polyps and adenomas) (344), there is also an augmented c-Src-specific activity in later stage of cancer development (345, 346), suggesting that c-Src also contributes to metastatic potential through their effects on adhesion, motility and invasion (347).

c-Src regulates focal adhesions and adherens junctions – two principal subcellular structures that control cell adhesion, invasion and motility (348, 349). c-Src is also responsible for blocking downstream integrin signaling resulting in the disassembly of focal-adhesion structures and in the reduction of cell clustering (350). On the contrary, the expression of kinase-dead v-Src leads to the exaggeration of focal adhesions formation (351). In addition, v-Src can induce tyrosine phosphorylation of Ras-related protein (RRAS), which then form a complex with v-Src to inhibit integrin activity and reduce cell-matrix adhesion (352). This suggests that c-Src could also affect the integrity of focal adhesion through RRAS. Data has also suggested that c-Src could regulate the stability of focal adhesion via its interaction of FAK.

Not only does c-Src promote the release of cells from the extracellular matix, it also promotes the release of cells from each other through its action on E-cadherin.

Specifically, c-Src disrupts adherens junctions by inhibiting the localization of E-cadherin, thus preventing its function at these contact points. Moreover, c-Src and other tyrosine kinases phosphorylate and promote ubiquitination of the E-cadherin complex, which leads to the endocytosis of E-cadherin (353). Of note, c-Src-induced tyrosine phosphorylation of FAK is equally important for the disruption of E-cadherin cell-cell contacts.

FAK is known as a non-receptor tyrosine kinase. It is important in the regulation of cell-cycle progression, cell survival, proliferation and migration (354). It controls both cell-cell and cell-matrix adhesion. It localizes to the focal adhesions that form between growing cells in the presence of extracellular matrix constituents, such as fibronectin. Being the first identified c-Src substrates, FAK is tyrosine phosphorylated by c-Src, which leads to its activation and the loss of focal adhesion (351). The FAK-c-Src complex, CAS, paxillin, Erk and myosin light-chain kinase (MLCK) are all important in the turnover of adhesion in the migrating cell front (355). The sequence of events is as follow. First, the FAK-c-Src complex phosphorylates paxillin and CAS. This is followed by the recruitment of other adhesion molecules and the regulation of cytoskeletal organization (356). Then, c-Src, FAK and CAS form a trimeric complex via the binding of c-Src SH2 domain to FAK and c-Src SH2 domain to CAS. FAK-c-Src signaling then results in the activation of Erk, which then phosphorylates and activates MLCK. All together, these steps lead to the disassembly of adhesion (261). Many cancer cells demonstrate concordant c-Src and FAK overexpression and activation, which can lead to increased invasion and metastasis (357).

1.11.4 The Role of c-Src in Tumorigenesis and Tumor Progression

It is a noticeable event when v-Src transforms normal fibroblasts. It results in marked morphological changes including cell rounding and disaggregation. As the cells lose their intercellular, integrin-based cytoskeletal attachment in an organized monolayer (261), they float in culture medium. Without cytoskeletal attachment, v-Src transformed cells are more motile and more prone to invade the basement membrane matrix. Within a few weeks, v-Src transformed cells begin to clump, forming cell foci where they lose their contact inhibition of growth (a hallmark of cancer cell). These cells are able to invade and metastasize (261).

Similar to oncogenic v-Src, activated mutants of c-Src can also transform cells in culture (358-360) and induce tumors in chickens (361). In gastrointestinal malignancies (hepatocellular, pancreatic, gastric and esophageal cancer (362)), breast (363), ovarian (364) and lung cancers (364), there is an increase in c-Src activity. More intriguingly, in hepatocellular cancers (365, 366) and colon cancers (367, 368), there is an overexpression of c-Src protein and an underexpression of CSK protein, resulting in augmented c-Src activation. In some cancers, despite having normal levels of c-Src protein expression, there is an increased specific activity of c-Src (261). Moreover, as cancer advances, both high levels of c-Src protein and c-Src kinase activity have been observed (364).

Interestingly, despite the requirement of c-Src in fibroblast cell division (231), and the potential role of c-Src in stimulating the proliferation of precancerous cells, the overexpression of c-Src in human colon carcinoma cells does not promote cell growth. Instead, it stimulates the assembly of integrin adhesions and augments the ability of cells

to spread on a substrate (369). Moreover, despite the co-transfection of EGFR and c-Src into fibroblasts causing increased proliferation, invasiveness and tumorigenesis (265), the combined effects of EGFR and c-Src only promote colon cancer cell metastasis but not proliferation. Together, these data imply that c-Src activation is involved in cell proliferation in the process of tumorigensis. However, in later stage of tumor development, c-Src activities control tumor cell adhesion and invasion (306).

More intriguingly, Yeatman *et al* has demonstrated the existence of a naturally occurring c-Src mutation in a subset of advanced human colon cancer (370). Likewise, Sugimura *et al* also has demonstrated the existence of the same c-Src mutation in endometrial carcinoma (302). They showed the existence of a truncated c-Src at amino acid 531. The truncated form of c-Src behaves like v-Src in that it is activating, transforming, tumorigenic and promotes metastasis (370). The truncation of c-Src at 531 prevents the autoinhibitory tyrosine 530 (equivalent to tyrosine 527 in chicken c-Src) from binding to its own SH2 domain, therefore, preventing it from forming a closed and inactive conformation. These data further support the notion of c-Src in the pathogenesis of tumor.

1.12 INTERACTION BETWEEN CASPASE-8 AND SFKS

Over the past few years, data has emerged that suggested the interaction between SFKs and caspase-8. Our group has shown that Lyn interacts with caspase-8 (unpublished data) while other groups have shown that c-Src interacts with caspase-8 (52, 53, 141). Various groups have studied the caspase-8 sites involved in the interaction with c-Src. Barbero *et al* showed that the linker region of caspase-8 where tyrosine 380 (caspase-8b isoform) is located is responsible for its interaction with c-Src. The $pY_{380}LEM$ motif is thought to act as a SH2 domain binding partner of c-Src's SH2 domain (52). Interestingly, another group has suggested that the RXDLL motif within the first DED domain of caspase-8 is responsible for its interaction with c-Src (53, 141).

Epidermal growth factor (EGF) is the cellular signal that promotes the interaction of caspase-8 with c-Src (53) and it has been suggested that upon EGF stimulation, the interaction between caspase-8 and c-Src promotes c-Src activation. This is based on the observation that upon EGF stimulation, the c-Src molecules that were immunoprecipitated with caspase-8 were phosphorylated at tyrosine 416 (53). Furthermore, it has been shown that caspase-8 is important in downstream Erk1/2 activation stimulated by EGF (53, 141).

1.13 BCL-2 FAMILY OF PROTEINS

1.13.1 Three Families of Mammalian Bcl-2 Proteins and Their Domain Structures

The Bcl-2 protein, encoded by the *BCL2* (B-cell lymphoma 2) gene, is an anti-apoptotic protein (371). Bcl-2 prevents most forms of apoptotic cell death as well as certain forms of necrotic cell death (372). In mammals, Bcl-2 has at least 20 relatives, all of which share at least one conserved Bcl-2 homology (BH) domain (Figure 11). The Bcl-2 family is further divided into three families: 1) the anti-apoptotic, 2) Bax, and 3) the BH3-only families. Both the Bax and the BH3-only families are pro-apoptotic. The anti-apoptotic Bcl-2 family includes Bcl-2, Bcl-x_L, Bcl-w, A1 (Bfl-1), Mcl1 and Boo. They share sequence homology within four regions, namely BH1, BH2, BH3 and BH4 domains. They potently inhibit apoptosis in response to many cytotoxic insults. Members of the pro-apoptotic Bax death family include Bax, Bak, Bok (Mtd) and Diva, and share sequence homology in BH1, BH2 and BH3 domains (373). Members of the BH3-only pro-apoptotic family only share sequence homology in BH3. They include Bid, Bim, Bik, Bad, Bmf, Hrk (DP5), Blk, Bnip3, Bnip3L, Noxa and Puma (192). Both types of pro-apoptotic proteins are crucial in the initiation of apoptosis. The BH3-only proteins serve as a sensor for any form of cellular damage and directly antagonize pro-survival proteins. The Bax-like proteins serve to disrupt mitochondrial membrane integrity (192).

Figure 11

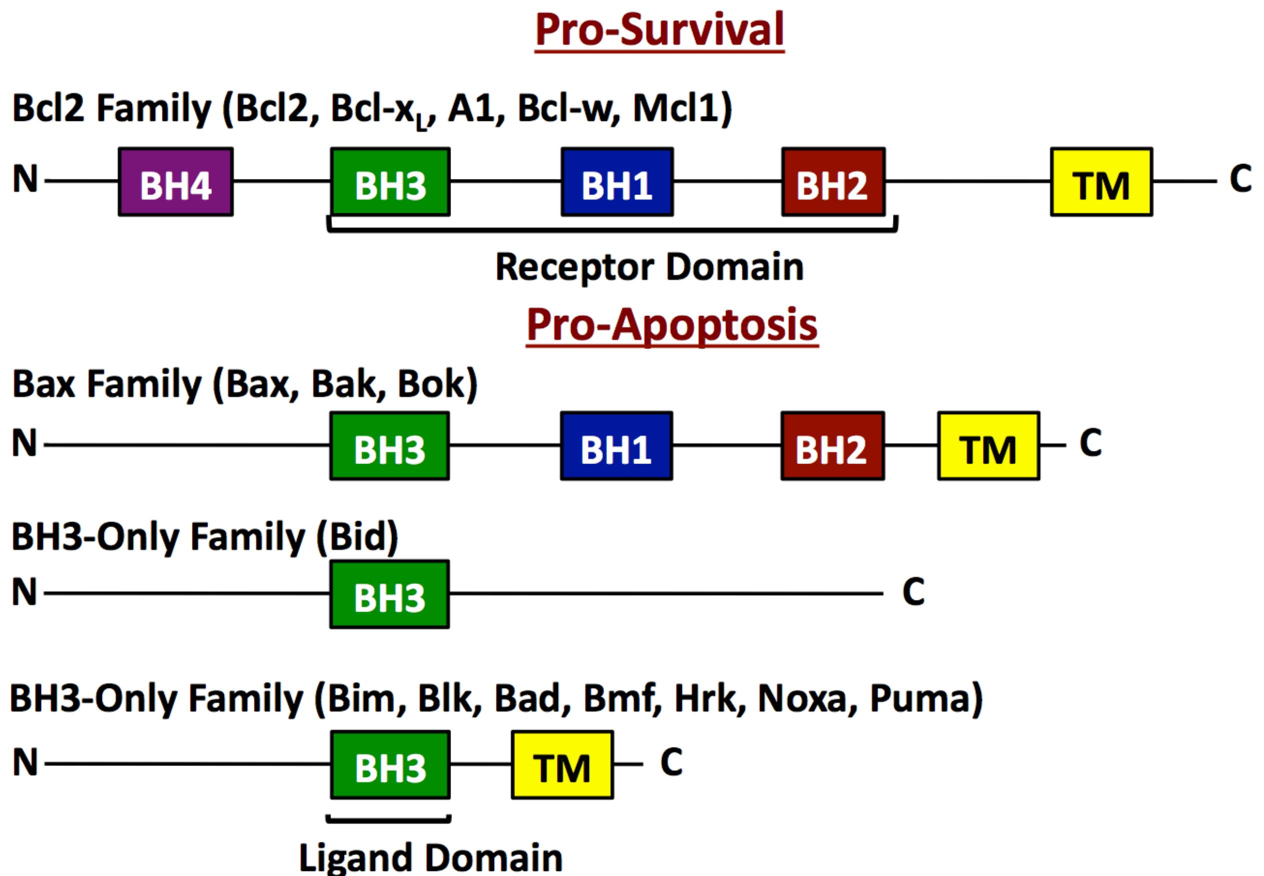


Figure 11. Bcl-2-Related Proteins and Their Structural Domains

Bcl-2-related proteins are divided into pro-survival and pro-apoptotic families. The pro-survival family, the Bcl-2 family includes Bcl-2, Bcl-x_L, A1, Bcl-w, and Mcl1. These members are consisted of BH1, 2, 3 and 4 domains and a transmembrane (TM) domain. The Bax family (pro-apoptotic) includes Bax, Bak and Bok and they are consisted of BH1, 2 and 3 domains and a TM domain. The last proapoptotic Bcl-2-related protein family is the BH3-only family. This family includes Bid, Bim, Blk, Bad, Bmf, Hrk, Noxa and Puma. As the name suggests, they only contain BH3 domain and the TM domain (except for Bid, which does not have the TM domain).

1.13.2 The Regulation of Apoptosis and Survival by Bcl-2 Family Proteins

The anti-apoptotic and pro-apoptotic Bcl-2 family proteins influence each other's biological functions by forming heterodimers (373, 374). The BH3 domain of a pro-apoptotic protein is inserted into a hydrophobic cleft formed by the BH1, BH2 and BH3 domains of an anti-apoptotic protein (375). The BH1, BH2 and BH4 domains are all required for anti-apoptotic activity (376) whereas the BH3 domain alone is sufficient in setting off pro-apoptotic activity (377).

The regulation of cell death and cell survival is an intricate matter. It requires a balance of anti-apoptotic proteins and pro-apoptotic proteins. Under normal circumstances, the anti-apoptotic Bcl-2 family keeps apoptosis in check at different regulatory levels. Firstly, Bcl-2-like proteins might control the activation of several apical caspases that act upstream or independently of any mitochondrial breach (192). They can also function at the mitochondrion to prevent Bax/Bak oligomerization (378). The BH3-only proteins, despite being pro-apoptotic, require Bax and Bak to carry out their apoptotic functions (379, 380). Moreover, individual BH3-only proteins are normally held in check by diverse mechanisms. For example, Bim and Bmf are not available for their apoptotic functions because they are normally sequestered by the dynein light chains. The dynein light chains are associated with microtubules in the case of Bim and actin cytoskeleton in the case of Bmf (381, 382). Moreover, Akt and protein kinase A can phosphorylate Bad, resulting in its binding to 14-3-3 scaffold proteins, making it unavailable for its apoptotic functions (383, 384). Bid requires proteolytic cleavage by either granzyme B or caspase-8 into truncated Bid in order to be active (36,

97). Lastly, Noxa, Puma and Hrk/DP5 are regulated primarily at the transcription level (385-388).

During apoptosis, the pro-apoptotic Bcl-2 family members are activated by conformational change (389). Through dephosphorylation (e.g. bad) (384) or proteolytic cleavage by caspases (e.g. bid) (36, 97), the pro-apoptotic BH3 domain becomes exposed to the surface. It is necessary for Bid to activate Bax in order to promote death. In addition, Bid and Bax act in concert to inactive pro-survival proteins (390). Moreover, Bid, Bad, Bax and Bim must translocate from the cytoplasm to the mitochondria to execute their pro-apoptotic activity (391-393). Bax and Bak insert themselves into the outer mitochondrial membrane (394, 395). They oligomerize there (396-399) to form pores that lead to the release of pro-apoptotic proteins such as cytochrome *c*. Different apoptotic proteins are responsible for cytochrome *c* release in different apoptotic settings. For example, Bid is involved in Fas-mediated apoptosis, Bax is involved in DNA damage-induced apoptosis and neurotrophin deprivation-induced death and Bad is involved in lymphokine deprivation-induced cell death in certain cells (372).

Given that the activities of anti-apoptotic and pro-apoptotic members of the Bcl-2 family are regulated by different mechanisms (such as various binding proteins with apparently distinct functions) and post-translational modifications (such as phosphorylation (400) and proteolytic cleavage (401)), the Bcl-2 family might constitute a convergent point for various apoptosis-regulating signals (372, 402) (Figure 12).

Figure 12

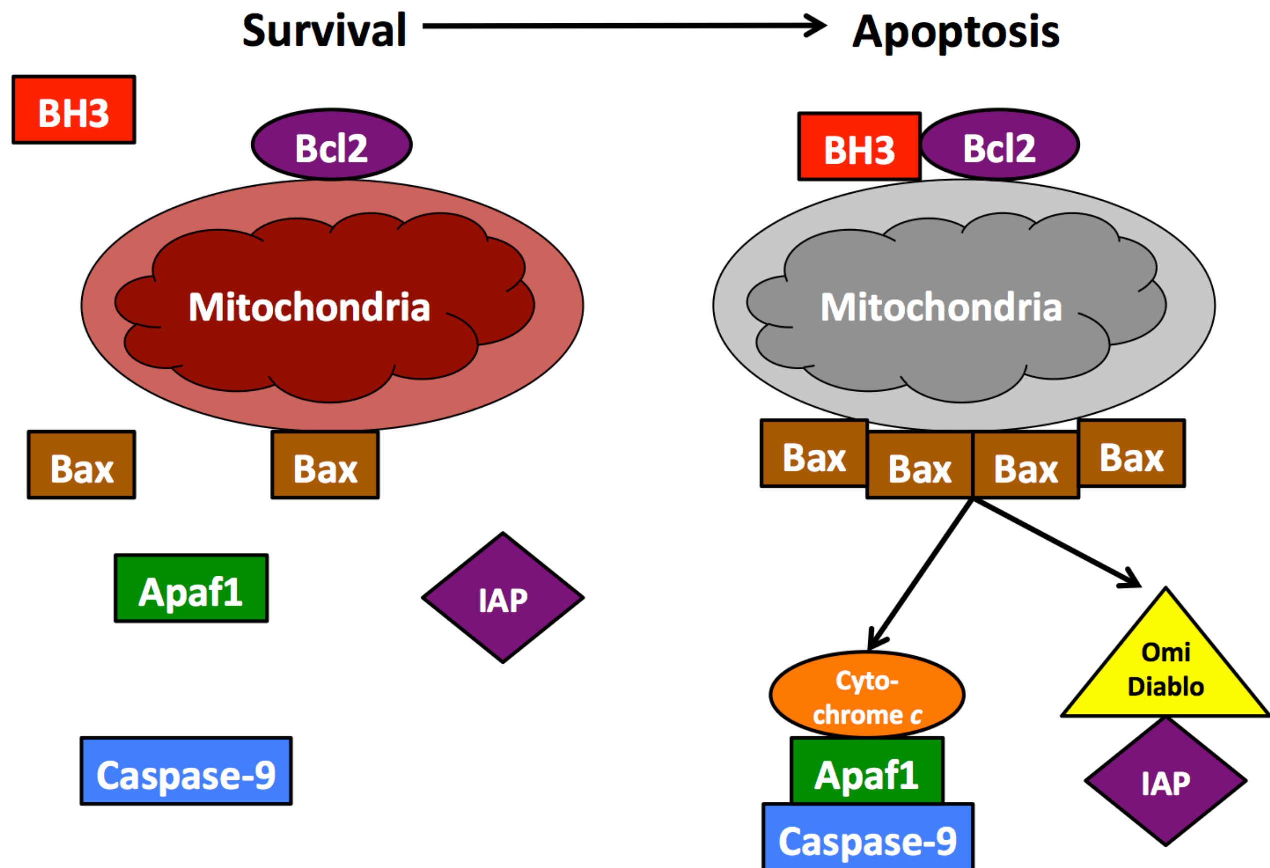


Figure 12. The Regulation of Cell Survival and Apoptosis by Bcl-2 Family Proteins

Bcl-2 protein serves to protect mitochondrial membrane integrity. However, when BH3-only protein neutralizes Bcl-2 protein, Bax and Bak can then form channels in mitochondrial outer membrane allowing the release of cytochrome *c* and Omi and Diablo. Cytochrome *c* then binds to Apaf 1, which then binds to caspase-9 resulting in its activation. Omi and Diablo antagonizes IAP. The end result is apoptosis.

1.14 SUMMARY

Here, I have discussed in detail the biology (structure, mechanisms of activation and functions) of two seemingly opposing molecules, caspase-8 and SFKs, specifically c-Src. I have also discussed the biology of apoptosis and the regulation of apoptosis and cell survival by Bcl proteins, demonstrating a switch between apoptosis and cell survival at distal stages of the cellular processes. I further alluded to the potential crosstalk between caspase-8 and SFKs by discussing the interaction between these two opposing molecules. In chapter 4 and 5 of this thesis, I will provide the evidence of a proximal regulatory switch between apoptosis and cell survival by demonstrating the reciprocal regulatory circuit between caspase-8 and c-Src.

Chapter 2. RATIONALE, AIMS AND FUNDAMENTAL RESEARCH QUESTIONS

2.1 RATIONALE

We and others have previously shown that caspase-8, a pro-apoptotic protein, could be tyrosine phosphorylated at tyrosine 397 (caspase-8a isoform) or tyrosine 380 (caspase-8b isoform) by Src family tyrosine kinases (SFKs) and this results in the suppression of apoptosis (177, 178). These data suggest a potential reciprocal regulation between caspase-8 (a pro-apoptotic protein) and SFKs (a pro-survival protein).

2.2 AIMS

The primary aim of my studies presented in this thesis was to explore the idea that two opposing cell signaling molecules, caspase-8 and c-Src, are able to negatively regulate each other, in order to allow the efficient propagation of either apoptosis or survival, respectively.

We hypothesized that caspase-8, when activated, could inhibit c-Src activity, and suppress downstream cell survival signaling. Conversely, we hypothesized that c-Src could tyrosine phosphorylate caspase-8 and prevent its activation, thereby suppressing apoptotic signaling. Moreover, we hypothesized that when caspase-8 is tyrosine phosphorylated, it could further activate c-Src activity and promote cell survival signaling.

2.3 FUNDAMENTAL RESEARCH QUESTIONS

In the first part of my thesis (Chapter 4), I aimed to answer the following questions:

1) Could caspase-8 impact on c-Src activation and interfere with the downstream effects of c-Src?
2) Is the regulation of c-Src activation by caspsae-8 dependent on the presence of caspase-8 enzymatic activity?
3) Is the ability of c-Src to phosphorylate caspase-8 regulated by caspase-8 enzymatic activity?
4) What other caspase-8 tyrosine sites are targeted by c-Src?

We approached these questions by employing caspase-8 constructs and a constitutively active c-Src (Y527F) construct. To eliminate caspase-8 enzymatic activity, we either used a caspase-8 inhibitor (Z-IETD-FMK) or we transfected an enzymatically inactive caspase-8 mutant (C377S).

In the second part of my thesis (Chapter 5), I aimed to answer the following questions:

1) Does tyrosine phosphorylation of caspase-8 abrogate its ability to be activated and to promote apoptosis?
2) Does tyrosine phosphorylation of caspase-8 promote c-Src activity and its downstream effects?
3) What are the potential mechanisms of the activation of c-Src by tyrosine phosphorylated caspase-8?

We approached these questions by using phosphomimetic and non-phosphorylatable caspase-8 mutants at Y465 and Y397 and constitutively active (Y527F) c-Src construct.

In summary, my work aimed at exploring the possibility of a reciprocal regulation between caspase-8 and c-Src and at delineating the potential mechanism of the regulation.

Chapter 3. MATERIALS AND METHODS

3.1 Cell Lines

Human embryonic kidney cells (HEK293 cells) were obtained from ATCC (number CRL-1573) and cultured in Dulbecco's modified Eagle's medium with high glucose (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin solution. HEK293 cells were cultured in a standard humidified incubator at 37°C in a 5% CO_2 atmosphere.

3.2 Antibodies, Inhibitors and Dyes

Table 3. Antibodies, Inhibitors and Dyes

Name	Description	Dilution	Source
Anti-active Caspase-3	Cleaved Caspase-3 (Asp175) (5A1E) Rabbit monoclonal	1:100	Cell Signaling Technology (CST)
Anti-Caspase-8	Caspase-8 (D35G2) Rabbit monoclonal	1:1000	CST
Anti-GFP	GFP (B-2) Mouse monoclonal	1:1000	Santa Cruz
Anti-Akt	Rabbit polyclonal	1:1000	CST
Anti-phospho-Akt (Thr308)	(244F9) Rabbit monoclonal	1:1000	CST
Anti-phospho-Akt (Ser473)	(587F11) Mouse monoclonal	1:1000	CST
Anti-Erk1/2	Rabbit polyclonal	1:1000	CST
Anti-phospho-Erk1/2 (Thr202/Tyr204)	Rabbit polyclonal	1:1000	CST
Anti-Src	(L4A1) Mouse monoclonal	1:1000	CST
Anti-phospho-Src (Tyr416)	(D49G4) Rabbit monoclonal	1:1000	CST
Anti-phospho-Tyr	Clone 4G10 Mouse monoclonal	1:2000	Millipore
Caspase-8 Inhibitor	Z-IETD-FMK		Calbiochem
Cy3-labelled rabbit anti-IgG		1:1000	Jackson Immunoresearch Laboratories

Name	Description	Dilution	Source
Peroxidase-conjugated anti-mouse IgG		1:5000	CST
Peroxidase-conjugated anti-mouse light chain specific IgG		1:5000	Jackson Immunoresearch Laboratories
Peroxidase-conjugated anti-rabbit IgG		1:5000	GE Health Care UK

3.3 DNA Construct

Constitutively active chicken Src (Y527F Src) in pCMV1 vector was provided by Dr. D. Flynn (West Virginia University).

3.4 Plasmid Construction

Total RNA from healthy human volunteer neutrophils was extracted using TRIzol reagent, and 1 μg of RNA was transcribed to first-strand cDNA using the Superscript II system (Invitrogen); the resultant cDNA was amplified by PCR using the Expand™ High Fidelity PCR System (Roche Molecular Diagnostic) with the following primer set: caspase-8 forward primer (containing a *Hin*dIII site and a Kozak sequence), 5’-GCAAGCTTCGATGGACTTCAGCAGAAATC-3’; caspase-8 reverse primer (containing an *Bam*HI site), 5'-GCGGATCCCAGATCCTCTTCTGAGATGAG-3'. Amplified fragments (Caspase-8a) were cloned into the pEGFP-C1 vector (Invitrogen) according to manufacturer’s instructions. The recombinant plasmids were transfected into DH5α competent cells (Invitrogen), and colonies were identified by restriction enzyme digestion and DNA sequencing (The Centre for Applied Genomics, Hospital for Sick Children, Toronto, Ontario, Canada).

Caspase-8a point mutations were generated as follows: GFP-Caspase-8a in pEGFP-C1 backbone was subjected to single rounds of site-directed mutagenesis using QuickChange Site-Directed Mutagensis kit (Stratagene), according to manufacturer's recommendations. The primers used are listed in Table 4. After *Dpn*I digestion of the amplified product, the mutant DNA was transfected to XL1-blue supercompetent cells, and colonies identified by restriction enzyme digestion and DNA sequencing (The Centre for Applied Genomics, Hospital for Sick Children, Toronto, Ontario, Canada).
All of the final constructs were verified by DNA sequencing (The Centre for Applied Genomics, Hospital for Sick Children, Toronto, Ontario, Canada).

Table 4. List of Primers Used for Point Mutations of GFP-Caspase-8 Constructs

Mutant	**Amino Acid Change**	**Primers Used**
Catalytically Inactive	C377S	5'-ATTCAGGCTAGTCAGGGGG-3' 5'-CCCCCTGACTAGCCTGAAT-3'
Phosphomimetic at Y397	Y397E	5'-GGAGCAACCCTTTTTAGAAATGG-3' 5'-CCATTTCTAAAAAGGGTTGCTCC-3'
Non-phosphorylatable at Y397	Y397F	5'-GGAGCAACCCGAGTTAGAAATGG-3' 5'-CCATTTCTAACTCGGGTTGCTCC-3'
Phosphomimetic at Y465	Y465E	5'-GAAGTGAACGAGGAAGTAAGC-3' 5'-GCTTACTTCCTCGTTCACTTC-3'
Non-phosphorylatable at Y465	Y465F	5'-GAAGTGAACTTTGAAGTAAGC-3' 5'-GCTTACTTCAAAGTTCACTTC-3'

We generated double mutant (Y397F+Y465E) by performing mutagenesis from Y397F construct with Y465E primers and double mutant (Y397E+Y465E) by performing mutagenesis from Y397E construct with Y465E primers.

Note, glutamic acid (E) does not give immunoreactivity to phospho-tyrosine antibody.

3.5 Transfection of Cell Line

The above plasmids, GFP-Caspase-8a (wild type or mutants) with or without Y527F Src, were transfected into HEK293 cells. 0.5 μg or 3 μg of each plasmid were transfected into 80-90% confluent well of 12-well plate or 10-cm dish (coated with poly-D-Lysine (Sigma)) respectively using 3 μL or 18 μL of ExtremeGENE 9 reagent (Roche Molecular Diagnostic) for 24 hours according to manufacturer's instructions. Transfection efficiency was approximately 30-40%.

For caspase-8 inhibitor study, cells were transfected with appropriate plasmids for 9 hours at 37°C, then they were treated with 20 μM of caspase-8 inhibitor (Z-IETD-FMK) (Calbiochem) and incubated for 15 hours at 37°C.

3.6 Protein Studies

For immunoprecipitation studies of exogenous GFP-caspase-8 from HEK293 cells, we lysed a confluent 10-cm dish of HEK293 cells for 10 minutes on ice in Triton X-100 lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF) with Complete Mini EDTA Free Protease Inhibitor Cocktail (Roche Molecular Diagnostic), PhosSTOP (Roche Molecular Diagnostic), 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium vanadate. We then centrifuged lysates at 4°C for 10 min at 12 000 rpm. Supernatants were pre-cleared with Pierce Protein G Agarose (Thermo Scientific) for one hour at 4°C, then centrifuged at 4°C for 1 minute at 12 000 rpm to remove agarose beads. Protein concentration in lysates was measured using the BCA protein assay (Thermo Scientific), then 2 μg of murine monoclonal GFP antibody (Santa Cruz) was added to 4 mg of protein and incubated for 1 hour at 4°C. Then 25 μL of

Pierce Protein G Agarose (Thermo Scientific) was added and incubated for 1 hour. Cell lysates were centrifuged and protein G agarose beads were washed three times in Triton X-100 buffer, then boiled in Laemmli buffer for 5 minutes prior to SDS-PAGE and Western analysis.

For Western blot analysis, we lyzed a confluent well of 12-well plate of HEK293 cells for 10 minutes on ice in Triton X-100 lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF) with Complete Mini EDTA Free Protease Inhibitor Cocktail (Roche Molecular Diagnostic), PhosSTOP (Roche Molecular Diagnostic), 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium vanadate. We then boiled lysates in Laemmli buffer for 5 minutes prior to SDS-PAGE and Western analysis with appropriate antibodies listed above.

3.7 Mass Spectrometry

Immunoprecipitated GFP-Caspase-8a (C377S or Y465E) was resolved on SDS-PAGE and bands corresponding to GFP-Caspase-8a were excised from the gel. The samples were submitted to the Mass Spectrometry Facility of the Hospital for Sick Children in Toronto, Ontario for in-gel trypsin digestion and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

3.8 Microscopy and Immunofluorescence Analysis

For immunofluorescence staining, cells were grown on glass coverslips coated with poly-D-Lysine (Sigma). Following transfection, cells were rapidly fixed by 4% paraformaldehyde for 30 minutes and washed with PBS. After quenching the paraformaldehyde with 100 mM glycine in PBS, the cells were permeabilized with 0.1% Triton-X-100 in 1% (w/v) albumin for 20 minutes and blocked with 3% (w/v) albumin

for 1 hour. The coverslips were then incubated with primary antibody (anti-active-Caspase-3) for 1 hour, followed by washing and incubation with secondary antibody (Cy3-labelled rabbit anti-IgG) for 1 hour. The coverslips were washed with PBS and mounted on glass slides using fluorescence mounting medium (Dako). The staining was visualized using an Olympus 1X81 microscope (Mellville) coupled to an Evolution QEi Monochrome camera controlled by the MetaMorph software. The percentage of GFP-positives cells that were also Cy3 positive (active caspase-3 positive) was quantified.

3.9 Statistical Analysis

Statistical significance was determined by Wilcoxon Signed Rank Test or Kruskal Wallis Test with Dunn's Multiple Comparison Test when there were more than two samples for comparison. GraphPad Prism 4.0 software was used.

Chapter 4. THE RECIPROCAL NEGATIVE REGULATION OF CASPASE-8 AND C-SRC

4.1 SUMMARY

The overall fate of a cell depends on the integration of two opposing inputs: apoptotic and survival signals. Caspase-8 and c-Src are key molecular determinants of the initiation of apoptotic and survival pathways, respectively. We hypothesized that these upstream mediators of the two opposing programs might negatively regulate each other to ensure full commitment to cell fate-determining process. Here, we show that caspase-8 can suppress c-Src and its downstream effector Erk1/2 but not Akt. This inhibitory effect requires the enzymatic activity of caspase-8. Conversely, we found that c-Src can induce tyrosine phosphorylation of caspase-8, which in turns abrogates its ability to inhibit c-Src. A large net increase in caspase-8 phosphorylation by c-Src becomes manifested in caspase-8 mutants, which lack enzymatic activity, and therefore are unable to inhibit c-Src. Using such mutants, we also show that tyrosine phosphorylation of caspase-8 may in fact turn this molecule from a c-Src inhibitor to a c-Src activator, i.e., it may convert this apoptosis inducer to a pro-survival mediator. In addition, we identified tyrosine 8, 243, 351 and 397 of caspase-8 as c-Src targets. Considered together, our data suggest a reciprocal regulatory mechanism between two proximal cell fate-signaling molecules – caspase-8 and c-Src. This mode of regulation may be a key mechanism in the efficient integration of death and survival signals and the fast execution of the dominant program.

4.2 INTRODUCTION

Caspase-8, an apical member of the caspase cascade, plays a central role in the initiation of apoptosis (83, 85, 89, 90). Accordingly, the regulation of caspase-8 represents a key mechanism in determining cell death and survival. Recent studies, including our own, have raised the possibility that caspase-8 might be an important integrator for various apoptosis-promoting and inhibiting signals (177, 178). In this regard, we (178) and others (52, 177) have shown that caspase-8 can be targeted by various Src family kinases (SFKs), including c-Src and Lyn. These kinases were shown to phosphorylate tyrosine 397 (caspase-8a isoform) and tyrosine 380 (caspase-8b isoform), which in turn resulted in reduced capase-8 activity and apoptosis (177, 178) and increased cell migration (52). These data suggest SFKs can exert anti-apoptotic effects, partly through inhibiting caspase-8. Conversely, casapse-3, an executioner caspase, was shown to cleave two SFKs, Fyn and Lyn at their unique N-terminal regions. These SFK family members are selective targets for caspase-3, since Lck, Hck or c-Src were not modified by this enzyme. The cleavage of Fyn and Lyn resulted in altered regulation of these kinases, reducing their membrane localization, but enhancing their *in vitro* catalytic activity (403). Overall, these changes were proposed to facilitate apoptosis (403). Together, these observations raise the possibility that certain SFKs and caspases may impact each other in a reciprocal manner, providing a link between apoptotic and survival mechanisms.

While the activation of specific SFKs (Lyn and c-Src) was shown to induce caspase-8 phosphorylation and inhibition, it remained to be established whether caspase-8 could modify SFK activity. We surmised that a bidirectional (reciprocal) regulatory

relationship might exist between caspase-8 and c-Src, the most abundant and ubiquitously expressed SFK. It is noteworthy in this regard to state that SFKs, in addition to their apoptosis-modulating effects, exert direct and strong pro-survival activity, by stimulating mitogenesis, cell growth and cell proliferation (231, 251, 306, 312-314). These processes are dependent on SFK-induced activation of phosphatidylinositol 3-kinase (PI3K), Akt, mitogen activated protein kinase kinase (MEK), and extracellular signal-regulated kinase (Erk) signaling pathways (304, 305, 334). Cognizant of this scenario, we first sought to confirm that c-Src could tyrosine phosphorylate caspase-8, and addressed which (additional) caspase-8 tyrosine residues that could be phosphorylated by c-Src. Further, we sought to investigate whether caspase-8 could impact on c-Src activation and might interfere with the downstream effects of c-Src, including global tyrosine phosphorylation, Erk and Akt activation. We also wished to address whether such c-Src-modulating effects might depend on the tyrosine phosphorylation of caspase-8, and whether tyrosine phosphorylated caspase-8 might not only lose its apoptosis-inducing potential but might also actively stimulate survival pathways. Here, we employed a constitutively active (Y527F) chicken c-Src to mimic the naturally occurring c-Src mutant (truncated at amino acid 531) in human colon cancer cells (370).

4.3 RESULTS

4.3.1 Caspase-8 Enzymatic Activity Regulates the Capacity of c-Src to Tyrosine Phosphorylate Caspase-8

It has been previously shown that SFKs were able to tyrosine phosphorylate caspase-8 (52, 177, 178). We first sought to confirm whether c-Src could tyrosine phosphorylate caspase-8. To this end, HEK293 cells were transfected with WT or C377S GFP-Caspase-8 with or without Y527F c-Src for 24 hours followed by Western blot analysis of whole cell lysates. Initially, we looked at tyrosine phosphorylation of caspase-8 with a general phospho-tyrosine antibody using Western blot analysis of whole cell lysates. The same blot was then stripped and reprobed for caspase-8, allowing the alignment of phospho-tyrosine band with the caspase-8 band. Without the expression of Y527F c-Src, GFP-Caspase-8, neither WT nor C377S were tyrosine phosphorylated (Figure 13A). However, in the presence of Y527F c-Src, the inactive mutant (C377S) but not the WT GFP-Caspase-8 was tyrosine phosphorylated. (Figure 13A).

We then investigated whether the caspase-8 enzymatic activity is the key factor regulating GFP-Caspase-8 tyrosine phosphorylation by Y527F c-Src. To assess this, HEK293 cells were co-transfected with WT GFP-Caspase-8 and Y527F c-Src for 9 hours then the cells were treated with 20 μM of caspase-8 inhibitor (Z-IETD-FMK) or its vehicle control (DMSO) for 15 hours. We observed tyrosine phosphorylation in the band corresponding to GFP-Caspase-8 only in the samples that had been exposed to the caspase-8 inhibitor (Figure 13B).

To confirm that the detected phosphorylation difference occurred indeed in the transfected GFP-Caspase-8, and to corroborate that this difference was due to suppressed

caspase-8 enzymatic activity and not any non-specific effect of the caspase-8 inhibitor, we used the catalytically inactive C377S mutant of GFP-Caspase-8. HEK293 cells were co-transfected with either WT or C377S caspase-8 along with Y527F c-Src for 24 hours, and then the GFP-Caspase-8 variants were immunoprecipitated using a GFP antibody. The precipitates were then analyzed, by Western blotting using a phospho-tyrosine antibody followed by caspase-8 antibody. Remarkably, Y527F c-Src induced the tyrosine phosphorylation of C377S but not WT GFP-Caspase-8 (Figure 13C). This suggests that caspase-8 enzymatic activity can inhibit c-Src activity thereby preventing its own tyrosine phosphorylation by the kinase. Although there was more GFP-Caspase-8 (uncleaved form) in the C377S mutant transfected cells compared to WT transfected cells, which is understandable since there was less cleavage in the C377S mutant, the mass effect of GFP-Caspase-8 (uncleaved form) alone cannot account for the difference in tyrosine phosphorylation level because in the WT transfected cells, there was absolutely no tyrosine phosphorylation of caspase-8.

Figure 13

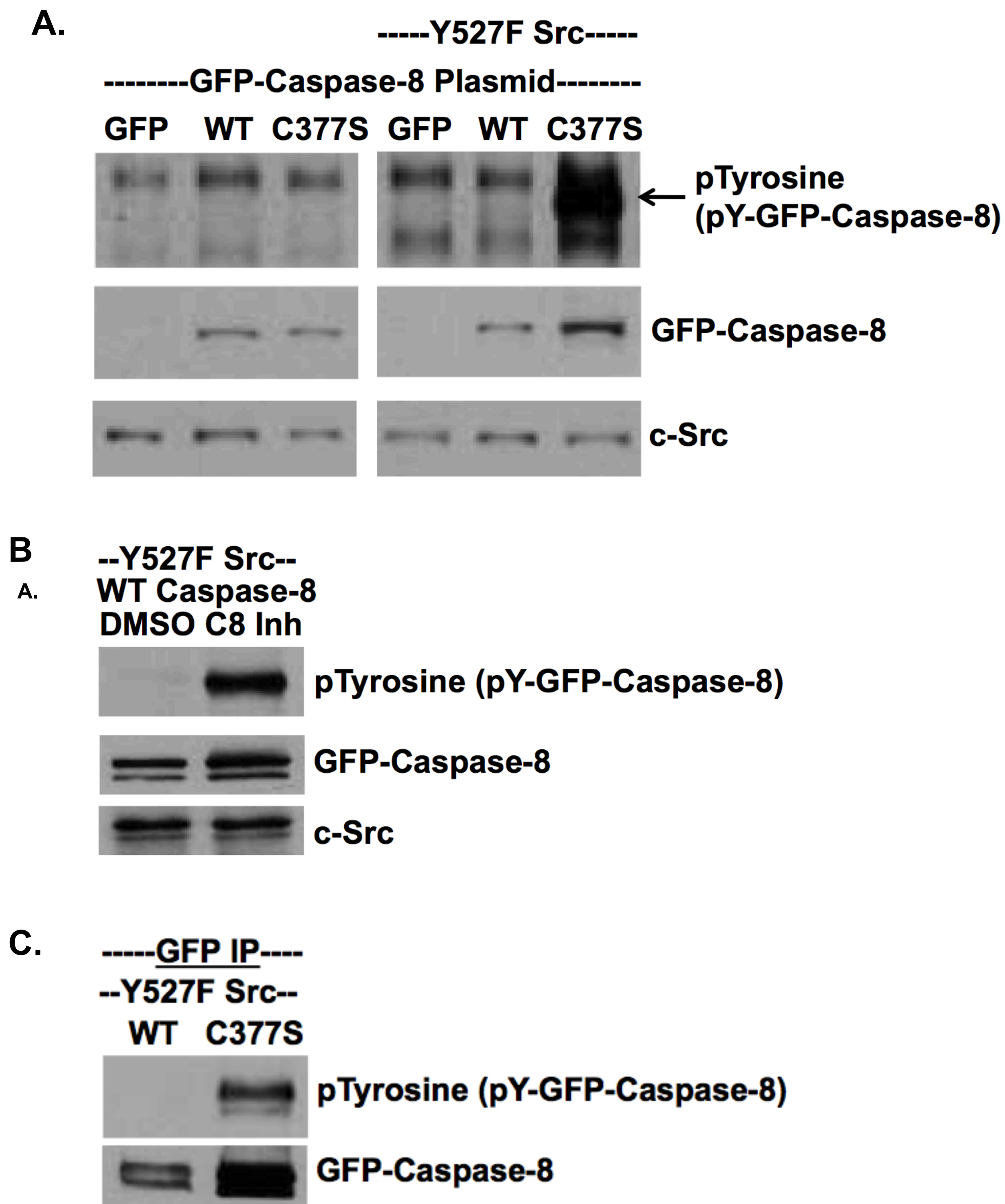


Figure 13. Caspase-8 Enzymatic Activity Regulates the Capacity of c-Src to Tyrosine Phosphorylate Caspase-8

A.) HEK293 cells were transfected with GFP-Caspase-8 plasmids (empty plasmid, WT or C377S mutant) with or without Y527F c-Src for 24 hours followed by Western blotting of whole cell lysates first with phospho-tyrosine antibody then with caspase-8 antibody to examine the tyrosine phosphorylation of GFP-Caspase-8. Only C377S inactive GFP-Caspase-8 mutant was tyrosine phosphorylated in the presence of Y527F c-Src. B.) HEK293 cells were co-transfected with GFP-Caspase-8 (WT) with Y527F c-Src for 9 hours followed by the treatment of 20 μM of Caspase-8 inhibitor for 15 hours. Western blotting of whole cell lysates was performed first with phospho-tyrosine antibody then with caspase-8 antibody to examine the tyrosine phosphorylation of GFP-Caspase-8. GFP-Caspase-8 was only tyrosine phosphorylated by Y527F c-Src in the presence of Caspase-8 inhibitor. C.) HEK293 cells were co-transfected with GFP-Caspase-8 plasmids (WT or C377S mutant) with Y527F c-Src for 24 hours followed by immunoprecipitation of GFP-Caspase-8 using a GFP antibody. Immunoprecipitates were probed with phospho-tyrosine antibody followed by caspase-8 antibody. Only C377S inactive mutant of GFP-Caspase-8 was tyrosine phosphorylated by Y527F c-Src.

4.3.2 Caspase-8's Tyrosine Phosphorylation Sites by Src Kinases

Previous studies have shown that SFKs could tyrosine phosphorylate caspase-8a at Y397 (178) or caspase-8b at Y380 (52, 177). We sought to investigate the specific tyrosine residues in caspase-8a that could be phosphorylated by c-Src. We first used a SFK phopshorylation prediction tool, GPS 2.1 (404) to identify potential tyrosine phosphorylation sites (Table 5).

Table 5

Amino Acid Position	Peptide Sequence
8	NDFSRBK**Y**DUGEQKD
243	SQTLDKV**Y**QMKSKPR
310	DCTVEQI**Y**EILKIYQ
397	TDSEEQP**Y**LEMDLSS
465	TILTEVN**Y**EVSNKDD

Table 5. Predicted Capsaes-8 Tyrosine Phosphorylated Sites

We found the following predicted tyrosine phosphorylation sites in caspase-8 by using a SFK phosphorylation prediction tool, GPS2.1. They include tyrosine 8, 243, 310, 397 and 465. The corresponding peptides surrounding the tyrosine sites are listed.

We then proceeded with mass spectrometry to determine which tyrosine residues could be phosphorylated by c-Src. To this end, we co-transfected HEK293 cells with C377S GFP-Caspase-8 along with Y527F c-Src for 24 hours. We then performed immunoprecipitation (IP) of the transfected GFP-Caspase-8 using a GFP antibody. The resultant precipitates were subjected to SDS-PAGE, and the band corresponding to the transfected GFP-Caspase-8 (the 80 kDa region) was excised from the gel and analyzed by mass spectrometry. The results showed that tyrosine 8, 243, 351 and 397 were phosphorylated by c-Src (Figure 14A & B). It is noteworthy to point out that the sequence coverage was only approximately 50%. Specifically, the peptide region surrounding the Y465 residues was hydrophobic preventing it from releasing from the SDS-PAGE gel for sequencing. Therefore, we were unable to confirm whether Y465 was phosphorylated by c-Src yet.

Figure 14

A.

```
       8
MDFSRNLYDI GEQLDSEDLA SLKFLSLDYI PQPKQEPIKD ALMLFQRLQE KRMLEESNLS

FLKELLFRIN RLDLLITYLN TRKEEMEREL QTPGRAQISA YRFHFCRMSW AEANSQCQTA

SVPFWRRVDH LLIRVMLYQI SEEVSRSELR SFKFLLQEEI SKCKLDDDMN LLDIFIEMEK

RVILGEGKLD ILKRVCAQIN KSLLKIINDY EEFSKGEELC GVMTISDSPR EQDSESQTLD
 243
KVYQMKSKPR GYCLIINNHN FAKAREKVPK LHSIRDRNGT HLDAGALTTT FEELHFEIKP
         310                                          351
HDDCTVEQIY EILKIYQLMD HSNMDCFICC ILSHGDKGII YGTDGQEAPI YELTSQFTGL
                                    397
KCPSLAGKPK VFFIQACQGD NYQKGIPVET DSEEQPYLEM DLSSPQTRYI PDEADFLLGM
                                               465
ATVNNCVSYR NPAEGTWYIQ SLCQSLRERC PRGDDILTIL TEVNYEVSNK DDKKNMGKQM

PQPTFTLRKK LVFPSD
```

Legends:
Phosphorylation

B.

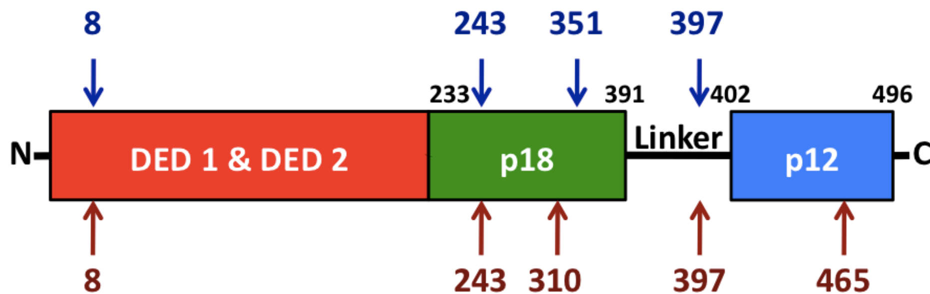


Figure 14. c-Src Tyrosine Phosphorylates Caspase-8 at Multiple Sites

A.) HEK293 cells were co-transfected with inactive C377S mutant of GFP-Caspase-8 and Y527F c-Src for 24 hours followed by immunoprecipitation of GFP-Caspase-8 using a GFP antibody. Immunoprecipitates were subjected to SDS-PAGE and the appropriate band (80 kDa region) was excised and sent for mass spectrometry analysis. Tyrosine 8, 243, 351 and 397 were phosphorylated by c-Src. Sequence coverage was only 50%. B.) In this schematic diagram, the tyrosine residues that were phosphorylated by c-Src (determined by mass spectrometry) and the tyrosine residues that were predicted to be phosphorylated by SFK were shown.

4.3.3 Caspase-8 Inhibits c-Src Kinase Activity and its Specific Downstream Signaling Pathway

Caspase-8's classic role is to initiate programmed cell death (apoptosis). SFKs, on the other hand, are known to be potent survival molecule. It has previously been demonstrated that during the induction of apoptosis, caspase-3 could cleave Fyn and Lyn but not Hck, Lck or c-Src, and alter their kinase activity (403). Here, we sought to study whether an apical apoptotic caspase, caspase-8, could inhibit c-Src activation and its downstream signaling pathways. The activation of c-Src involves the dephosphorylation of negative-regulatory tyrosine residue (Y527) (249, 405, 406) and the autophosphorylation of activation loop tyrosine residue (Y416) (261, 407). To assess the impact of caspase-8 on c-Src, we used a strategy that took advantage of this "double modification" activation mechanism. Specifically, here we used a constitutively active version of c-Src (Y527F) as an inducer of c-Src activity, and the phosphorylation of c-Src at Y416 as an indicator of additional c-Src activation. To assess the downstream effects of c-Src, we also followed global tyrosine phosphorylation as well as the activation of MEK/Erk1/2 (304, 305) and PI3K/Akt (334, 337), pathways that have been involved in c-Src-dependent mitogenesis, growth and survival.

To study the effect of caspase-8 on c-Src activation, we co-transfected wild type (WT) GFP-Caspase-8 or pEGFP-C1 empty plasmid, with constitutively active (Y527F) c-Src into HEK293 cells for 24 hours. We then resolved the whole cell lysates on SDS-PAGE to quantitate 1) Y416 phosphorylation of c-Src; 2) global protein tyrosine phosphorylation; 3) threonine (T202)/Y204 phosphorylation of Erk1/2, an indicator of

Erk1/2 activation; and 4) T308 or Serine (S473) phosphorylation of Akt, both indicators of Akt activation.

We found that compared to empty plasmid, WT caspase-8 significantly prevented the autophosphorylation of Y416 of c-Src (Figure 15A & B) and tyrosine phosphorylation of many protein substrates (Figure 15C), suggesting that caspase-8 could inhibit c-Src activation and activity. Moreover, compared to empty plasmid, WT caspase-8 also downregulated T202/Y204 phosphorylation of Erk1/2 (Figure 16A & B), suggesting that caspase-8 could also inhibit the activation of Erk1/2. However, WT caspase-8 did not significantly suppress T308 or S473 (Figure 16C & D) phosphorylation of Akt. These data together suggest that caspase-8 could suppress c-Src activation and the phosphorylation of some c-Src target molecules.

Figure 15

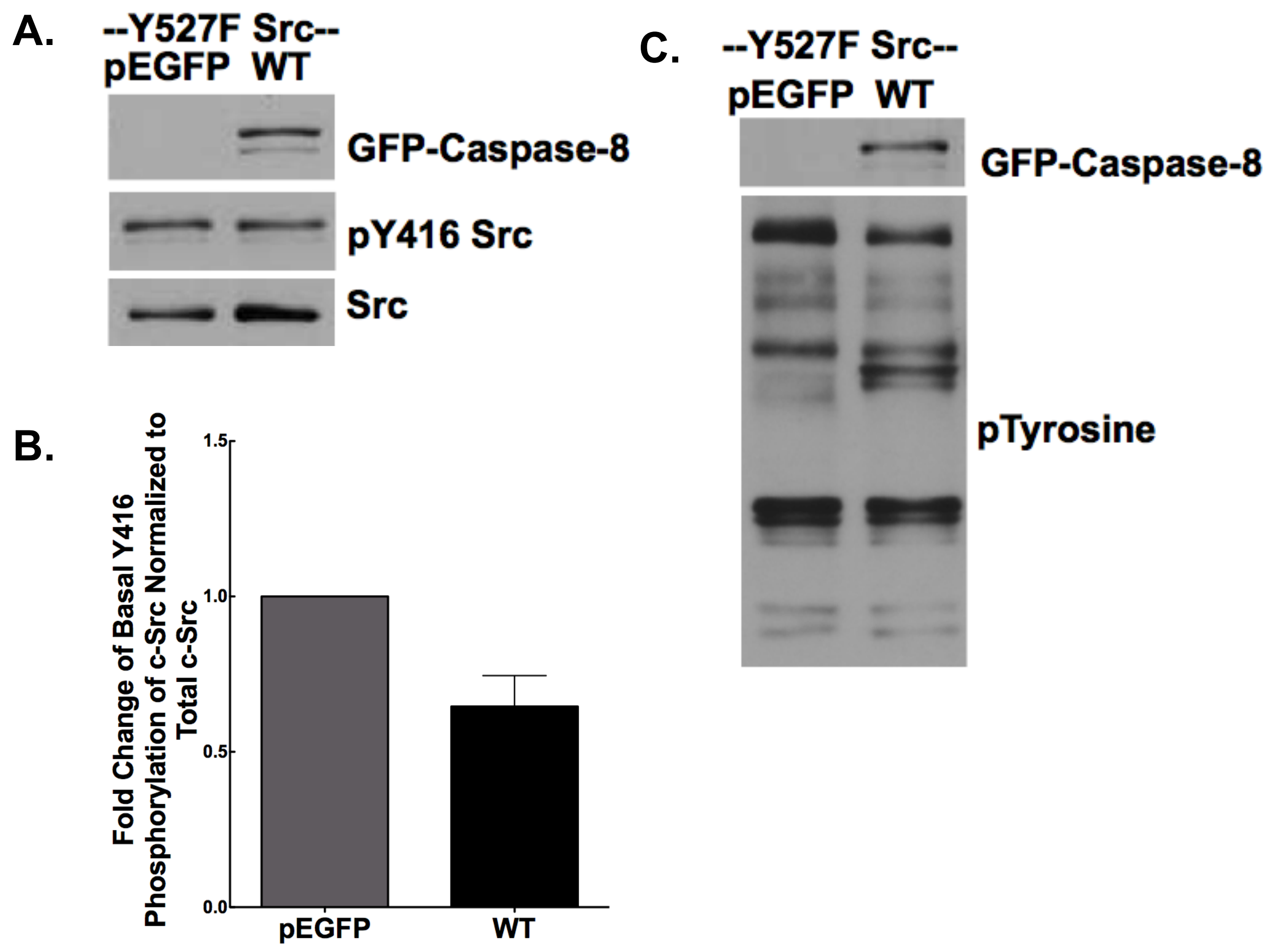


Figure 15. Caspase-8 Inhibits c-Src Activation and its Activity

HEK293 cells were co-transfected with cDNAs encoding pEGFP-C1 empty plasmid or GFP-tagged Caspase-8 and constitutively active (Y527) c-Src for 24 hours. Total lysates were developed with 1) GFP antibody to monitor the transfection level of GFP-Caspase-8, 80 kDa; 2) pY416 c-Src antibody to study the activation of c-Src, 60 kDa; 3) c-Src antibody to detect the total c-Src, 60 kDa; and 4) phospho-tyrosine antibody to examine global protein tyrosine phosphorylation. A.) Y416 phosphorylation of c-Src was suppressed by the expression of GFP-Caspase-8 compared to pEGFP-C1 empty plasmid. B.) Densitometry analysis was performed to represent the Y416 phosphorylation level of c-Src normalized to the expression level of total c-Src (N = 12, $p < 0.01$). C.) c-Src activity, reflected by global protein tyrosine phosphorylation was inhibited by the expression of GFP-Caspase-8 compared to pEGFP-C1 empty plasmid.

Figure 16

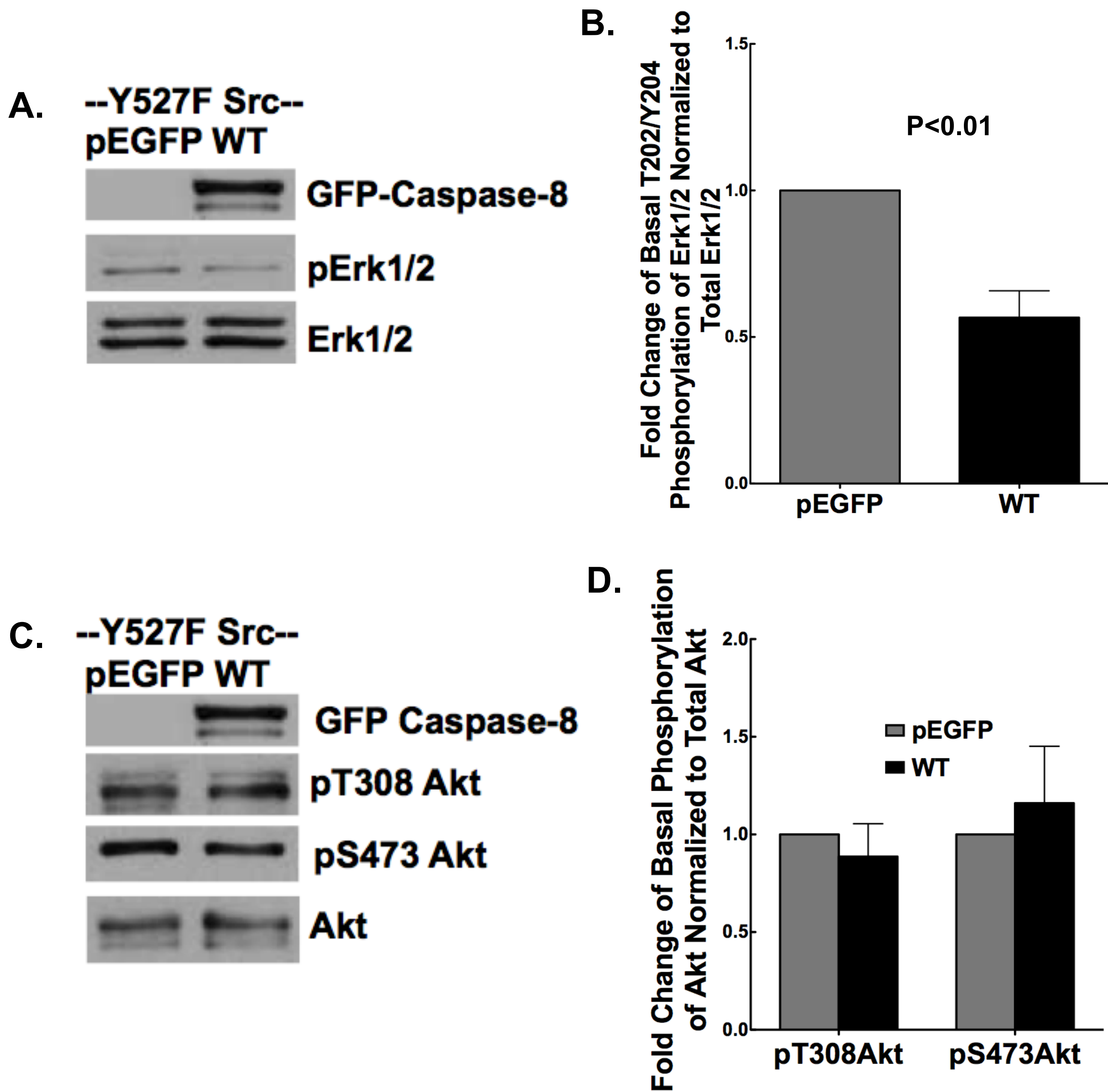


Figure 16. Caspase-8 Inhibits Erk1/2 Activation but not Akt Activation

HEK293 cells were co-transfected with cDNAs encoding pEGFP-C1 empty plasmid or GFP-tagged Caspase-8 and constitutively active (Y527) c-Src for 24 hours. Total lysates were developed with 1) GFP antibody to monitor the transfection level of GFP-Caspase-8, 80 kDa; 2) pT202/Y204 Erk1/2 antibody to study the activation of Erk1/2, 42 & 44 kDa; 3) Erk1/2 antibody to detect the total Erk1/2, 42 & 44 kDa; 4) pT308 Akt and 5) pS473 Akt antibodies to study the activation of Akt, 60 kDa; and 6) Akt antibody to detect the total Akt, 60 kDa. A.) T202/204 phosphorylation of Erk1/2 was suppressed by the expression of GFP-Caspase-8 compared to pEGFP-C1 empty plasmid. B.) Densitometry analysis was performed to represent the pT202/Y204 phosphorylation level of Erk1/2 normalized to the expression level of total Erk1/2 (N = 8, $p < 0.01$). C.) T308 and S473 phosphorylation of Akt were not altered by the expression of GFP-Caspase-8 compared to pEGFP-C1 empty plasmid. D.) Densitometry analysis was performed to represent the pT308 and pS473 phosphorylation level of Akt normalized to the expression level of total Akt.

4.3.4 The Inhibition of c-Src by Caspase-8 is Caspase-8 Enzymatic Activity Dependent

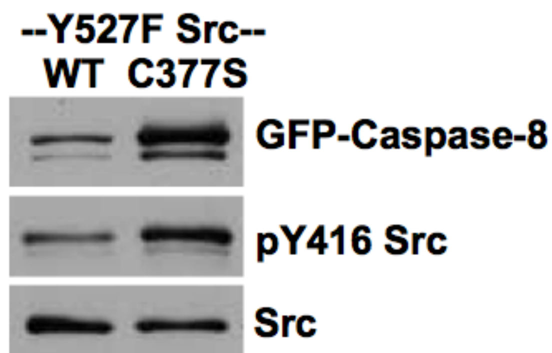
Caspase-8 is expressed as a zymogen and becomes activated when caspase-8 molecules are brought together in close proximity by adaptor molecules that bind to the death receptor upon ligation by death ligand. This model is called the induced proximity model (15). The transfection of caspase-8 alone could lead to the activation of caspase-8 by virtue of bringing caspase-8 molecules close together due to protein overexpression. We have shown that the transfection of caspase-8 was sufficient to induce activation of caspase-3 (see next chapter). We sought to investigate whether caspase-8 enzymatic activity is required in the suppression of c-Src activation and activity.

To this end, we employed a known catalytically inactive mutant of caspase-8, C377S (26, 45) to eliminate the enzymatic activity of caspase-8. HEK293 cells were transfected with either WT or C377S caspase-8 with Y527F Src for 24 hours and we examined the phosphorylation of Y416 of c-Src in whole cell lysates. Interestingly, in contrast to WT caspase-8, the C377S caspase-8 mutant not only failed to decrease the phosphorylation of Y416 of c-Src, but also significantly increased it (Figure 17A & B). This observation supports the notion that the inhibition of c-Src activation was dependent on caspase-8 enzymatic activity and may suggest a direct activation of c-Src activity by uncleaved caspase-8. Similar phenomenon was observed with global protein tyrosine phosphorylation suggesting that the suppression of global protein tyrosine phosphorylation by caspase-8 is also caspase-8 enzymatic activity dependent (Figure 17C).

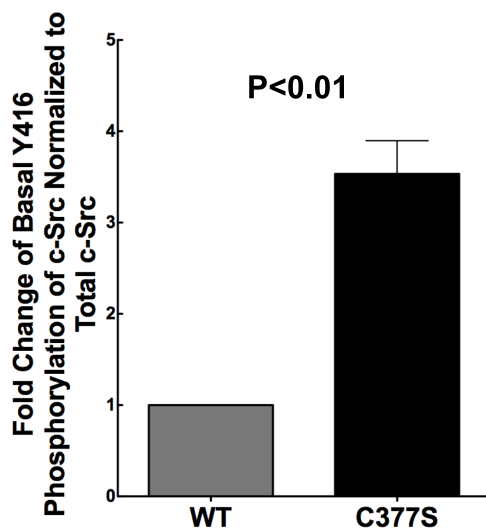
We then used the same whole cell lysates to examine for activation of Erk1/2 and Akt. We found that C377S caspase-8, compared to WT caspase-8, also stimulated the phosphorylation of T202/Y204 of Erk1/2 suggesting that the inhibition of Erk1/2 activation by caspase-8 is also caspase-8 enzymatic activity dependent (Figure 18A & B). As expected, eliminating caspase-8 enzymatic activity by using C377S mutant of caspase-8 did not influence phosphorylation of T308 or S473 (Figure 18C & D) of Akt. Together these data suggest that an enzymatically active caspase-8 is needed for caspase-8-mediated inhibition of c-Src activation and the consequent phosphorylation of some downstream c-Src targets.

Figure 17

A.



B.



C.

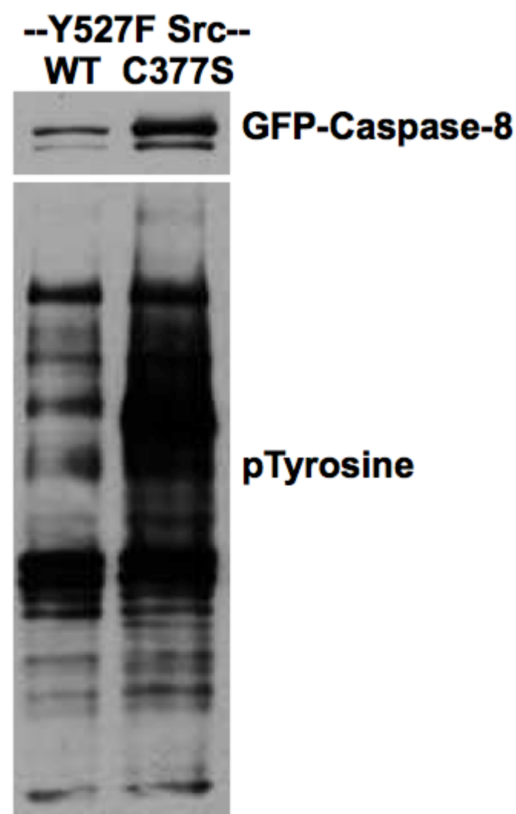


Figure 17. Inactive Caspase-8 (C377S) Results in Enhanced c-Src Activation and Activity

HEK293 cells were co-transfected with cDNAs encoding GFP-tagged Caspase-8 (WT or C377S mutant) and constitutively active (Y527) c-Src for 24 hours. Total lysates were developed with 1) GFP antibody to monitor the transfection level of GFP-Caspase-8, 80 kDa; 2) pY416 c-Src antibody to study the activation of c-Src, 60 kDa; 3) c-Src antibody to detect the total c-Src, 60 kDa; and 4) phospho-tyrosine antibody to examine global protein tyrosine phosphorylation. A.) Y416 phosphorylation of c-Src was significantly enhanced by the expression of C377S caspase-8 compared to WT caspase-8. B.) Densitometry analysis was performed to represent the Y416 phosphorylation level of c-Src normalized to the expression level of total c-Src (N = 12, $p < 0.01$). C.) c-Src activity, reflected by global protein tyrosine phosphorylation was upregulated by the expression of C377S caspase-8 compared to WT caspase-8.

Figure 18

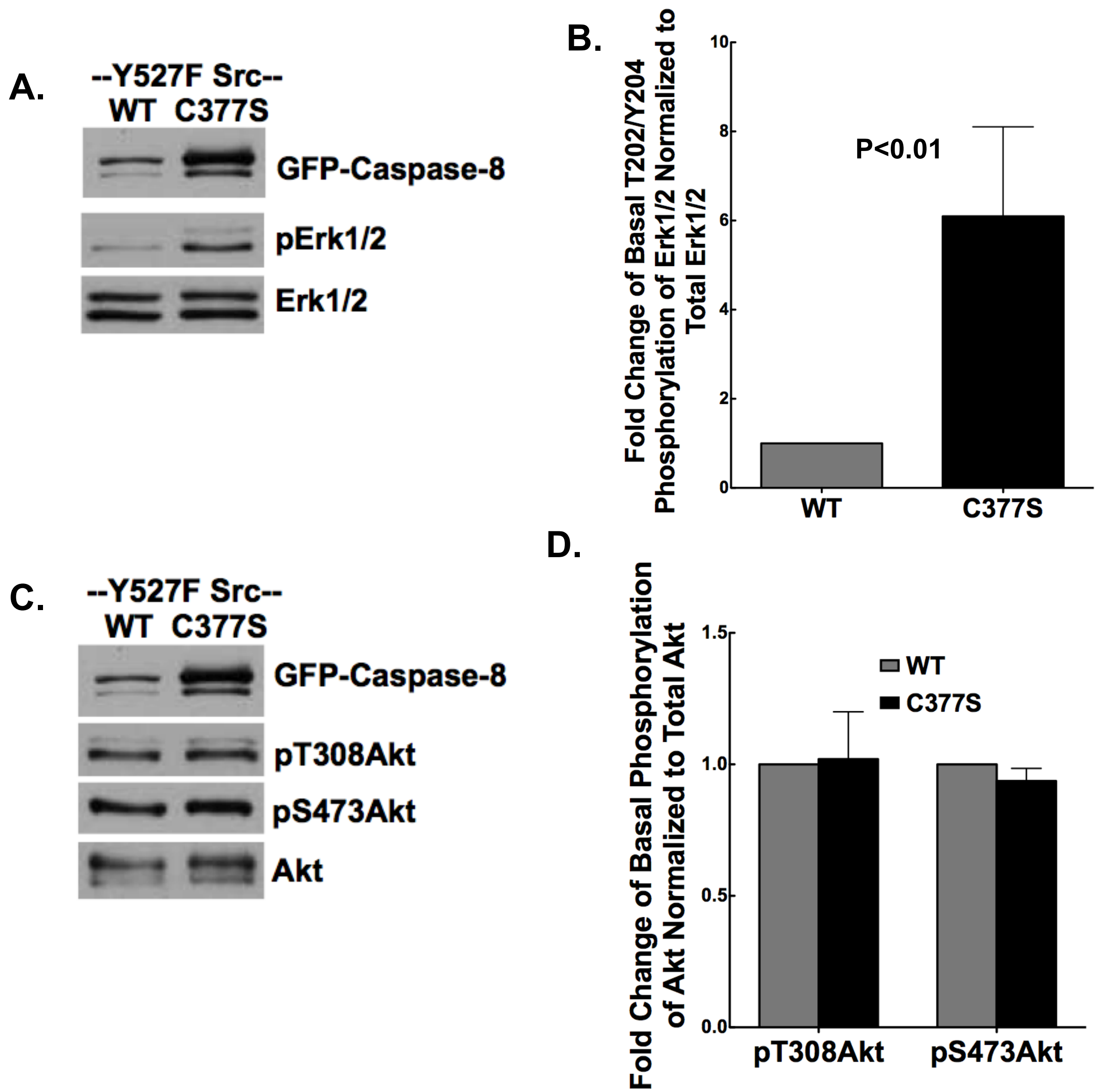


Figure 18. Inactive Caspase-8 (C377S) Results in Enhanced Erk1/2 Activation but not Akt Activation

Total lysates from experiments described in Figure 17 were developed with 1) GFP antibody to monitor the transfection level of GFP-Caspase-8, 80 kDa; 2) pT202/Y204 Erk1/2 antibody to study the activation of Erk1/2, 42 & 44 kDa; 3) Erk1/2 antibody to detect the total Erk1/2, 42 & 44 kDa; 4) pT308 Akt and 5) pS473 Akt antibodies to study the activation of Akt, 60 kDa; and 6) Akt antibody to detect the total Akt, 60 kDa. A.) T202/204 phosphorylation of Erk1/2 was significantly enhanced by the expression of C377S caspase-8 compared to WT caspase-8. B.) Densitometry analysis was performed to represent the pT202/Y204 phosphorylation level of Erk1/2 normalized to the expression level of total Erk1/2 ($N = 8$, $p < 0.01$). C.) T308 and S473 phosphorylation of Akt were not altered by the expression of C377S caspase-8 compared to WT caspase-8. D.) Densitometry analysis was performed to represent the pT308 and pS473 phosphorylation level of Akt normalized to the expression level of total Akt.

To corroborate our findings that caspase-8 enzymatic activity is necessary for the inhibition of c-Src and Erk1/2 activation, we used a caspase-8 inhibitor to inhibit caspase-8 enzymatic activity. To this end, we co-transfected HEK293 cells with WT GFP-Caspase-8 and Y527F c-Src for 9 hours then the cells were treated with 20 μM of caspase-8 inhibitor (Z-IETD-FMK) or vehicle control (DMSO) for 15 hours. Caspase-8 inhibition led to decrease in caspase-8 cleavage, hence, resulted in an increased expression of full-length caspase-8. Compared to vehicle control, caspase-8 inhibitor abrogated the suppression of c-Src activation by caspase-8 (trend), suggesting that caspase-8 enzymatic activity is required to inhibit c-Src activity (Figure 19A & B). The caspase-8 inhibitor also abrogated the suppression of global protein tyrosine phosphorylation by caspase-8 overexpression (Figure 19C).

We then used the same whole cell lysates to examine for activation of Erk1/2 and Akt. We found that the caspase-8 inhibitor also prevented the caspase-8 overexpression-induced Erk1/2 inhibition (trend), suggesting that the inhibition of Erk1/2 activation by caspase-8 overexpression is also caspase-8 enzymatic activity dependent (Figure 20A & B). However, the caspase-8 inhibitor did not result in the upregulation of phosphorylation of T308 or S473 of Akt (Figure 20C & D) suggesting that activation of Akt is not regulated by caspase-8 enzymatic activity.

Figure 19

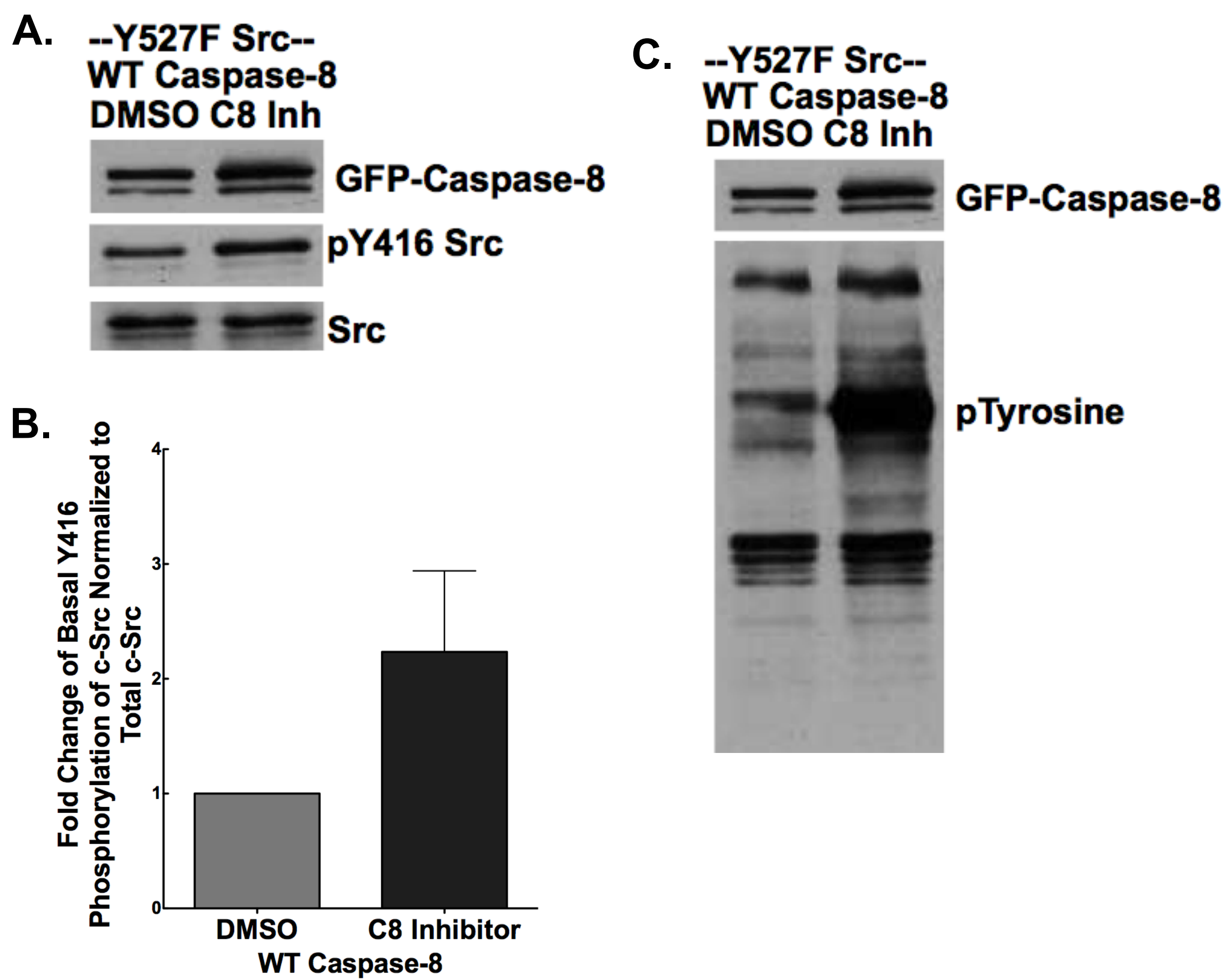


Figure 19. Caspase-8 Enzyme Inhibition Results in Enhanced c-Src Activation and Activity

HEK293 cells were co-transfected with cDNAs encoding GFP-tagged Caspase-8 and constitutively active (Y527) c-Src for 9 hours then the cells were treated with 20 μM of caspase-8 inhibitor or DMSO (vehicle control) for 15 hours. Total lysates were developed with 1) GFP antibody to monitor the transfection level of GFP-Caspase-8, 80 kDa; 2) pY416 c-Src antibody to study the activation of c-Src, 60 kDa; 3) c-Src antibody to detect the total c-Src, 60 kDa; and 4) phospho-tyrosine antibody to examine global protein tyrosine phosphorylation. A.) Compared to vehicle control, Y416 phosphorylation of c-Src was enhanced (trend) in the presence of Caspase-8 inhibitor. B.) Densitometry analysis was performed to represent the Y416 phosphorylation level of c-Src normalized to the expression level of total c-Src (N = 3, $p = 0.5$). C.) c-Src activity, reflected by global protein tyrosine phosphorylation was enhanced in the presence of Caspase-8 inhibitor.

Figure 20

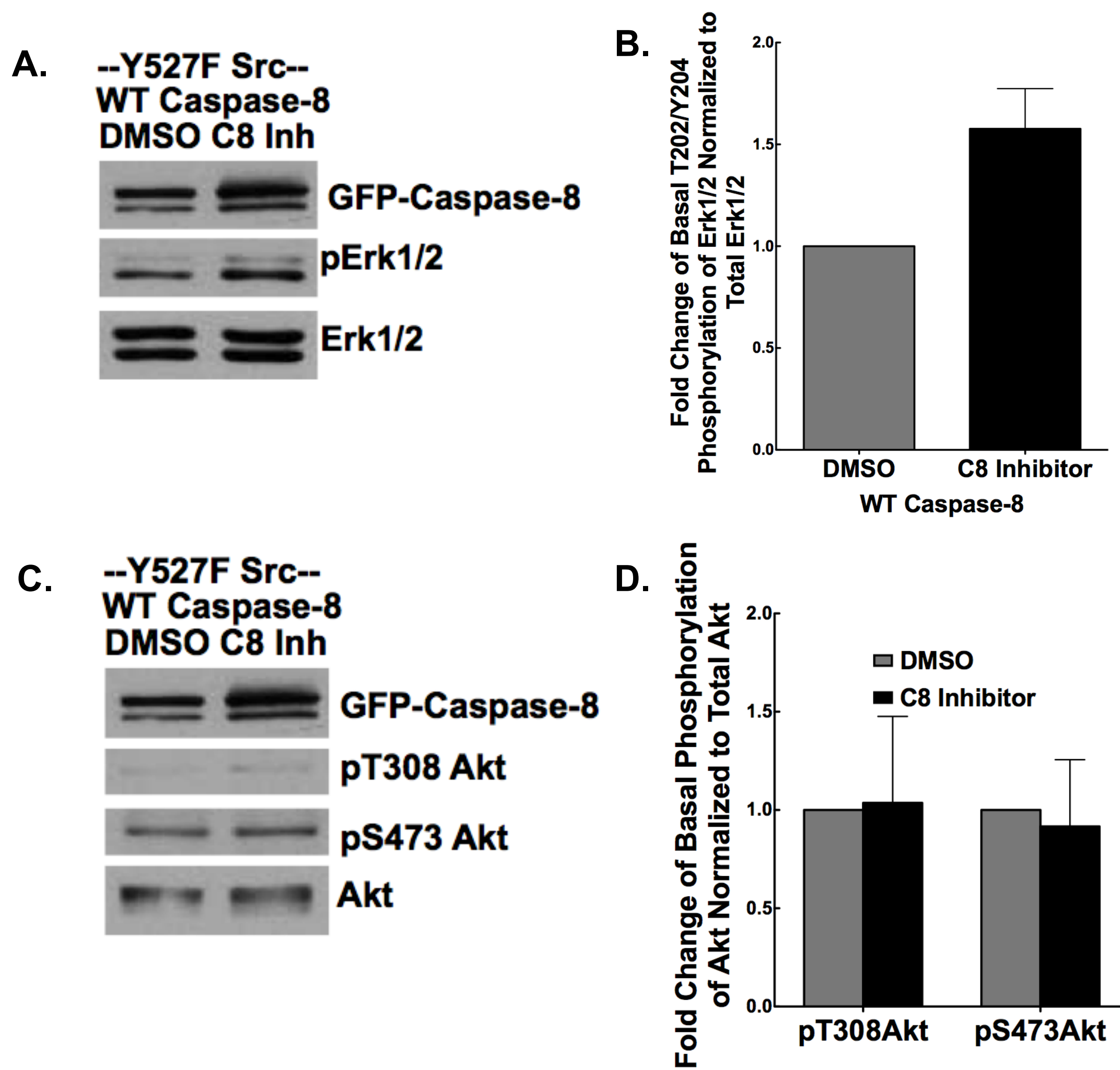


Figure 20. Caspase-8 Enzyme Inhibition Results in Enhanced Erk1/2 Activation but not Akt Activation

Total lysates from experiments described in Figure 19 were developed with 1) GFP antibody to monitor the transfection level of GFP-Caspase-8, 80 kDa; 2) pT202/Y204 Erk1/2 antibody to study the activation of Erk1/2, 42 & 44 kDa; 3) Erk1/2 antibody to detect the total Erk1/2, 42 & 44 kDa; 4) pT308 Akt and 5) pS473 Akt antibodies to study the activation of Akt, 60 kDa; and 6) Akt antibody to detect the total Akt, 60 kDa. A.) T202/Y204 phosphorylation of Erk1/2 was enhanced (trend) in the presence of Caspase-8 inhibitor. B.) Densitometry analysis was performed to represent the pT202/Y204 phosphorylation level of Erk1/2 normalized to the expression level of total Erk1/2 ($N = 3$, $p = 0.25$). C.) T308 and S473 phosphorylation of Akt were not altered by the presence of Caspase-8 inhibitor. D.) Densitometry analysis was performed to represent the pT308 and pS473 phosphorylation level of Akt normalized to the expression level of total Akt.

4.4 DISCUSSION

c-Src and caspase-8 pathways are seemingly two parallel processes, one governing cell survival, and the other cell death. We hypothesized that the c-Src pathway can regulate caspase-8 pathway and vice versa. Specifically, we hypothesized that when a cell commits to a survival pathway, the death pathway is suppressed and when a cell commits to a death pathway, the survival pathway is suppressed.

Several observations in the literature suggest the interplay between the regulation of SFKs and caspases. SFKs have been shown to upregulate the expression of an anti-apoptotic Bcl-2 family protein, Bcl-x_L (340). Anti-apoptotic Bcl-2 family proteins, in turn, can antagonize the initiator caspases (192) and pro-apoptotic Bcl-2 family proteins (378). On the other hand, caspase-9 and -7-mediated cleavage of Lyn led to the formation of a truncated kinase that inhibited the apoptotic program (408) and promoted inflammatory processes (409), suggesting a complex interplay between SFKs and caspases, involving negative and positive feedbacks, depending on the specific isoforms of both groups.

In this chapter, we have shown that caspase-8 activity suppressed c-Src activation and some of its downstream targets including global tyrosine phosphorylation, Erk1/2 activation and tyrosine phosphorylation of caspase-8. Our results also suggest that in the absence of caspase-8 enzymatic activity, the full-length caspase-8, when tyrosine phosphorylated, could further enhance the activation of c-Src and Erk1/2. Together, these data imply a bidirectional (reciprocal) regulatory relationship between caspase-8 and c-Src.

We speculate that in the setting of **apoptosis**, i.e., in the presence of caspase-8 enzymatic activity, c-Src activation and some of its downstream activities are suppressed, in order to allow the cells to commit to cell death. In this chapter, we demonstrated that caspase-8 enzymatic activity negatively regulates c-Src activation, activity and its capacity to tyrosine phosphorylate caspase-8. It is noteworthy to mention that the transfection efficiency of GFP-Caspase-8 was only about 30-40%. Therefore, the effect of caspase-8 suppression of c-Src activation and activity was largely underestimated since whole cell lysates were used for their quantification. We propose that caspase-8 acts as an enzyme rather than (or in addition to) as an adaptor in the suppression of c-Src. This acts as a fail-safe mechanism to prevent unnecessary inhibition of survival pathway in the absence of an apoptotic signal. We further propose that caspase-8 might directly or indirectly cleave c-Src, thus negatively regulating its activation. Other groups have, for example, shown that caspase-3 could cleave Fyn and Lyn in their N-terminus, altering their subcellular localization, thus alter their functions (403).

We further speculate that in the setting of **survival**, c-Src could antagonize caspase-8 in order to downregulate the apoptotic pathway. Previously, it has been shown that SFKs could tyrosine phosphorylate caspase-8 (52, 177, 178). It has also been suggested that tyrosine phosphorylation of caspase-8 at Y397 (caspase-8a) (178) or at Y380 (caspase-8b) (177) could suppress apoptosis. We found that in the presence of caspase-8 enzymatic activity, c-Src was unable to tyrosine phosphorylate caspsae-8. Conversely, only in the absence of caspase-8 enzymatic activity, achieved by the use of caspase-8 inhibitor or transfection with C377S inactive caspase-8 mutant, could c-Src tyrosine phosphorylate caspase-8. In addition, in the absence of caspase-8 enzymatic activity,

full-length caspase-8, when tyrosine phosphorylated, seemed to enhance c-Src activation and some of its downstream targets. This suggests that only in the presence of a pro-survival signal could caspase-8 be turned into a pro-survival molecule and further activates c-Src. Moreover, in contrary to previous studies (52, 177, 178), we demonstrated that c-Src not only could phosphorylate caspase-8a at Y397, it could also phosphorylate it at Y8, Y243, Y351 (mass spectrometry data) and potentially at Y310 and Y465. This suggests that other tyrosine residues might be important in regulating caspase-8 activity when they are phosphorylated. This will be further explored in the next chapter.

Together, these data suggest that if an apoptotic program, initiated by caspase-8 activity, were activated, it would support the suppression of pro-survival signaling. Conversely, if a pro-survival signaling, initiated by c-Src activity, were activated, it would support the suppression of apoptotic signaling. In other words, the interplay between caspase-8 and c-Src regulates the balance of apoptosis and survival, respectively, in a mutually reciprocal manner. And it is the balance of apoptosis and pro-survival signaling that will set the final outcome of the cell.

In summary, the decision of a cell to undergo apoptosis versus survival depends on the balance of death and survival signals. In the setting where there is a predominant death signal, enough to activate caspase-8, survival pathway is inhibited at a relatively proximal level. Likewise, in the setting where there is a predominant survival signal, enough to allow activation of c-Src to tyrosine phosphorylate caspase-8, apoptotic pathway is inhibited, also at a relatively proximal level (Figure 21). These reciprocal inhibitory interactions could act as positive feedback mechanisms both towards survival

and cell death, enhancing the speed and efficiency of the dominant, cell fate-determining processes.

Figure 21

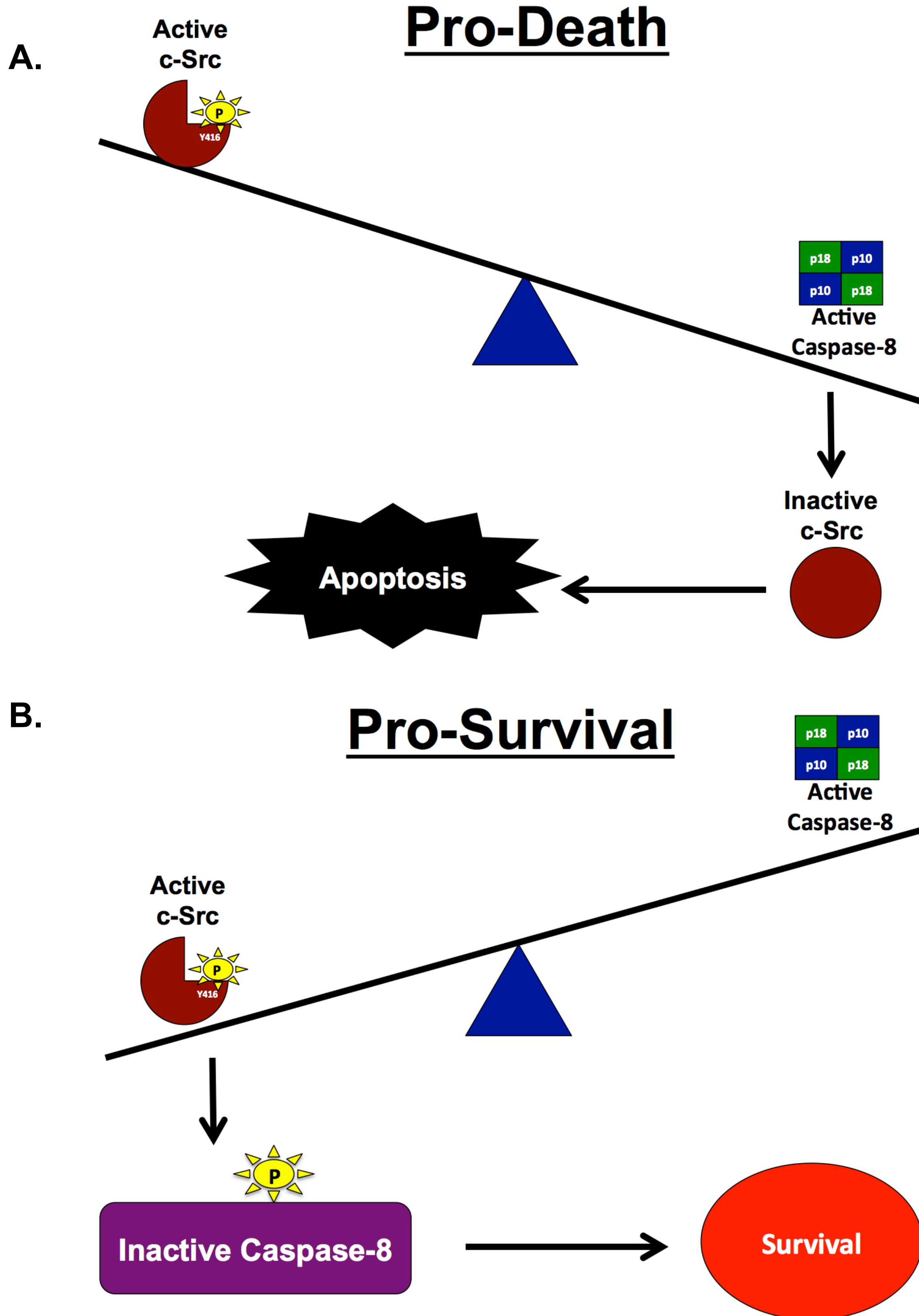


Figure 21. The Balance of Death and Survival

A) When the balance between caspase-8 enzymatic activity and c-Src activity favors caspase-8 enzymatic activity, c-Src activity is inhibited by caspase-8 enzymatic activity and further suppression of survival pathways ensues, resulting in the augmentation of apoptosis. B) When the balance between caspase-8 enzymatic activity and c-Src activity favors c-Src activity, caspase-8 is tyrosine phosphorylated and its activity inhibited, resulting in the suppression of apoptosis.

Chapter 5. MULTIPLE TYROSINE PHOSPHORYLATION SITES SWITCH CASPASE-8 FROM AN APOPTOTIC TO A PRO-SURVIVAL MOLECULE

5.1 SUMMARY

Caspase-8, a major regulator of extrinsic apoptosis, can be tyrosine (Y) phosphorylated by Src family tyrosine kinases (SFKs) and this event has been implicated in the suppression of its apoptotic functions. However, the mechanism of this action has not been explored. Moreover, although the notion that caspase-8 can act as a pro-survival molecule in certain situations has been raised, the molecular details of such potential function have not been elucidated. To explore the mechanism of apoptosis inhibition upon caspase-8 tyrosine phosphorylation, we undertook a series of experiments using co-transfection of GFP-tagged WT Caspase-8 or its various phosphomimetic or non-phosphorylatable mutants along with a constitutively active (Y527F) c-Src construct. As readout, we followed the cleavage, tyrosine phosphorylation, and apoptotic activity of the GFP-Caspase-8 protein as well as their capacity to induce c-Src phosphorylation at Y416 and activation of downstream c-Src targets. We found that phosphomimetic modification of caspase-8 at Y465, but not at Y397 prevented its cleavage and apoptotic activities. Moreover, phosphomimetic modification of caspase-8 at Y465 resulted in the activation of two survival pathways, c-Src and Erk1/2. Intriguingly, despite the lack of anti-apoptotic and pro-survival effect of single phosphomimetic modification at Y397, the pro-survival effect of phosphomimetic modification at Y465 was dependent on the phosphomimetic modification at Y397. These results suggest that multiple tyrosine phosphorylation sites together switch caspase-8 from an apoptotic to a pro-survival molecule.

5.2 INTRODUCTION

Caspase-8 is an apical caspase, which plays a central role in apoptosis induced by the ligation of death receptors (extrinsic apoptosis) (83, 85, 89, 90). Caspase-8 is expressed as a zymogen comprised of an N-terminal pro-domain, with two death effector domains (DED) (14-16, 24, 65), followed by a large (p18) and a small (p12) enzyme subunit. In order to be fully activated, it must undergo a multi-step cleavage process to generate a mature enzyme composed of two p18 and two p12 subunits (23, 26, 27, 65). The mature caspase-8 enzyme then cleaves and activates downstream proteases including caspase-3, to allow apoptosis to ensue.

Caspase-8 has been shown to be tyrosine phosphorylated by Src family tyrosine kinases (SFKs) at tyrosine (Y) 397 in caspase-8a (178) and Y380 in caspase-8b (52, 177). It has also been shown to be tyrosine phosphorylated upon epidermal growth factor (EGF) stimulation (177), which led to the activation of extracellular signal-regulated kinase (Erk) (53). Tyrosine phosphorylation of caspase-8 resulted in the suppression of caspase-8 activity and Fas-induced apoptosis (178, 410) and promotion of cell migration (52). Furthermore, when tyrosine phosphorylated, caspase-8 interacted with c-Src via its Src homology 2 (SH2) domains (52, 141) and p85 subunit of phosphatidylinositol 3-kinase (PI3K) (140).

Together these data suggest that growth factor signaling could potentially influence caspase-8 activity by tyrosine phosphorylation. However, the exact mechanism whereby caspase-8 tyrosine phosphorylation suppresses apoptosis remains to be clarified. Moreover, it is conceivable that tyrosine phosphorylation not only mitigates the apoptotic function of this caspase but also induces direct pro-survival mechanisms. While such a

possibility has been raised by previous studies (53, 141) and our work in chapter 4, this intriguing question has not been addressed.

In this study, we sought to first investigate whether other potential tyrosine phosphorylation sites (in addition to Y397) play essential roles in suppressing caspase-8 apoptotic functions. Moreover, we sought to investigate whether these potential tyrosine phosphorylation sites can enhance c-Src activation and activity and to examine the potential mechanism of c-Src activation upon tyrosine phosphorylation of caspase-8. From our work in chapter 4, we identified multiple potential tyrosine phosphorylation sites. There were one (Y8) within the N-terminal domain, three (Y243, Y310 and Y351) within the p18 subunit, one (Y397) within the linker region and one (Y465) within the p12 subunit. Since the p12 subunit of caspase-8 is crucial in its dimerization, we decided to examine Y465 residue. We also decided to examine Y397 within the linker region, which has been previously studied. During our studies, we observed that Y465 residue regulated caspase-8 activity in conjunction with Y397 residue; therefore, we proceeded with the generation of computational mutation of these residues to further examine the mechanism of caspase-8 regulation by tyrosine phosphorylation.

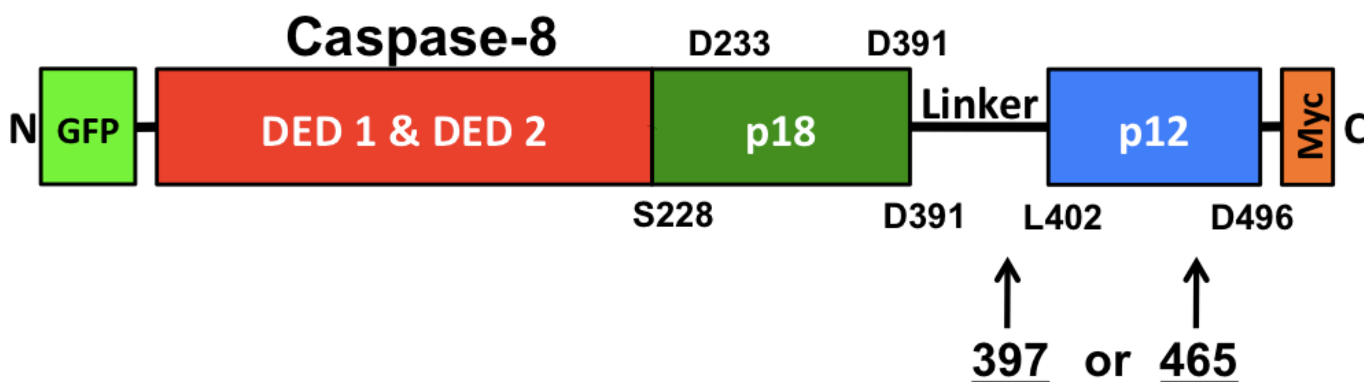
Here, we show that Y465, when modified into a phosphomimetic residue, is the key residue in the suppression of apoptosis by inhibiting caspase-8's ability to undergo cleavage, whereas Y397 is the key residue in the activation of c-Src and Erk1/2, suggesting that tyrosine phosphorylation of Y397 and Y465 turns caspase-8 from an apoptotic protein into a pro-survival protein.

5.3 RESULTS

5.3.1 Generation of Phosphomimetic and Non-Phosphorylatable Mutants

In order to investigate the function and regulation of caspase-8, we generated phosphomimetic and non-phosphorylatable caspase-8 constructs at Y397 and Y465 sites. In order to facilitate the tracking of transfection efficiency and the study of caspase-8 cleavage, we labeled our caspase-8 construct with GFP in the N-terminus and Myc in the C-terminus (Figure 22).

Figure 22



Phosphomimetic	Tyrosine (Y) -> Glutamic Acid (E)
Non-Phosphorylatable	Tyrosine (Y) -> Phenylalanine (F)

Figure 22: Generation of Caspase-8 Mutants

GFP-tagged caspase-8 structure is shown. Both phosphomimetic (tyrosine (Y) -> glutamic acid (E)) and non-phosphorylatable (tyrosine (Y) -> phenylalanine (F)) mutants were generated at residue 397 or 465.

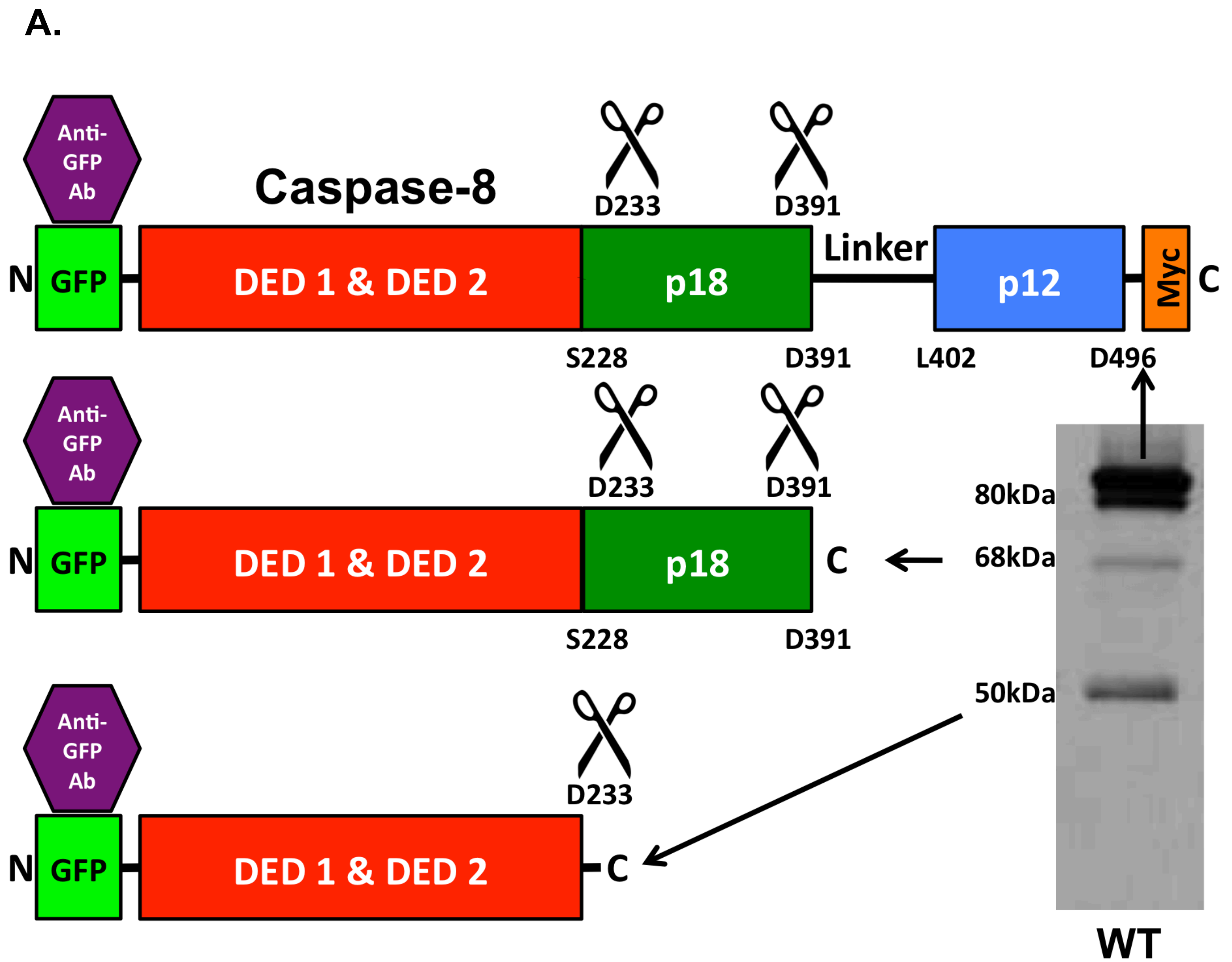
5.3.2 Phosphomimetic Modification at Y465 but not Y397 of Caspase-8a Prevents its Cleavage

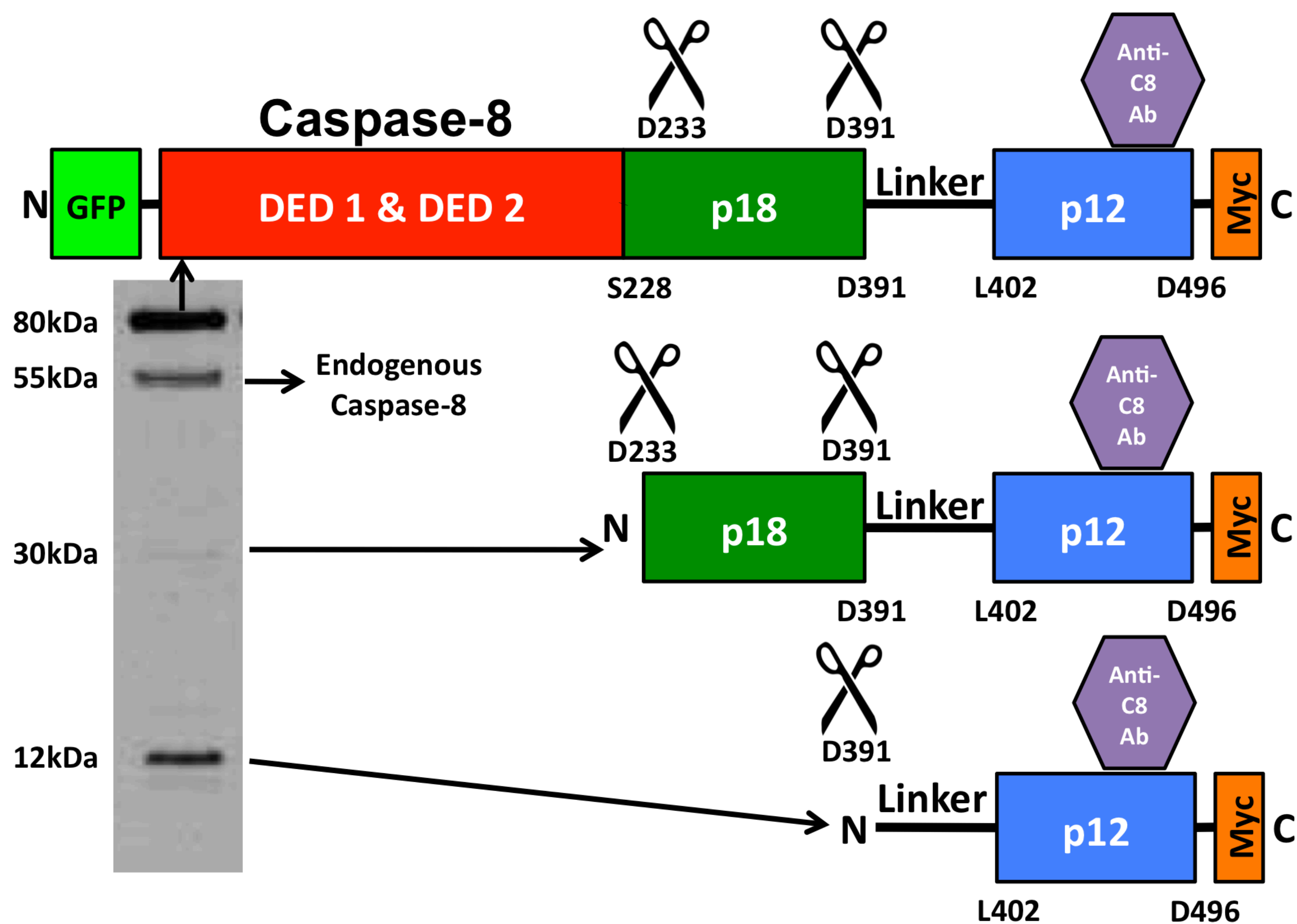
Caspase-8 is classically known as an initiator caspase. Its activation requires its dimerization (in proximity) followed by a 2-step cleavage after aspartic acid (D) residues. Cleavage occurs first after D391 within the linker region and then after D233 at the beginning of p18 subunit (23, 26, 27, 65). Cleavage after D391 is important in the activation of caspase-8 whereas cleavage after D233 is dispensible in the activation of caspase-8 but it allows the release of active caspase-8 from the pro-domain. To investigate the mechanism whereby tyrosine phosphorylation suppresses caspase-8 enzyme activity, we examined the cleavage pattern of wild type (WT) and various mutants of caspase-8. To this end, HEK293 cells were transfected with these GFP-Caspase-8 constructs and 24 hours later whole cell lysates were obtained and analyzed by Western blotting. We probed the blots both with a GFP antibody, which recognizes the N-terminus of the heterologously expressed caspase-8 and with a caspase-8 antibody, which recognizes the C-terminus (p12 subunit) of both the endogenous and the heterologously expressed caspase-8 protein. Although a Myc-tag was present in the C-terminus of the caspase-8 construct, it did not provide a good epitope for Western blot detection. Therefore, a caspase-8 antibody that recognizes the p12 subunit was used instead.

By using a GFP antibody, we verified that WT caspase-8 could be cleaved after D391, resulting in a 68-kDa N-terminal fragment; and after D233, resulting in a 50-kDa N-terminal fragment (Figure 23A). In accordance with this, caspase-8 antibody visualized the full-length protein as well as a 30-kDa and 12-kDa C-terminal fragments

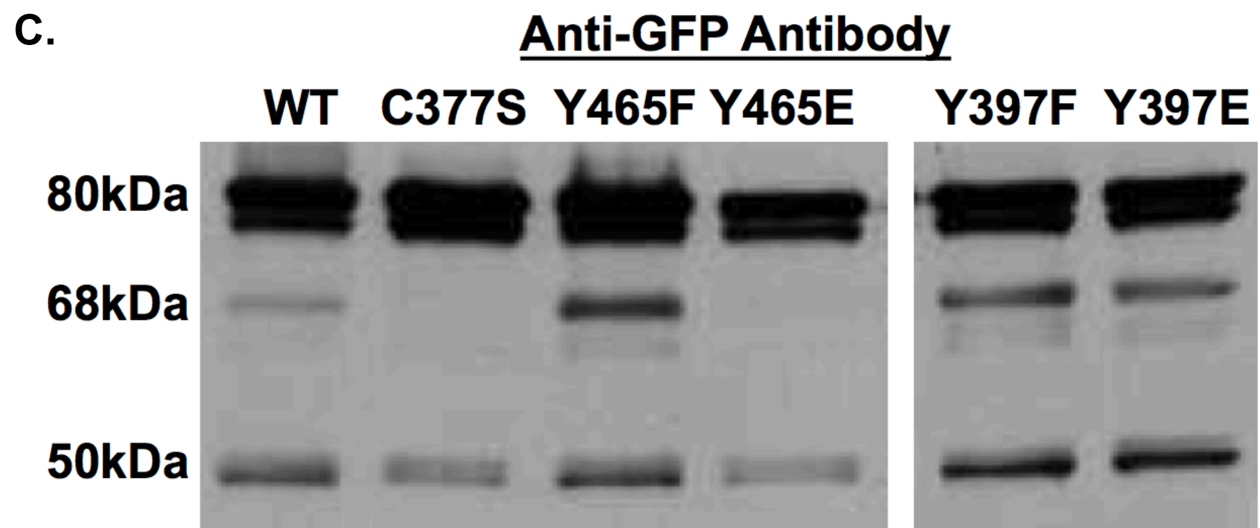
corresponding to cleavage after D233 and D391 respectively (Figure 23B). Similar cleavage patterns were observed in Y465F, Y397F and Y397E mutants, indicating that these mutations do not alter the processing of caspase-8. However, similar to the C377S inactive mutant, Y465E (phosphomimetic) mutant was not cleaved after D391, as indicated by the fact that it failed to produce a 68-kDa N-terminal fragment and a 12-kDa C-terminal fragment (Figure 23C & D). Together, these data imply that phosphomimetic modification of Y465 prevented caspase-8 from cleavage after D391 (Figure 23E). Both the phosphomimetic and non-phosphorylatable modification of Y397 and non-phosphorylatable modification of Y465 residue had no effect on cleavability after D391 (Figure 23C & D).

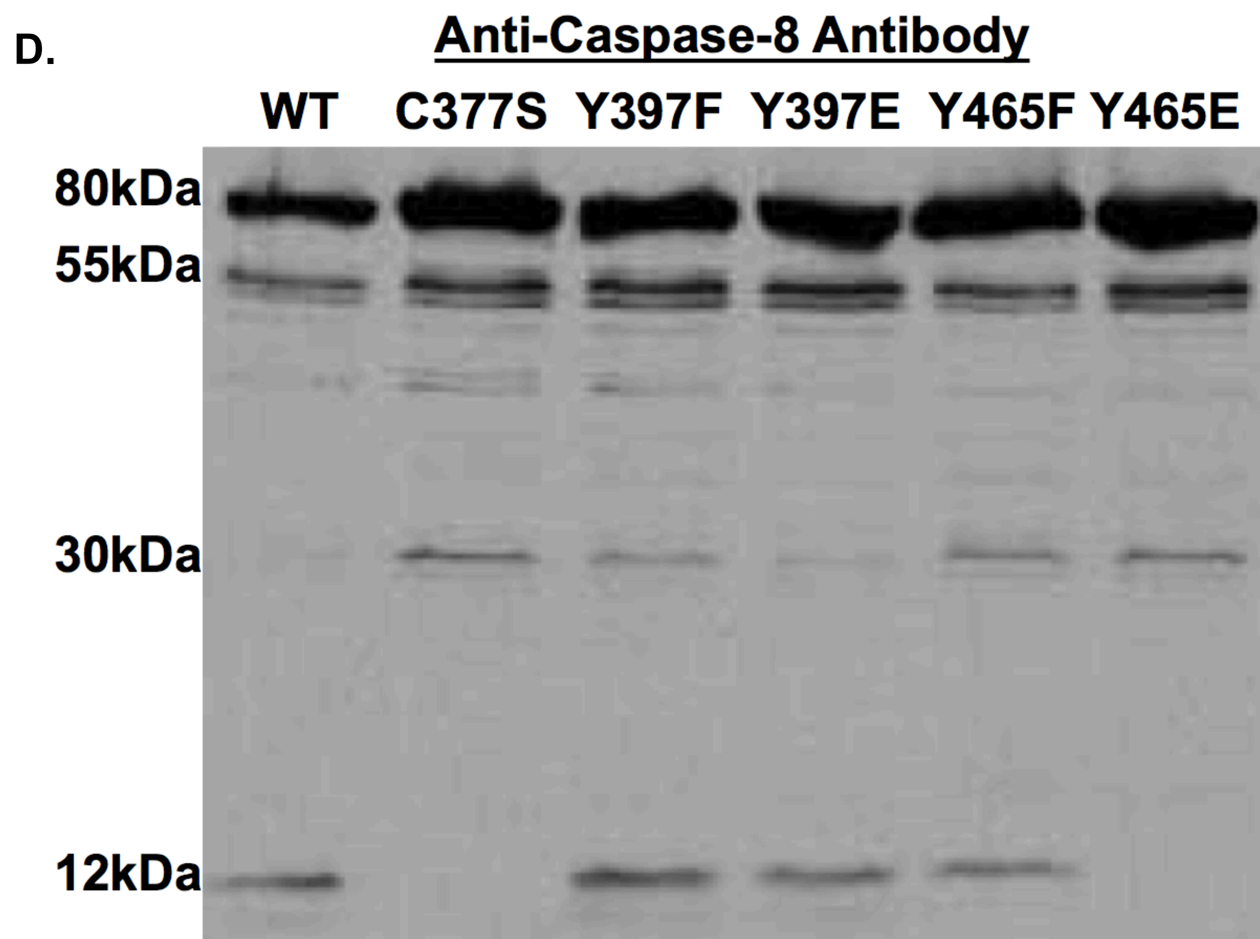
Figure 23





B.
Caspase-8
D233
D391
Anti-C8 Ab
N
GFP
DED 1 & DED 2
p18
Linker
p12
Myc
C
S228
D391
L402
D496
80kDa
55kDa
30kDa
12kDa
Endogenous Caspase-8
D233
D391
Anti-C8 Ab
N
p18
Linker
p12
Myc
C
D391
L402
D496
D391
Anti-C8 Ab
N
Linker
p12
Myc
C
L402
D496


C.
Anti-GFP Antibody
WT
C377S
Y465F
Y465E
Y397F
Y397E
80kDa
68kDa
50kDa


D.
Anti-Caspase-8 Antibody
WT
C377S
Y397F
Y397E
Y465F
Y465E
80kDa
55kDa
30kDa
12kDa

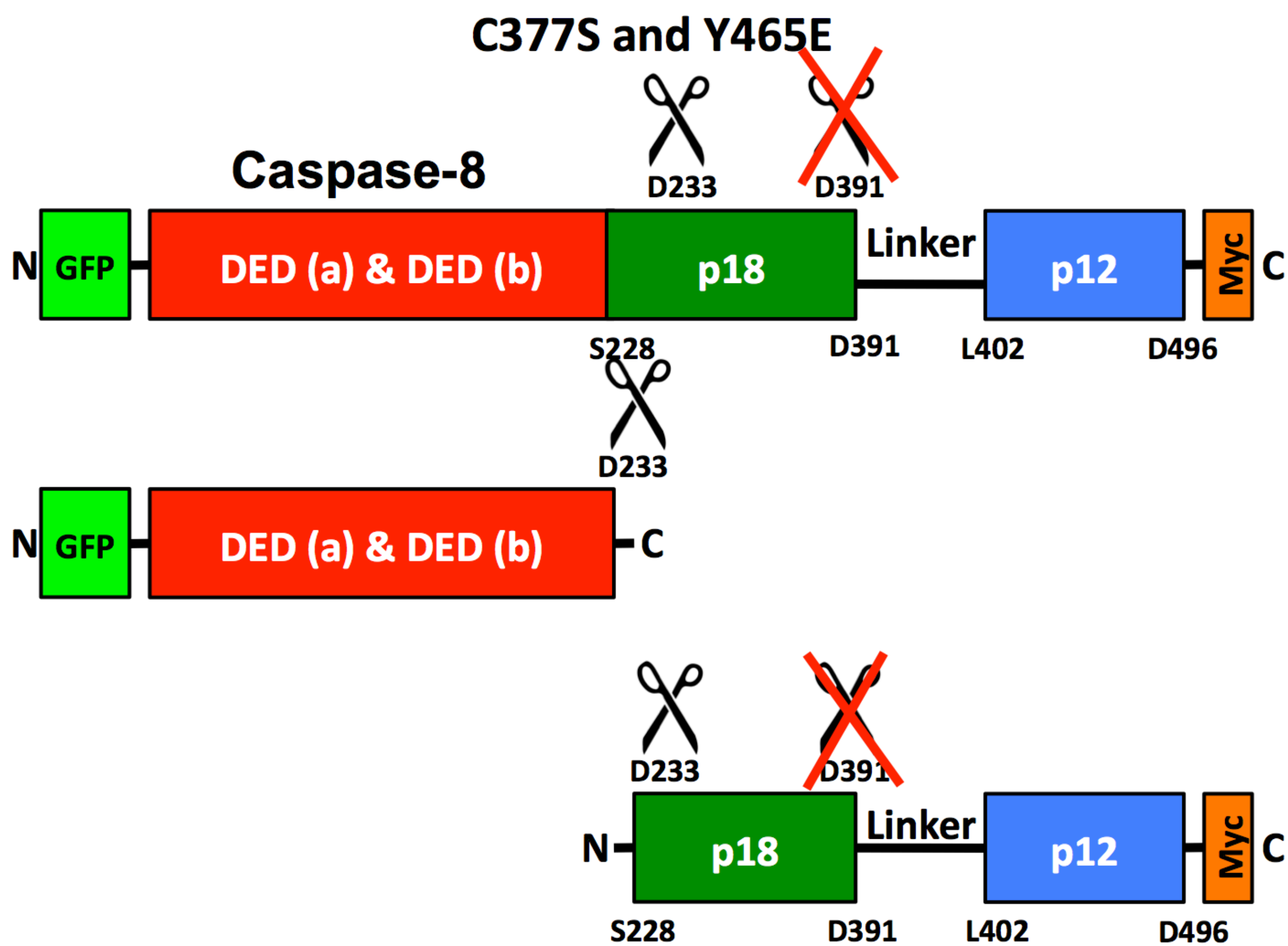

E.
C377S and Y465E
Caspase-8
D233
D391
N
GFP
DED (a) & DED (b)
p18
Linker
p12
Myc
C
S228
D391
L402
D496
D233
N
GFP
DED (a) & DED (b)
C
D233
D391
N
p18
Linker
p12
Myc
C
S228
D391
L402
D496

Figure 23. Wild Type, Y397F, Y397E, Y465F Caspase-8 Mutants were Cleaved after D233 and D391 whereas C377S and Y465E Caspase-8 Mutants were only Cleaved after D233

HEK293 cells were transfected with various GFP-Caspase-8 mutants (WT, C377S, Y397F, Y397E, Y465F and Y465E) for 24 hours. Whole cell lysates were subjected to Western blot analysis with either a GFP or a caspase-8 antibody. A) With a GFP antibody (recognized the N-terminal end of GFP-caspase-8), we found that GFP-caspase-8 WT was cleaved into three bands. The 80-kDa band represented full size GFP-caspase-8, the 68-kDa band represented a N-terminal cleavage product after D391 whereas the 50-kDa band represented a N-terminal cleavage product after D233. B) With a caspase-8 antibody (recognized the C-terminal end of GFP-Caspase-8 and endogenous caspase-8), we found that GFP-Caspase-8 WT was cleaved into three bands. The 80-kDa band represented full size GFP-Caspase-8, the 30-kDa band represented a C-terminal cleavage product after D233 and the 12-kDa band represented a C-terminal cleavage product after D391. The 55-kDa band represented endogenous caspase-8. C) With the use of a GFP antibody, we showed that WT, Y397F, Y397E and Y465F cleaved into three N-terminal bands (80-, 68- and 50-kDa) whereas C377S and Y465E only cleaved into two N-terminal bands (80- and 50-kDa). D) With the use of a caspase-8 antibody, we showed that WT, Y397F, Y397E and Y465F cleaved into three C-terminal bands (80-, 30- and 12-kDa) whereas C377S and Y465E only cleaved into two C-terminal bands (80- and 30-kDa). E) A schematic diagram demonstrating that C377S and Y465E were cleaved after D233 but not after D391 is shown.

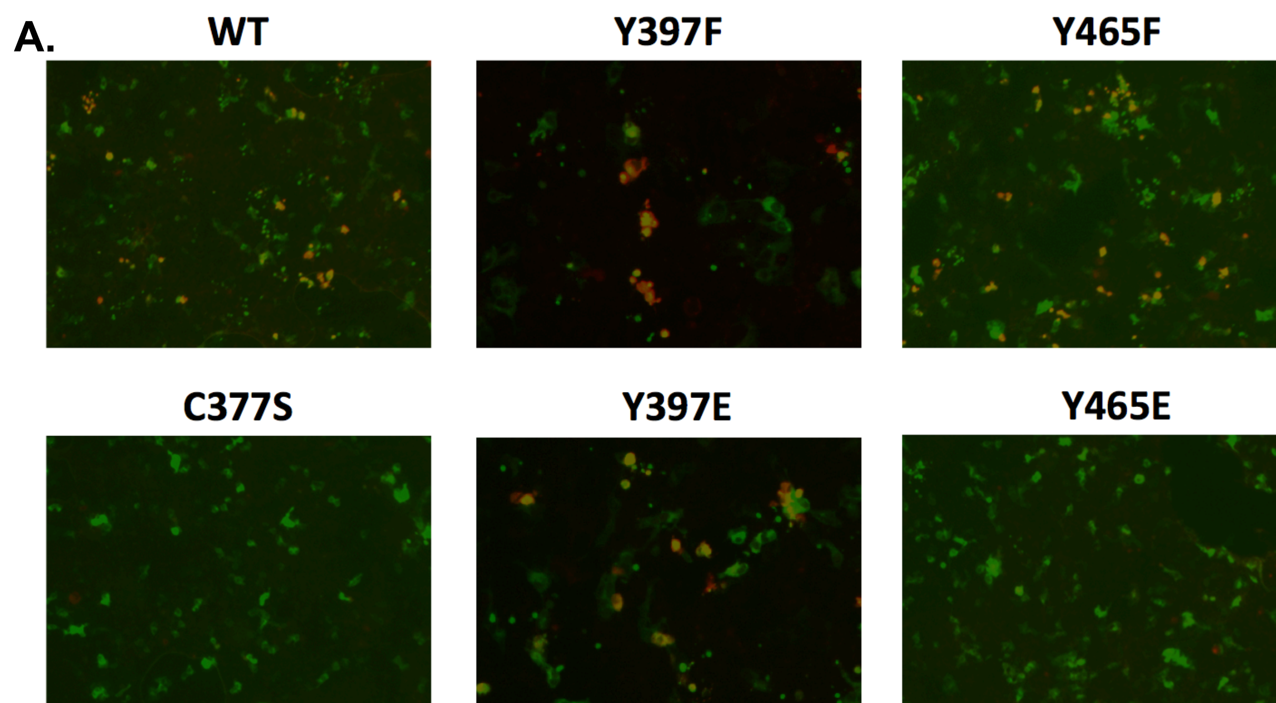
5.3.3 Phosphomimetic Modification of Y465 but not Y397 of Caspase-8 Abolishes its Capacity to Activate Caspase-3 and to Induce Apoptosis

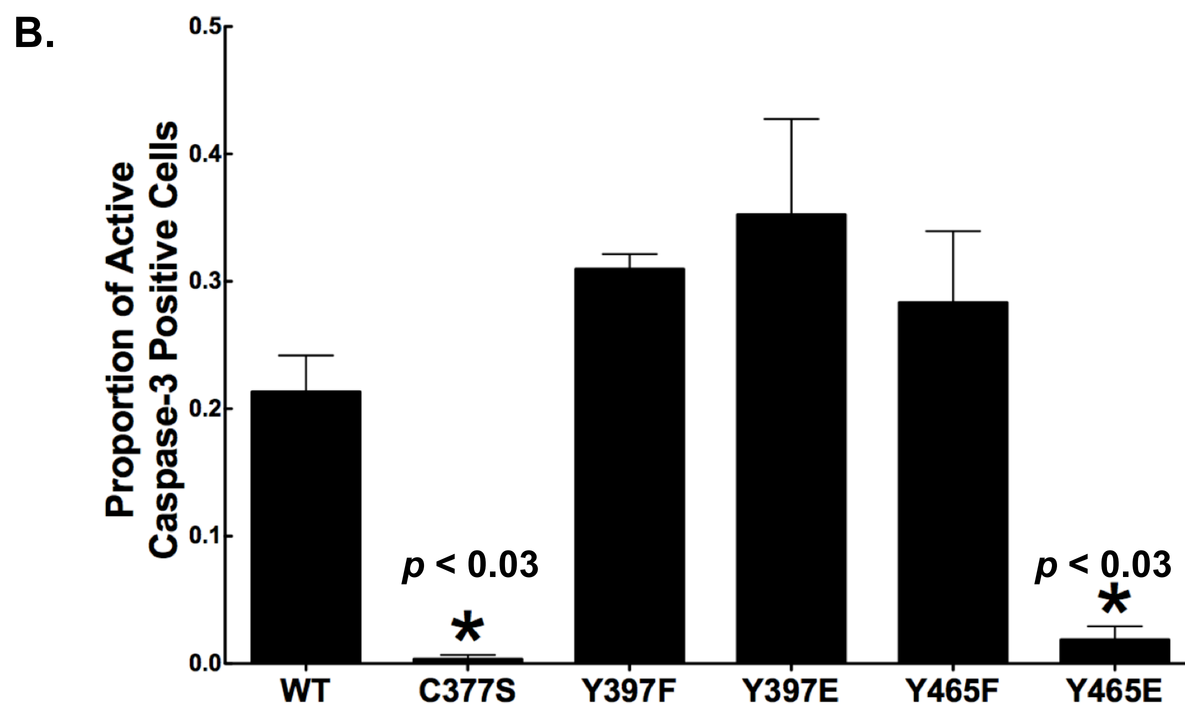
After being cleaved, caspase-8 becomes active and proceeds to cleave downstream proteases including caspase-3, thereby promoting apoptosis. Since phosphomimetic modification of Y465 of caspase-8 prevented its cleavage after D391, we asked whether this modification would indeed disrupt the ability of caspase-8 to activate caspase-3. To test this, we transfected WT and various mutants of caspase-8 into HEK293 cells for 24 hours, then immunostained them for active (cleaved) caspase-3. We then counted the number of active caspase-3 positive cells in caspase-8 transfected cells (red merged with green into orange). Approximately 20% of the WT GFP-Caspase-8-expressing cells were positive for active caspase-3. Consistent with their ability to be cleaved, the expression of Y397F, Y397E, Y465F resulted in caspase-3 cleavage in 30-35% of the transfected cells. However, less than 1% of the cells expressing C377S, the inactive mutant, or the Y465E phosphomimetic mutant exhibited active caspase-3 staining. This finding is consistent with the inability of these mutants to be cleaved (Figure 24A & B).

After showing that phosphomimetic mutation at Y465 of caspase-8 prevented its cleavage and its capacity to activate caspase-3, we wanted to investigate whether the phosphomimetic modification of Y465 fails to cause cell morphological changes that could be indicative of cell death, specifically, rounding of cells or formation of apoptotic bodies. To this end, we transfected WT or various mutants of caspase-8 into HEK293 cells for 24 hours then cells were imaged using fluorescence microscopy. We demonstrated that WT GFP-Caspase-8 caused significant changes in cell morphology with the occurrence of cell rounding. Similar changes in cell morphology were seen in

Y397F, Y397E and Y465F mutants. However, both C377S and phosphomimetic mutant at Y465 prevented changes in cell morphology seen in WT GFP-Caspase-8 (Figure 24C). These data together suggest that phosphorylation at Y465, just like inactive mutant (C377S) of caspase-8, abolishes the ability of caspase-8 to cause cell morphological changes.

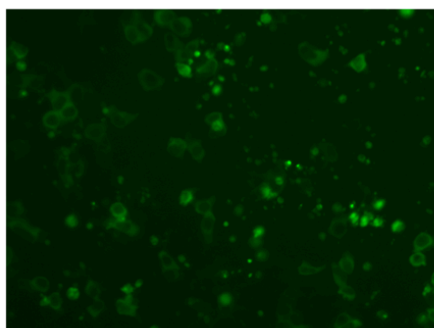
Figure 24



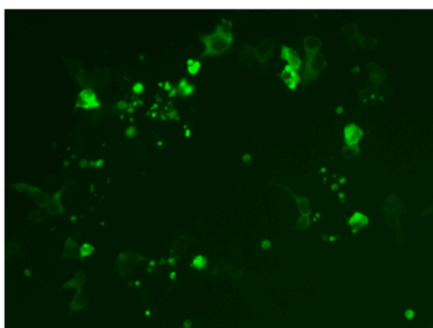


C.

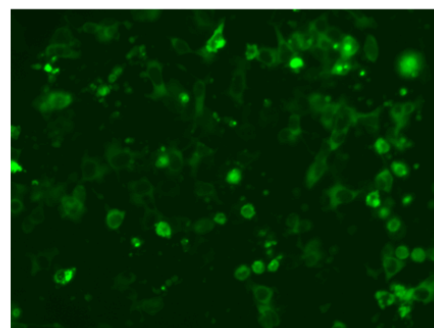
WT



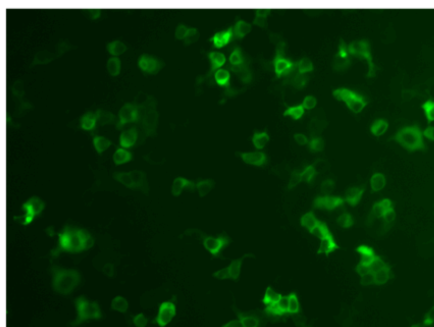
Y397F



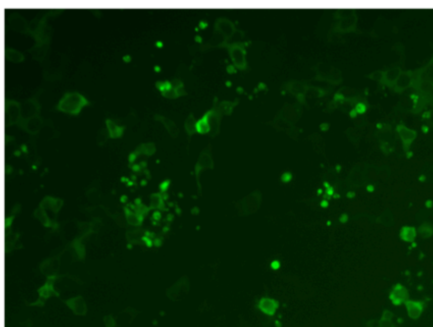
Y465F



C377S



Y397E



Y465E

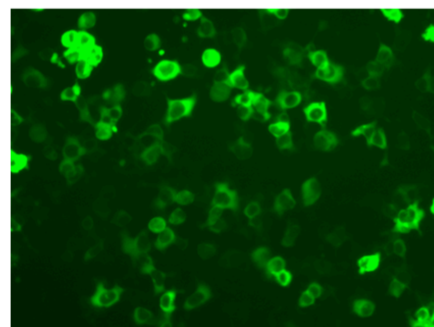


Figure 24. C377S and Y465E Caspase-8 Fail to Activate Caspase-3 and Fail to Induce Cell Morphological Changes

HEK293 cells were transfected with various GFP-caspase-8 mutants (WT, C377S, Y397F, Y397E, Y465F and Y465E) for 24 hours. A) To examine for activation of caspase-3, we fixed the transfected cells on coverslips and immunostained them with an active caspase-3 antibody then with secondary antibody conjugated with Cy3 fluorochrome. The representative fluorescence microscopy images of each construct were shown. B) To quantify the percentage of active caspase-3 positive cells in GFP-caspase-8 transfected cells, the number of cells that were transfected with GFP-caspase-8 (green) and the number of cells that were also stained with anti-active caspase-3 antibody (orange) were counted. The percentage of active caspase-3 positive cells that were transfected with GFP-caspase-8 was displayed. C377S and Y465E caspase-8 mutants failed to activate caspase-3 (N = 3, * $p < 0.03$). C) Cell morphology of transfected cells was examined using live fluorescence microscopy. C377S and Y465E GFP-Caspase-8 mutants failed to cause cell rounding that is consistent with cell death.

5.3.4 Phosphomimetic Modification at Y465 of Caspase-8 Enhances Activation of c-Src

Tyrosine phosphorylation of caspase-8 at Y380 (caspase-8b) has been shown to promote the interaction between caspase-8 with the SH2 domain of c-Src (52, 140). Furthermore, caspase-8 has been reported to contribute to growth factor signaling (53). So far we have shown that phosphomimetic modification of Y465 of caspase-8 prevented its activation (cleavage) and activity, implying that this modification rendered the molecule incapable of inducing apoptosis. We however wonder whether it might also confer a pro-survival activity to the caspase-8 molecule. This idea stemmed from the fact that several c-Src substrates also act as c-Src activators, such as actin filament-associated protein (AFAP-110) (411) and XB-130 (412), because they promote or stabilize the open (active) conformation of the kinase. The activation of c-Src involves the dephosphorylation of an inhibitory tyrosine residue (Y527) (249, 405, 406) and the autophosphorylation of an activating loop tyrosine residue (Y416) (261, 407). Here, we applied a strategy, which allowed us to simultaneously induce c-Src activation-mediated effects on caspase-8 and to study the impact of these on (further) activation of c-Src. We used a constitutively active c-Src (Y527F) (mimicking the dephosphorylation of the inhibitory site), and studied the phosphorylation of Y416 as an indicator of c-Src activation in the presence of various caspase-8 mutants, as detailed below. As an additional marker of c-Src activation, we also followed global protein tyrosine phosphorylation upon Y527F c-Src expression.

Specifically, we co-transfected HEK293 cells with Y527F c-Src along with pEGFP-C1 empty plasmid or GFP fusion plasmid encoding for WT or mutant GFP-

Caspase-8 including C377S (inactive) or Y465E (phosphomimetic) or Y465F (non-phosphorylatable). After 24 hours, the cells were lysed and Western blot analysis was performed to assess phosphorylation of Y416 of c-Src and global protein tyrosine phosphorylation. Compared to empty plasmid, phosphomimetic mutant at Y465 increased the phosphorylation of Y416 of c-Src. However, the non-phosphorylatable mutant at Y465, not only failed but it significantly decreased the phosphorylation of Y416 of c-Src (Figure 25A & B). Considered together these data suggest that phosphorylation of Y465 promoted activation of c-Src while dephosphorylation of Y465 inhibited c-Src activation.

In chapter 4, we have shown that WT caspase-8 suppressed phosphorylation at Y416 of c-Src. Since Y397 of caspase-8 was reported to be a major target of SFKs, we studied the impact of the modification of this residue on c-Src phosphorylation at Y416. In contrast to Y465, neither the phosphomimetic nor the non-phosphorylatable mutants at Y397 altered the capacity of caspase-8 to suppress Y416 c-Src phosphorylation. Thus, changes in the phosphorylation status of Y397 did not abrogate the negative effect of caspase-8 on c-Src activation as seen in WT caspase-8 (Figure 25C). This is consistent with our findings shown in sections 5.3.2 and 5.3.3 that both phosphomimetic and non-phosphorylatable mutants at Y397 harbored caspase-8 activity reflected by their ability to undergo cleavage, to activate caspase-3 and to induce morphological changes. The presence of caspase-8 activity in these mutants might explain the suppression of c-Src activity.

In accordance with the impact of Y465 modification on c-Src phosphorylation, the phosphomimetic mutant at Y465 increased global protein tyrosine phosphorylation.

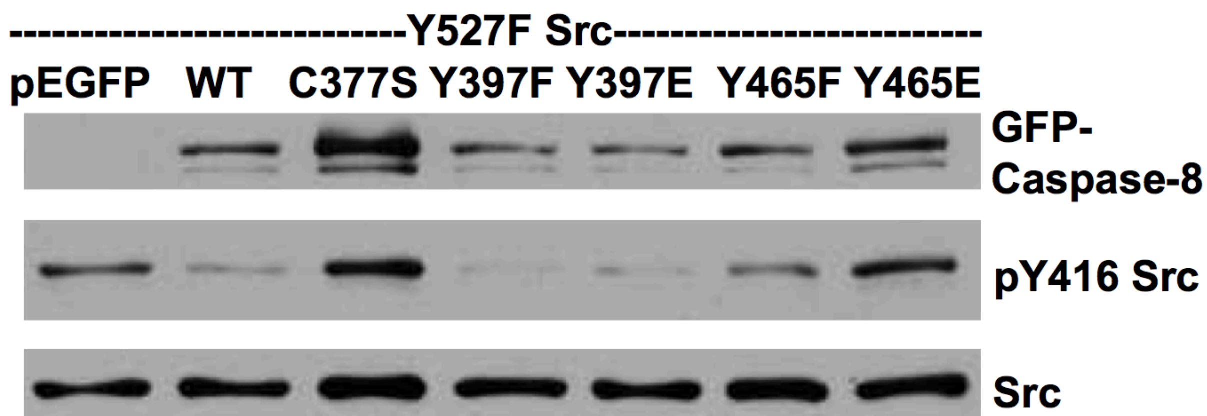
Similar results were obtained with C377S, the inactive mutant (Figure 25D). However, other than tyrosine phosphorylation of caspase-8 itself, the non-phosphorylatable mutant at Y465 of caspase-8 failed to increase global protein tyrosine phosphorylation of other protein substrates (see red arrows in Figure 25D). These data suggest that phosphorylation at Y465 of caspase-8 is important in activating c-Src.

The ability of Y465F to be tyrosine phosphorylated was a surprising finding. Based on this result, we suspect that a protein tyrosine phosphatase (PTP) might be involved in the dephosphorylation of WT, Y397F and Y397E caspase-8. In that we mean active caspase-8 might harbor the capability to activate a PTP and this PTP might interact directly with caspase-8 molecule in order to dephosphorylate it. Moreover, we suspect that the interaction of this PTP with caspase-8 is dependent on the presence of Y465 residue. Therefore, in the Y465F mutant, despite its ability to activate a PTP, the Y465F mutant failed to interact with it, rendering the PTP incapable of dephosphorylating caspase-8.

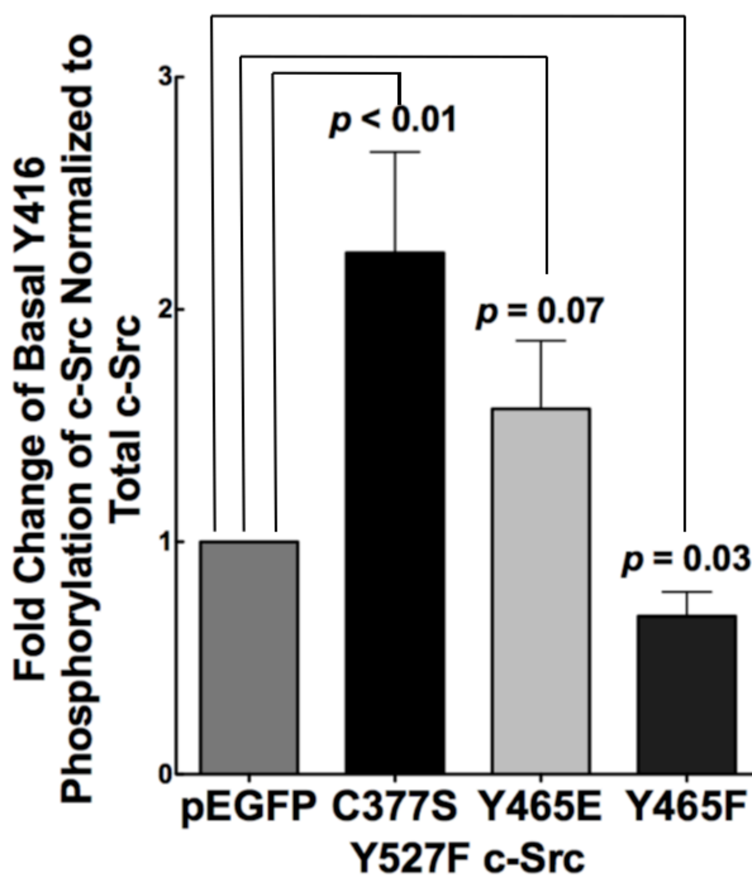
Of note, the effects of Y465E and C377S caspase-8 on Y416 phosphorylation of c-Src and global protein tyrosine phosphorylation were dependent on the presence of Y527F c-Src. Single transfection of GFP-caspase-8 (Y465E or C377S) did not lead to increase in Y416 phosphorylation of c-Src (Figure 25E & F) or global protein tyrosine phosphorylation (Figure 25G).

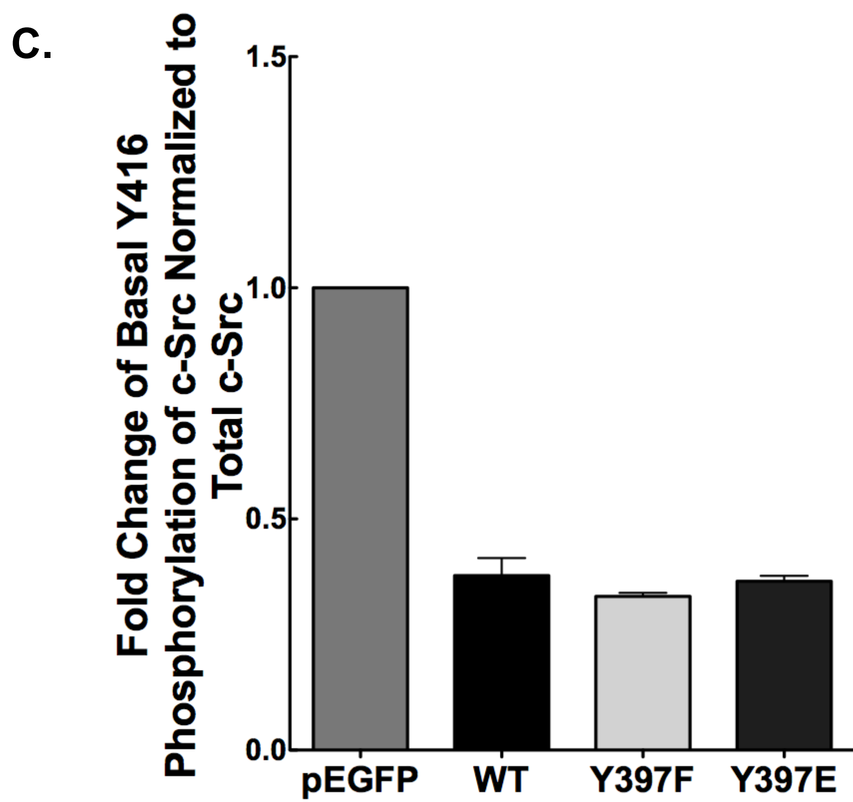
Figure 25

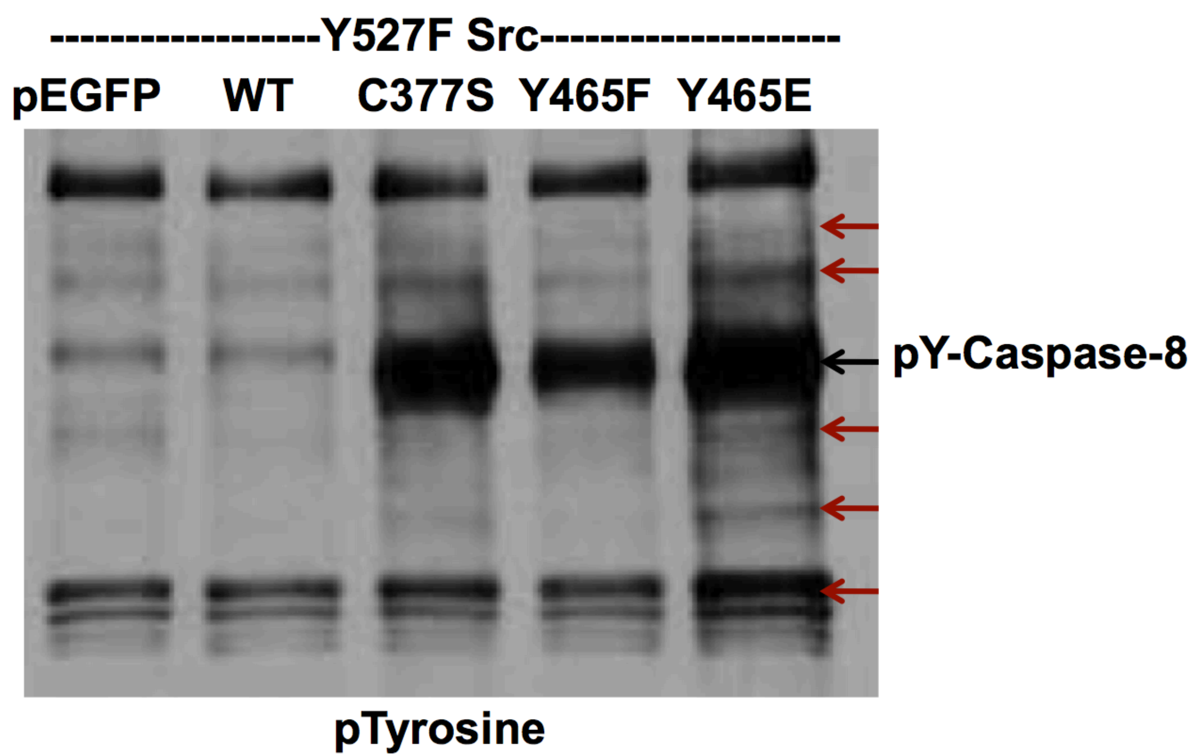
A.



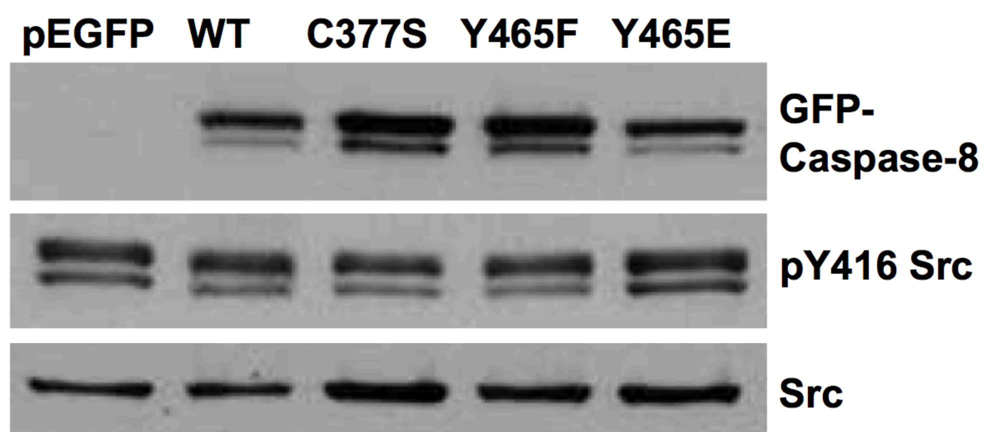
B.



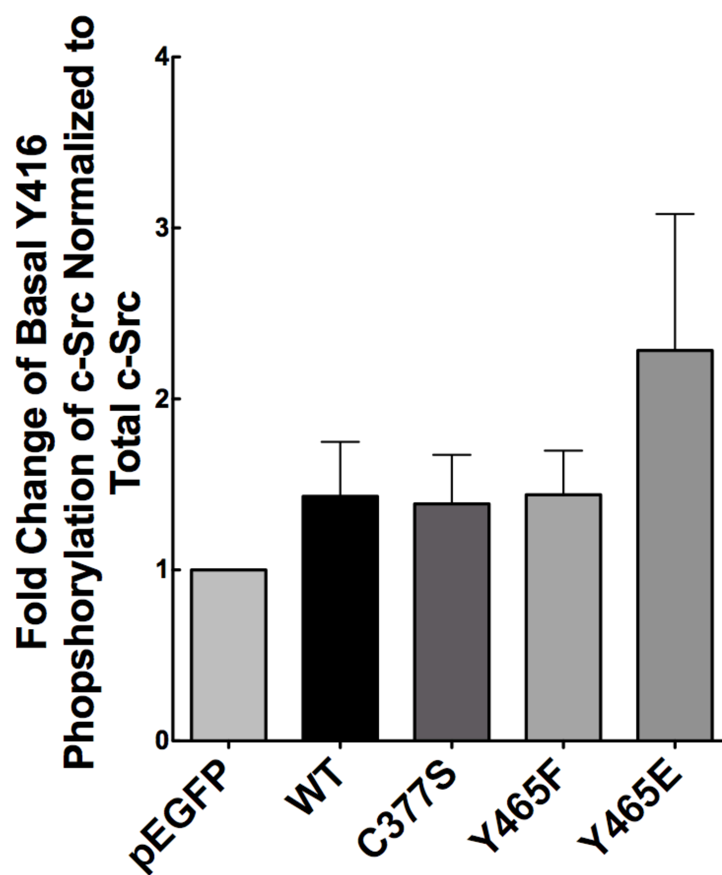

C.
Fold Change of Basal Y416 Phosphorylation of c-Src Normalized to Total c-Src
1.5
1.0
0.5
0.0
pEGFP
WT
Y397F
Y397E


D.
-------------------Y527F Src---------------------
pEGFP
WT
C377S
Y465F
Y465E
pY-Caspase-8
pTyrosine

E.



F.



G.

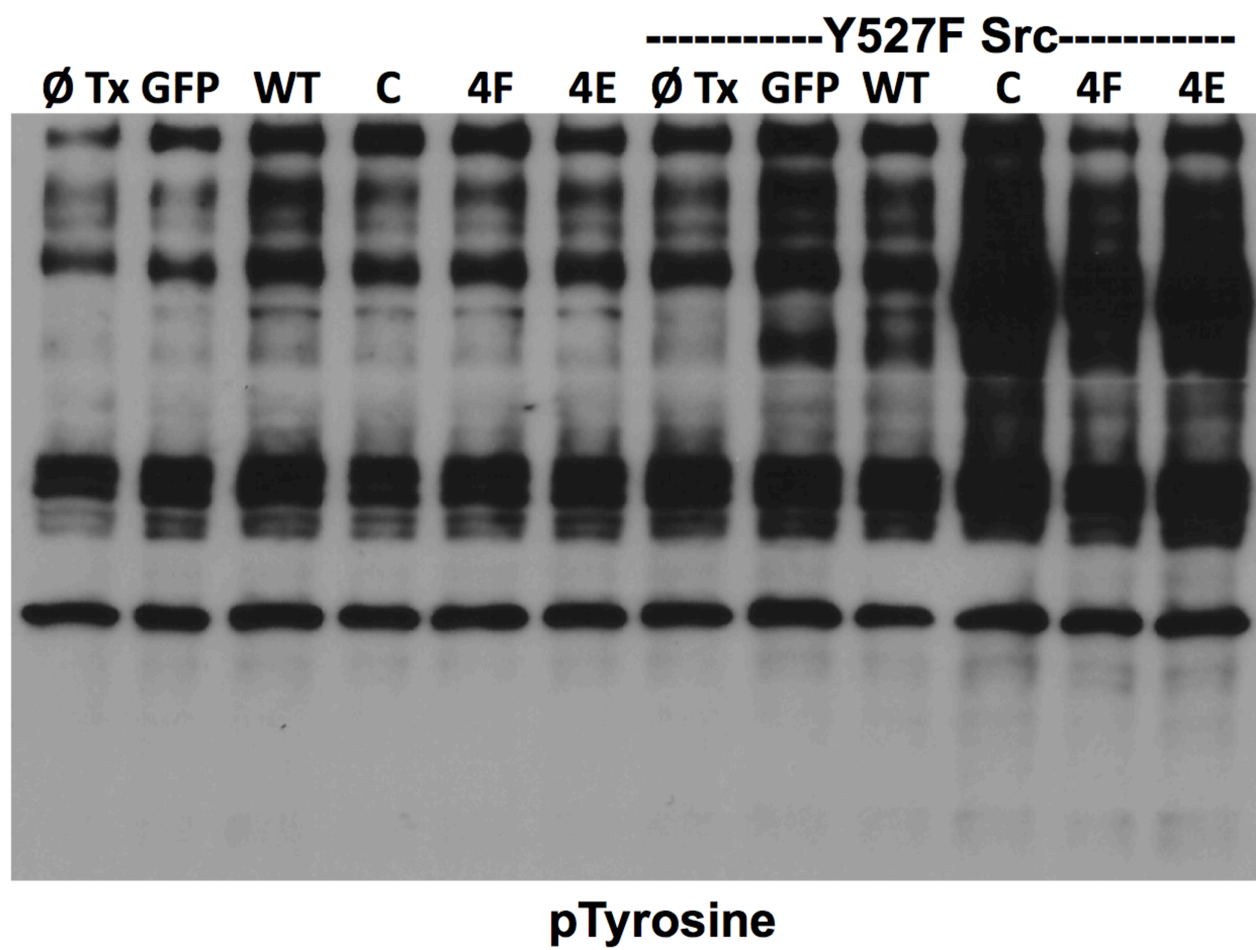


Figure 25. C377S and Y465E Caspase-8 Mutants Augment whereas WT, Y397F, Y397E and Y465F Suppresses the Activation and Activity of c-Src

HEK293 cells were co-transfected with empty GFP plasmid or GFP-tagged caspase-8 (WT and various mutants) and Y527F c-Src for 24 hours. Whole cell lysates were subjected to Western blot analysis. A. & B.) Compared to empty GFP plasmid, the expression of C377S and Y465E GFP-caspase-8 with Y527F c-Src resulted in an increase in phosphorylation of Y416 of c-Src (normalized to total expressed c-Src level) (N = 12, $p < 0.01$ for C377S, $p = 0.07$ for Y465E). Meanwhile, compared to empty GFP plasmid, the expression of Y465F GFP-caspase-8 with Y527F c-Src resulted in a decrease in phosphorylation of Y416 of c-Src (normalized to total expressed c-Src level) (N = 12, $p = 0.03$ for Y465F). A. & C.) However, compared to empty GFP plasmid, the expression of WT, Y397F and Y397E GFP-caspase-8 with Y527F c-Src resulted in a decrease (trend) in phosphorylation of Y416 of c-Src (normalized to total expressed c-Src level) (N = 4, $p = 0.125$). D) The expression of C377S and Y465E GFP-caspase-8 also resulted in an increase in global protein tyrosine phosphorylation. However, the expression of Y465F GFP-caspase-8 failed to increase protein tyrosine phosphorylation of substrates (see red arrows) other than GFP-caspase-8 itself (see black arrow). E. & F.) To determine whether the above phosphorylation of Y416 c-Src effect was dependent on the expression of Y527F c-Src, HEK 293 cells were transfected with only empty GFP plasmid or GFP-tagged caspase-8 (WT and various mutants) for 24 hours. Whole cell lysates were subjected to Western blot analysis. Compared to empty GFP plasmid, there was no significant difference in phosphorylation of Y416 of c-Src (normalized to total expressed c-Src level) (N = 3). G.) To confirm the effect of Y527F c-Src on inducing increase in global protein tyrosine

phosphorylation and to confirm the difference in global protein tyrosine phosphorylation seen in different GFP-caspase-8 mutants was dependent on Y527F c-Src activity, HEK293 cells were transfected with GFP-empty plasmid or GFP-caspase-8 (WT and various mutants) with or without Y527F c-Src. Global protein tyrosine phosphorylation was observed with Western blotting. There was a significantly less global protein tyrosine phosphorylation in cells without Y527F c-Src compared to with Y527F c-Src. The increase in global protein tyrosine phosphorylation seen with C377S and Y465E mutants was dependent on the presence of Y527F c-Src.

5.3.5 Phosphomimetic Modification at Y465 of Caspase-8 Enhances the Activation of Erk1/2 but not Akt

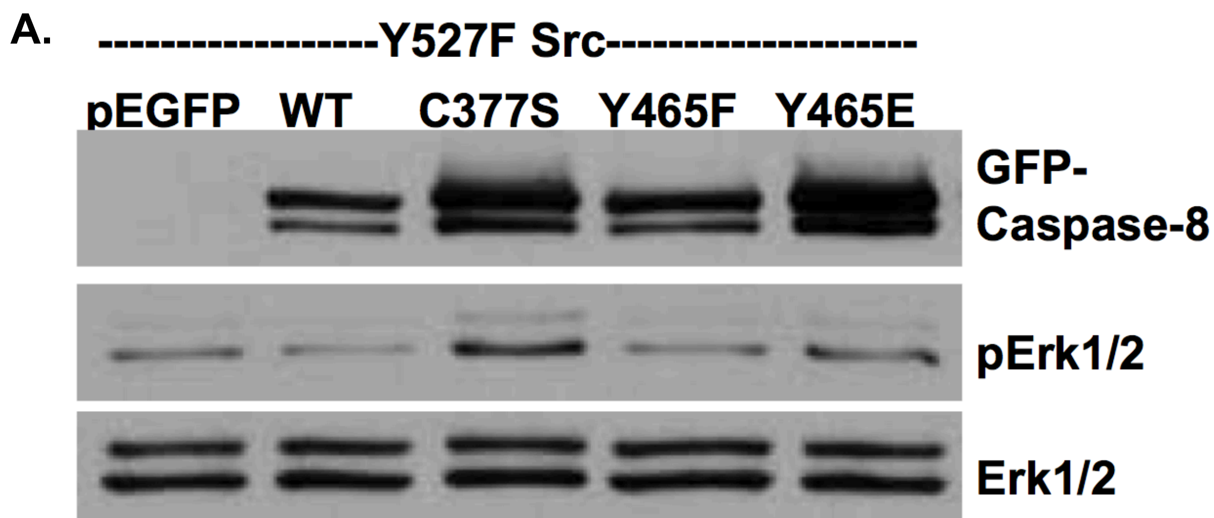
It has been shown that caspase-8 was involved in EGF-induced Erk1/2 activation (53) and that EGF could induce tyrosine phosphorylation of caspase-8 (177). Moreover, it has been demonstrated that when caspase-8 was tyrosine phosphorylated at Y380 (Caspase-8b), it was able to interact with the p85 subunit of PI3K (140). Therefore, we sought to investigate whether phosphorylation at Y465 could induce activation of Erk1/2 and Akt (a PI3K downstream signaling molecule).

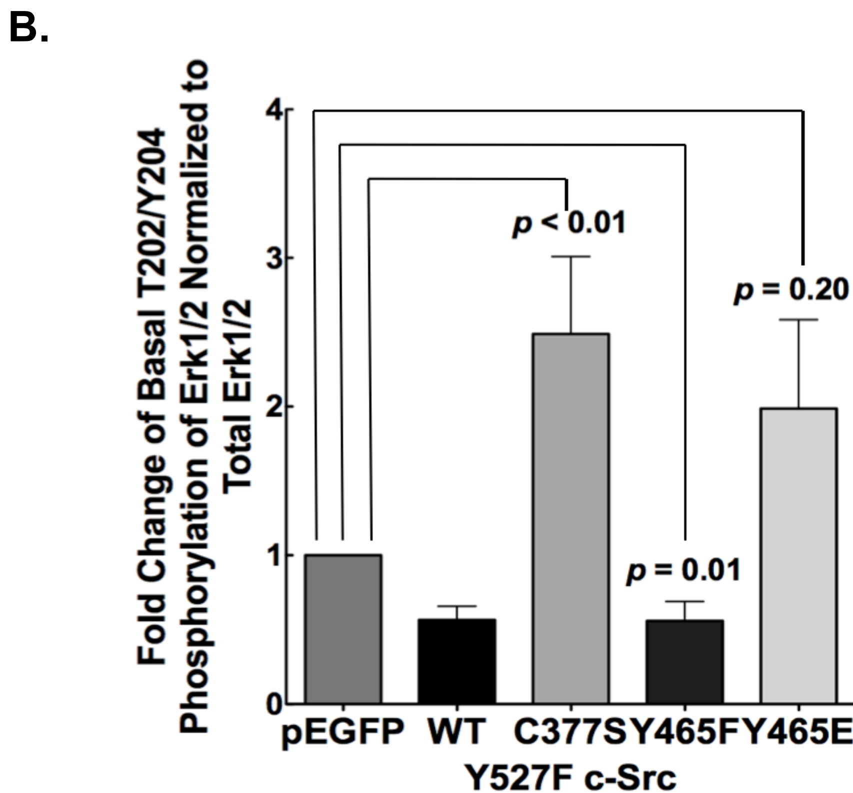
To this end, we co-transfected HEK293 cells with pEGFP-C1 empty plasmid, C377S inactive mutant, phosphomimetic mutant of Y465 or non-phosphorylatable mutant of Y465 along with Y527F c-Src construct for 24 hours followed by whole cell lysis and Western blot analysis using antibodies against phospho-Erk1/2 (pT202/Y204) or phospho-Akt (pT308 or pS473). We found that phosphomimetic mutant of Y465 increased (trend) phosphorylation of Erk1/2, similar to C377S inactive mutant. However, non-phosphorylatable mutant of Y465 failed to increase phosphorylation of Erk1/2 (Figure 26A & B). These data together suggest that phosphorylation at Y465 and the non-cleavability of caspase-8 are important for activation of Erk1/2.

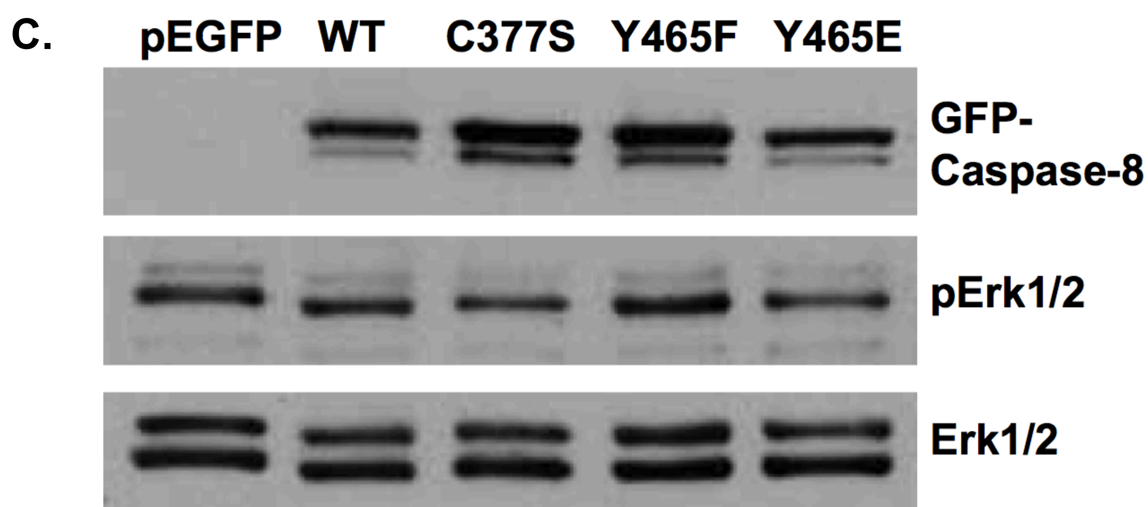
Of note, the effect of Y465E and C377S caspase-8 on phosphorylation of Erk1/2 was dependent on the presence of Y527F c-Src. Single transfection of GFP-caspase-8 (Y465E or C377S) did not lead to increase in phosphorylation of Erk1/2 (Figure 26C). This further suggests the potential requirement of phosphorylation of another tyrosine residue on caspase-8 in order to promote c-Src activation.

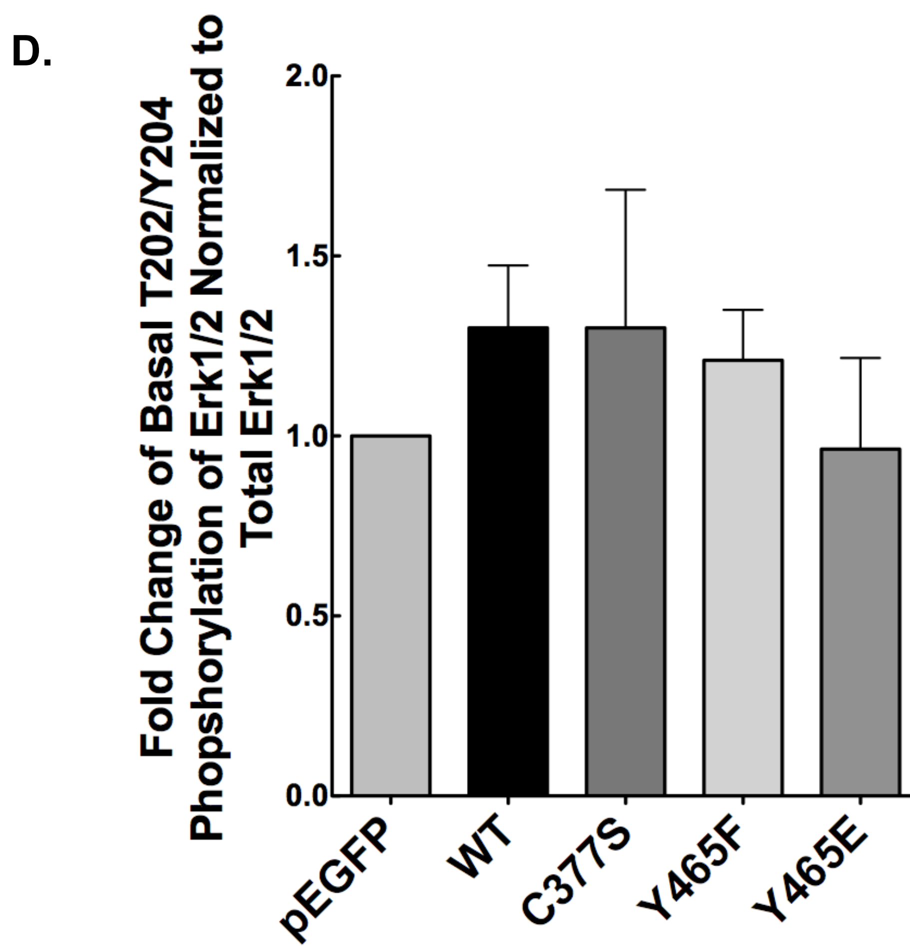
Unlike Erk1/2 phosphorylation, phosphomimetic modification at Y465 of caspase-8 did not induce activation of Akt (phosphorylation of T308 & S473 Akt) (Figure 26D & E).

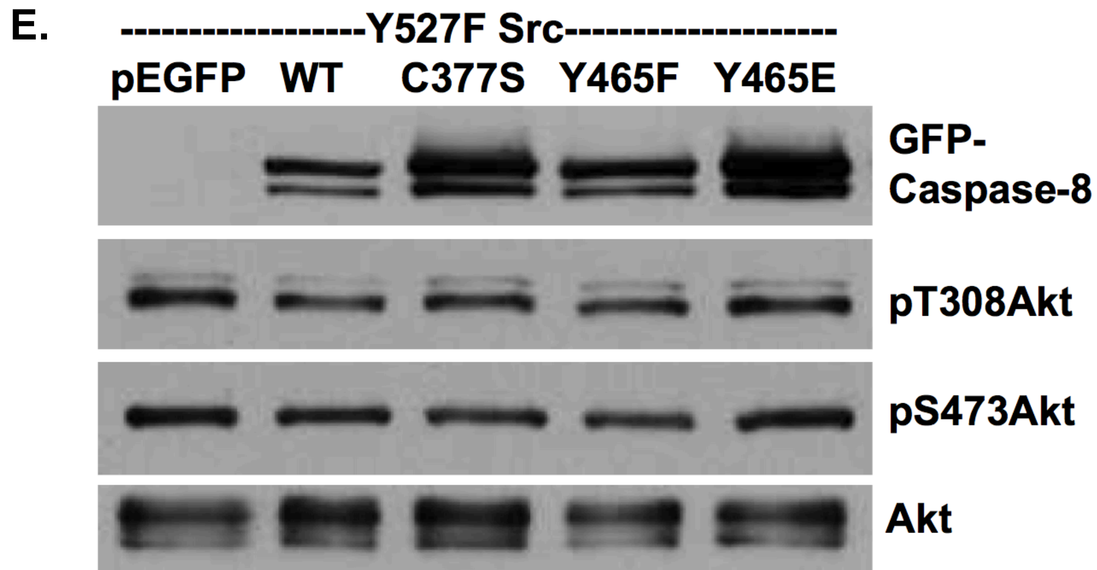
Figure 26






C.
pEGFP
WT
C377S
Y465F
Y465E
GFP-
Caspase-8
pErk1/2
Erk1/2


D.
Fold Change of Basal T202/Y204
Phopshorylation of Erk1/2 Normalized to
Total Erk1/2
2.0
1.5
1.0
0.5
0.0
pEGFP
WT
C377S
Y465F
Y465E


E.
------------------Y527F Src--------------------
pEGFP WT C377S Y465F Y465E
GFP-
Caspase-8
pT308Akt
pS473Akt
Akt

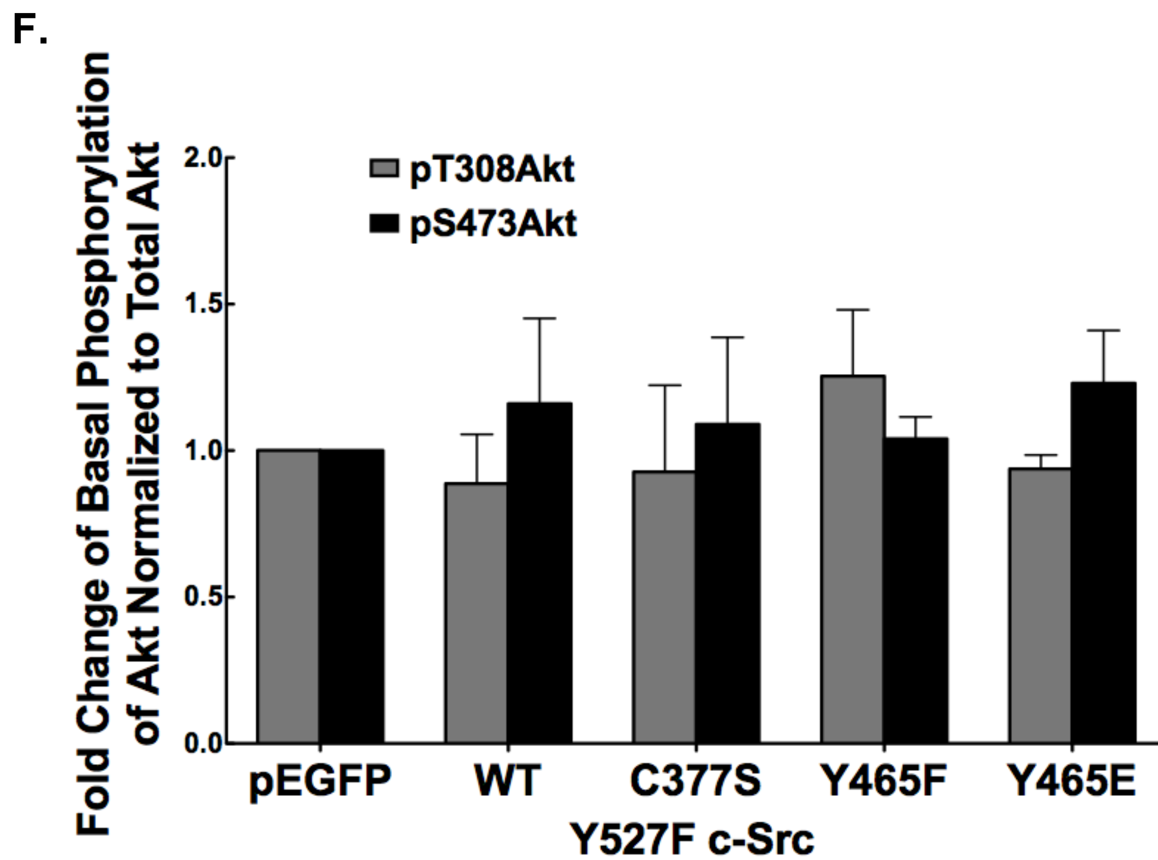

F.
Fold Change of Basal Phosphorylation of Akt Normalized to Total Akt
pT308Akt
pS473Akt
2.0
1.5
1.0
0.5
0.0
pEGFP
WT
C377S
Y465F
Y465E
Y527F c-Src

Figure 26. C377S and Y465E Caspase-8 Mutants Augment the Activation of Erk1/2 but not Akt

We co-transfected HEK293 cells with empty GFP plasmid or GFP-tagged caspse-8 (WT and various mutants) and Y527F c-Src for 24 hours. Whole cell lysates were subjected to Western blot analysis. A. & B.) The expression of C377S and Y465E GFP-caspase-8 resulted in an increase in phosphorylation of T202/Y204 of Erk1/2 (normalized to total expressed Erk1/2 level) (N = 8, $p < 0.01$ for C377S, $p = 0.20$ for Y465E). However, the expression of WT and Y465F GFP-caspase-8 resulted in a decrease in phosphorylation of T202/Y204 of Erk1/2 (normalized to total expressed Erk1/2 level) (N =8, $p = 0.01$). C. & D.) To determine whether the above phosphorylation of Erk1/2 effect was dependent on the expression of Y527F c-Src, HEK 293 cells were transfected with only empty GFP plasmid or GFP-tagged caspase-8 (WT and various mutants) for 24 hours. Whole cell lysates were subjected to Western blot analysis. Compared to empty GFP plasmid, there was no significant difference in phosphorylation of Erk1/2 (normalized to total expressed Erk1/2 level) (N = 3). E. & F.) The expression of GFP-caspase-8 (WT and various mutants) did not alter phosphorylation of T308 and S473 of Akt (normalized to total expressed Akt level) (N = 3).

5.3.6 The Activation of c-Src by Y465E Phosphomimetic Caspase-8 Mutant is Dependent on Phosphorylation of Y397

We observed that when Y465E mutant was cotransfected with Y527F c-Src, the mutant caspase-8 exhibited substantial tyrosine phosphorylation (Figure 27A). We wondered whether phosphorylation at Y465 alone is sufficient for activating c-Src and Erk1/2 or phosphorylation of other tyrosine residues is also necessary. Knowing that Y397 could be a target of c-Src (see chapter 4), we asked whether phosphorylation at Y397 is also important in caspase-8 dependent c-Src and Erk1/2 activation.

While we were actively pursuing the confirmation of tyrosine phosphorylation of Y465 with a different technique to overcome the challenges we faced with the sequencing of peptide surrounding Y465 residue (discussed in chapter 4), we examined whether Y397 is tyrosine phosphorylated in the Y465E mutant by using mass spectrometry. To this end, we co-transfected HEK293 cells with Y465E mutant of GFP-Caspase-8 and Y527F c-Src for 24 hours followed by immunoprecipitation of transfected GFP-Caspase-8 from whole cell lysates using a GFP antibody. We then resolved the GFP-Caspase-8 IP on SDS-PAGE followed by in-gel trypsin digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. We found that in Y465E caspase-8 mutant, Y397 was phosphorylated by c-Src (Figure 27B).

After identifying Y397 to be a residue phosphorylated by c-Src in Y465E caspase-8 mutant, we sought to investigate whether phosphorylation of Y397 contributes to the activation of c-Src and Erk1/2. To this end, we generated two double mutants, namely non-phosphorylatable mutant of Y397 in the background of phosphomimetic mutant of Y465 (Y397F+Y465E) and phosphomimetic mutants of both Y397 and Y465

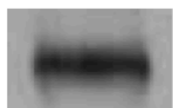
(Y397E+Y465E) (Figure 27C). We then examine the effect of the double mutants on c-Src and Erk1/2 activation. We observed that in cells that expressed Y397F+Y465E double mutant, compared to Y465E single mutant, there was a decrease (trend) in phosphorylation of Y416 c-Src (Figure 27D & E), and Erk1/2 phosphorylation (Figure 27F & G). These data suggest that in the presence of Y465 phosphorylation, phosphorylation of Y397 was needed for the activation of c-Src and Erk1/2. Moreover, we have shown above that Y397E single mutant not only failed to augment activation of c-Src, but it suppressed c-Src activation (Figure 25A & C). This suggests that in order for caspase-8 to be able to augment activation of c-Src, both Y397 and Y465 must be phosphorylated.

Figure 27

A.

Y465E

GFP-Caspase-8



pYCaspase-8 (pTyrosine)

B.

```
        8
MDFSRNLYDI GEQLDSEDLA SLKFLSLDYI PQPKQEPIKD ALMLFQRLQE KRMLEESNLS
FLKELLFRIN RLDLLITYLN TRKEEMEREL QTPGRAQISA YRFHFCRMSW AEANSQCQTA
SVPFWRRVDH LLIRVMLYQI SEEVSRSELR SFKFLLQEEI SKCKLDDDMN LLDIFIEMEK
RVILGEGKLD ILKRVCAQIN KSLLKIINDY EEFSKGEELC GVMTISDSPR EQDSESQTLD
 243
KVYQMKSKPR GYCLIINNHN FAKAREKVPK LHSIRDRNGT HLDAGALTTT FEELHFEIKP
           310
HDDCTVEQIY EILKIYQLMD HSNMDCFICC ILSHGDKGII YGTDGQEAPI YELTSQFTGL
                                         397
KCPSLAGKPK VFFIQACQGD NYQKGIPVET DSEEQPYLEM DLSSPQTRYI PDEADFLLGM
                                                    465
ATVNNCVSYR NPAEGTWYIQ SLCQSLRERC PRGDDILTIL TEVNEEVSNK DDKKNMGKQM
PQPTFTLRKK LVFPSD
```

Covered by mass spectrometry (bold underlined in original): NLYDI, GEQLDSEDLA, SLKFLSLDYI, PQPKQEPIKD, ALMLFQR, MLEESNLS, FLK, KEEMEREL, QTPGRAQISA, YR, RVDH, LLIRVMLYQI, SEEVSRSELR, FLLQEEI, SK, VILGEGKLD, ILKRVCAQIN, KSLLKIINDY, EEFSKGEELC, GVMTISDSPR, EQDSESQTLD, KVYQMK, GYCLIINNHN, FAK, VFFIQACQGD, NYQKGIPVET, DSEEQPYLEM, DLSSPQTR, NMGKQM, PQPTFTLRK. Phosphorylation (red): Y397.

Legends:

Sequence covered by Mass Spectrometry

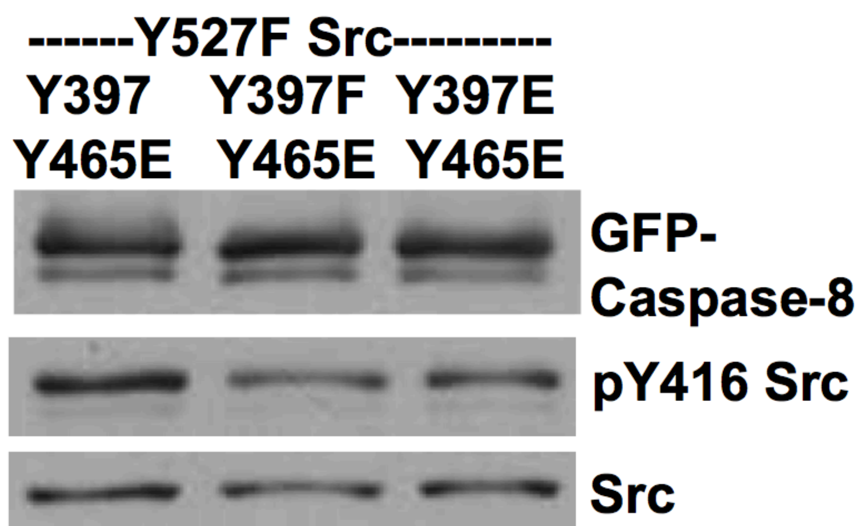
Sequence not covered by Mass Spectrometry

Phosphorylation

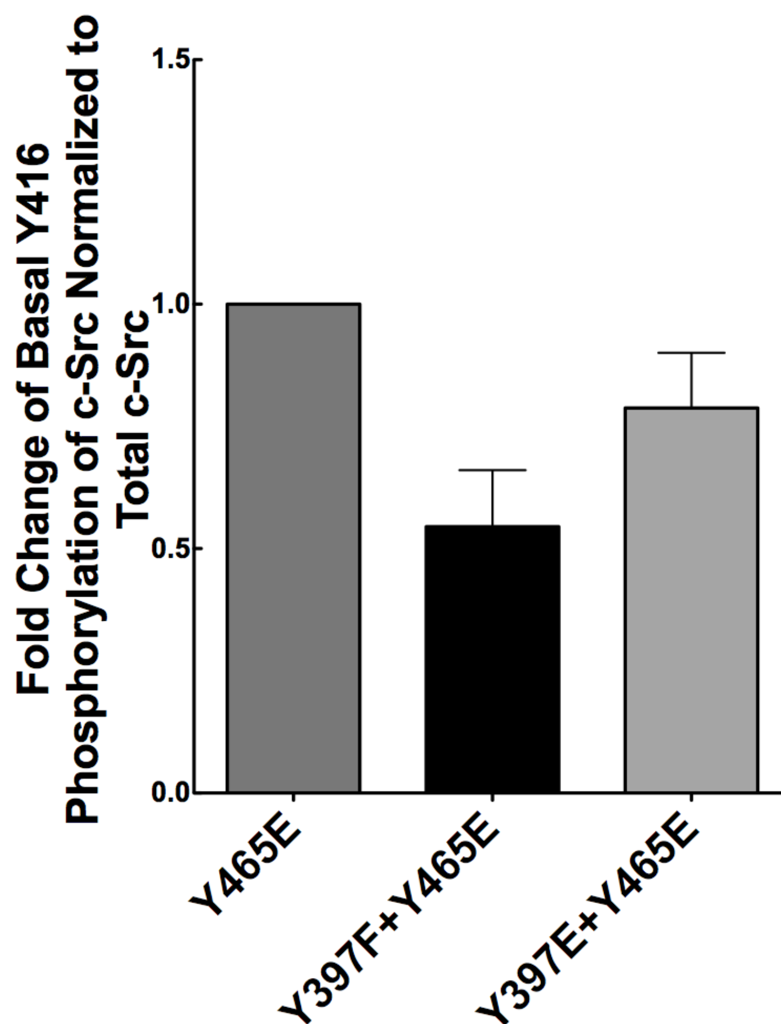
C.

Y397F/Y465E
Y397E/Y465E

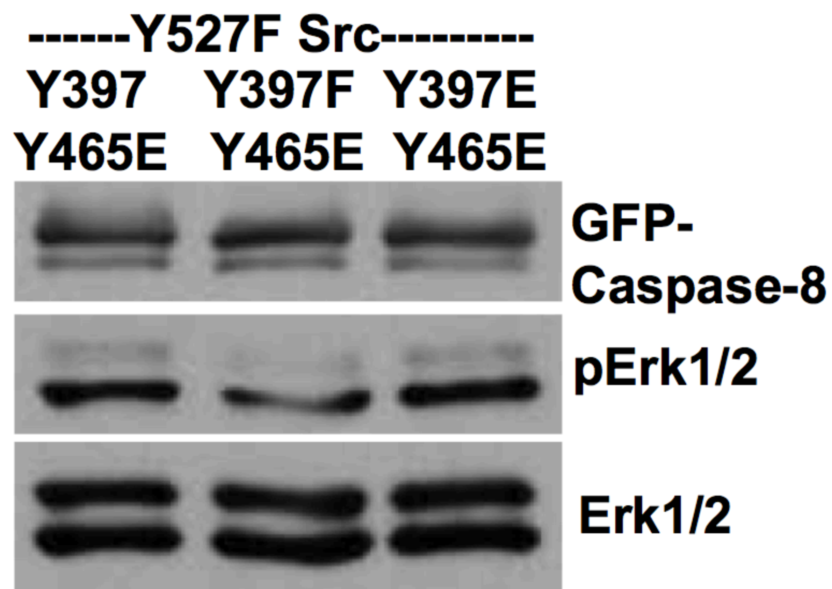
D.



E.



F.



G.

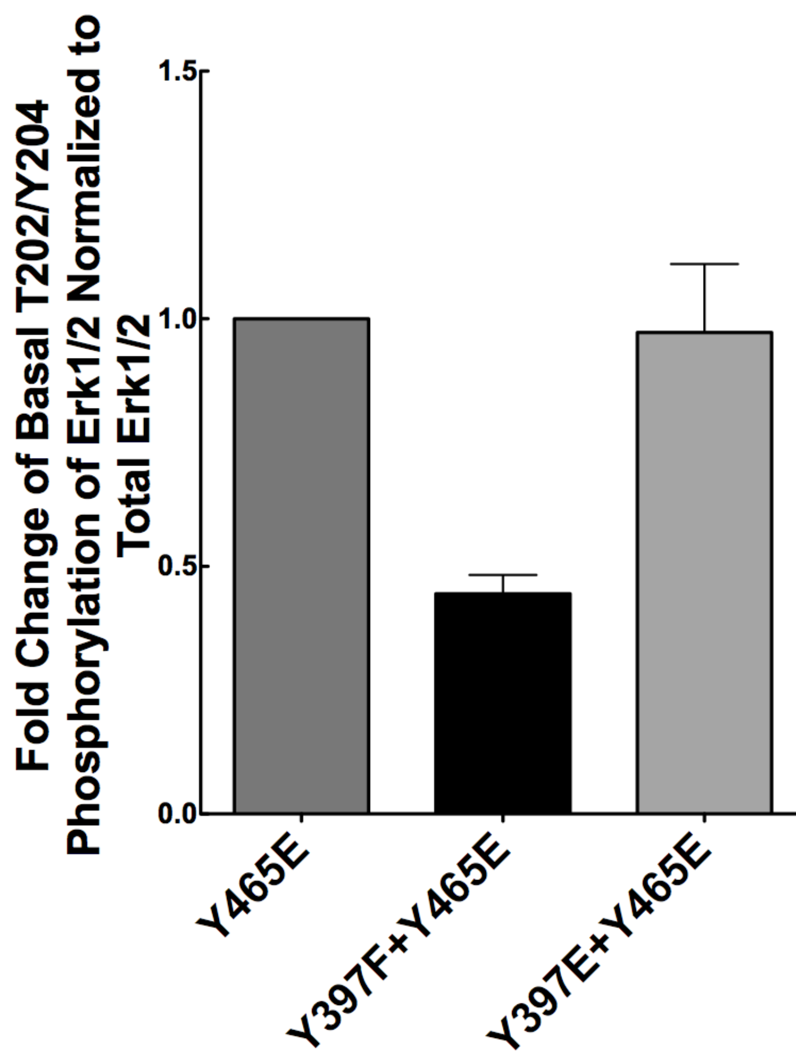


Figure 27. The Pro-Survival Function of Y465E Caspase-8 Mutant is Dependent on the Phosphomimetic Modification at Y397

A.) HEK293 cells were co-transfected withY465E GFP-Caspase-8 mutant and Y527F c-Src for 24 hours. Whole cell lysates were subjected to Western blot analysis with a phosphotyrosine antibody and a GFP antibody. Y465E was tyrosine phosphorylated. B.) To examine for phosphorylation at additional tyrosine site in Y465E mutant, HEK293 cells were co-transfected withY465E GFP-Caspase-8 mutant and Y527F c-Src for 24 hours. GFP-Caspase-8 mutant was immunoprecipitated from whole cell lysates using a GFP antibody. Immunoprecipitates were resolved on SDS-PAGE and stained with Coomassie blue. Protein band of 80-kDa was excised and sent for LC-MS/MS analysis. Y397 of Y465E mutant was phosphorylated by c-Src. C.) We generated Y397F/Y465E and Y397E/Y465E GFP-Caspase-8 double mutants. We co-transfected GFP-tagged Caspse-8 (Y465E, Y397F/Y465E or Y397E/Y465E) and Y527F c-Src into HEK293 cells for 24 hours. We then obtained whole cell lysates and resolved the total protein on SDS-PAGE for Western blot analysis. D. & E.) Compared to Y397E/Y465E GFP-Caspase-8 double mutant, the expression of Y397F/Y465E GFP-Caspase-8 resulted in a decrease (trend) in phosphorylation of Y416 of c-Src (normalized to total expressed c-Src level) (N = 4). F. & G.) Compared to Y397E/Y465E GFP-caspase-8 double mutant, the expression of Y397F/Y465E GFP-Caspase-8 resulted in a decrease (trend) in phosphorylation of T202/Y204 of Erk1/2 (normalized to total expressed Erk1/2 level) (N = 4).

5.4 DISCUSSION

Caspase-8 has been known as a pro-apoptotic protein. Its activation leads to cell death. However, recently, many groups have shown that caspase-8 has many non-apoptotic functions, including the regulation of cellular inflammation (121), immune cells proliferation and activation (69, 125, 137), cell migration (140, 142), endosome trafficking (55, 143), NF-κB activation (126, 146) and others. Traditionally, caspase-8 gene and protein expression were thought to be suppressed in tumor cells, contributing to tumor cell survival and proliferation. However, in recent years, data has shown that deletion or silencing of caspase-8 gene occurs extremely infrequently in cancers (181); but rather some has shown that there is an increased expression of caspase-8 in lung cancers (182). Moreover, a significant fraction of aggressive stage IV neuroblastoma (10-30%) maintains caspase-8 expression, and inactivation mutations are surprisingly rare (162, 173, 179, 180). Together, these suggest caspase-8 is an important player in the pathogenesis of cancer development such as promoting cell proliferation and cell migration. More intriguingly, tyrosine phosphorylation of caspase-8 has also been shown in colon cancer cells (177), suggesting that post-translational modification of caspase-8 protein may play a role in the pathogenesis of cancer development and progression.

SFKs, in particular c-Src, has been shown to promote mitogenesis, cell growth and survival (311-314). It is also involved in tumorigenesis and tumor progression by promoting cell growth and cell migration (306, 362-369). Moreover, it has been shown that naturally occurring c-Src mutation (truncated at amino acid 531) is seen in both advanced colon cancer and endometrial carcinoma cells. The truncated form of c-Src has increased c-Src activity because the autoinhibitory Y530 (equivalent to Y527 in chicken

c-Src) fails to interact with its own SH2 domain, therefore preventing it from staying in an inactive close conformation (302, 370). This naturally occurring c-Src truncated mutation is similar to our Y527F constitutively active c-Src. Our group (178) and other groups (52, 177) have shown that SFKs could tyrosine phosphorylate caspase-8 which in turn alters its activity and impacting downstream apoptotic pathway.

Here, we demonstrated that phosphomimetic modification of caspase-8, specifically at Y465, prevented cleavage and subsequent activation of caspase-3 and cell morphological changes. Unlike previous studies (52, 177, 178), we showed that phosphomimetic modification at Y397 alone did not prevent cleavage and subsequent activation of caspase-8 and cell morphological changes. We further demonstrated that not only did phosphomimetic of Y465 of caspase-8 prevent apoptosis, it also promoted cell survival by increasing c-Src and Erk1/2 activation. Moreover, we demonstrated that both Y465 and Y397 residues are required for the pro-survival function of caspase-8.

In chapter 4, we showed that in the presence of caspase-8 enzymatic activity, c-Src activation and function are suppressed. Here we propose that in the presence of a pro-survival signal, caspase-8 becomes phosphorylated at Y465. When Y465 of caspase-8 is phosphorylated, it prevents cleavage and activation. Hence it fails to suppress c-Src activation. c-Src then phosphorylates caspase-8 at Y397. The phosphorylation of Y397 of caspase-8 provides a phosphotyrosine motif (pYLEM) to interact with SH2 domain of c-Src and allows c-Src to assume an open conformation and thus promotes autophosphorylation of Y416 of c-Src for full activation. This contributes to the suppression of apoptosis and the propagation of survival pathways in the setting of a pro-survival environment, acting as a positive feedback loop (Figure 28). Thus, we propose

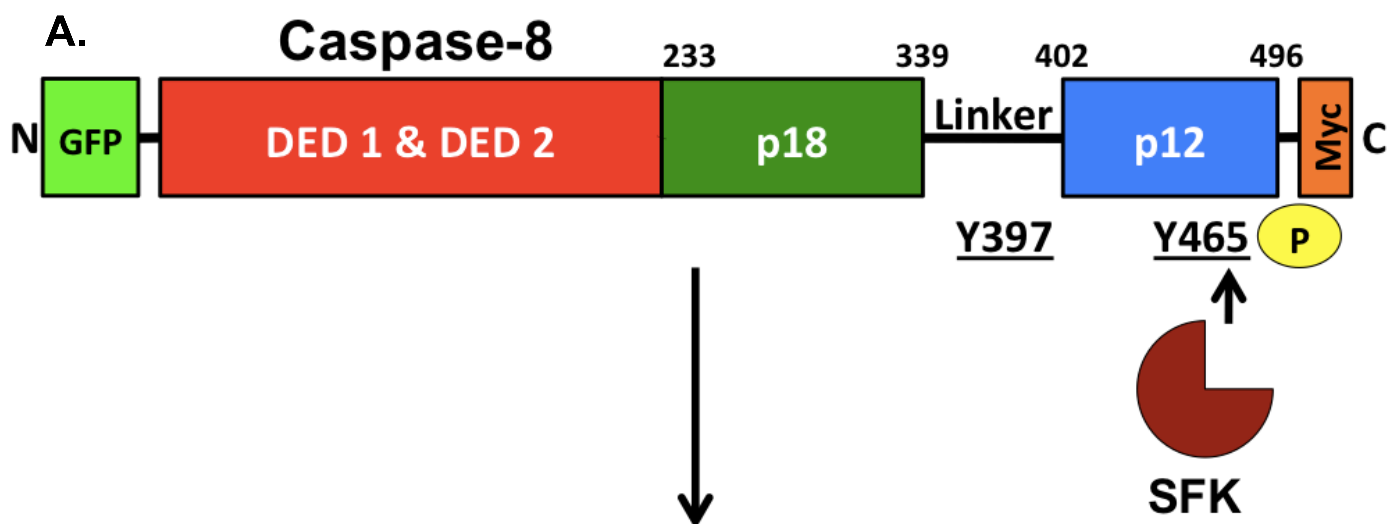
that caspase-8 acts both as a substrate and an activator (as an adaptor) of c-Src, a molecular phenomenon that has been observed with focal adhesion kinase (FAK) (413), AFAP-100 (411) and XB-130 (412).

This scenario might potentially explain the dual apoptotic and survival effect of tumor necrosis factor (TNF) (414). Hughes *et al* demonstrated that when caspase-8 failed to undergo cleavage after binding to death receptor complex (DISC), it failed to induce apoptosis. Our data suggest that if caspase-8 were phosphorylated at Y465, which prevented its cleavage, its recruitment to DISC would not allow it to induce apoptosis or to suppress c-Src activity. In turn, the uncleavable capase-8 could then be further phosphorylated by c-Src at Y397 and thus to further activate c-Src and propagate pro-survival signaling.

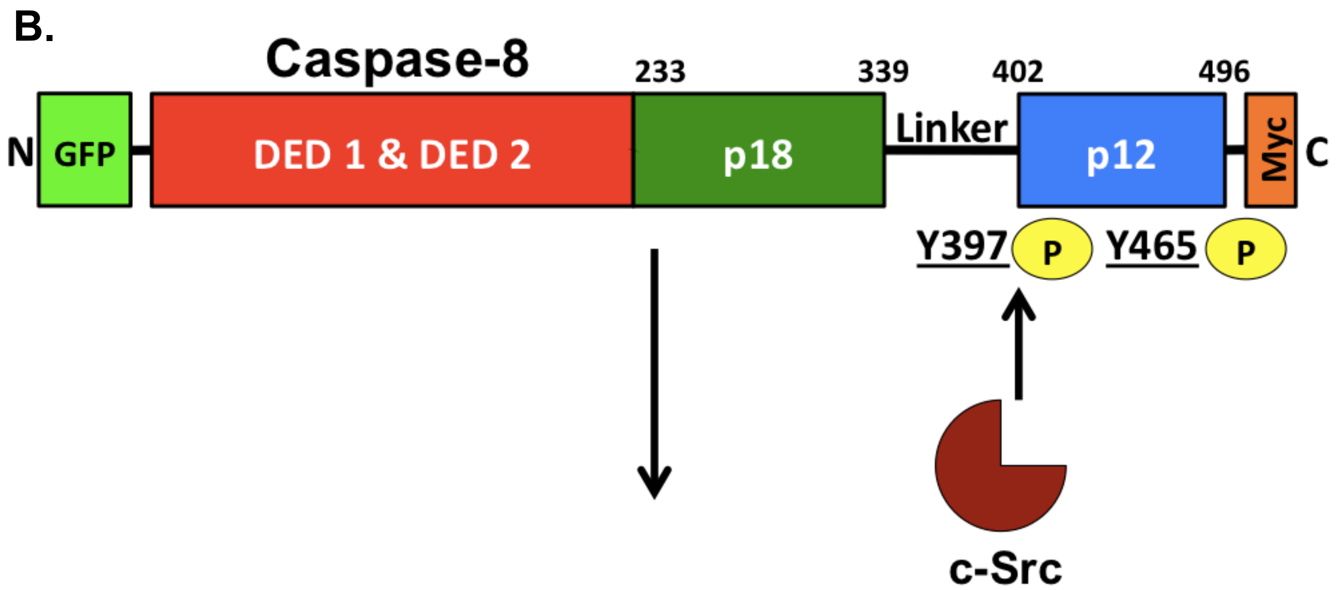
The limitation of our study is that we have not proven as yet that Y465 is phosphorylated by c-Src. This is due to the technical difficulty associated with the mass spectrometry analysis. Namely, the peptide surrounding Y465 is hydrophobic, which precluded it from being released from SDS-PAGE gel for sequencing. We are actively pursuing other approaches to allow the sequencing of the peptide surrounding Y465 to determine whether Y465 is phosphorylated. However, irrespective of the phosphorylation status of Y465, this residue in and of itself is a crucial determinant of caspase-8 activity since identifying Y465 as a residue necessary for caspase-8 cleavage provides insight into the mechanism of caspase-8 cleavage and activation and serves as the basis of further studies aimed at elucidating the exact molecular mechanism of auto-cleavage of caspase-8.

In summary our data provide insight into the molecular mechanisms of caspase-8 activation. Moreover, our data support the notion of tyrosine phosphorylation at Y397 and Y465 acting as a molecular switch to convert caspase-8 into a pro-survival molecule. Based on our findings, we propose a coherent model wherein the interplay between caspase-8 and c-Src allows switching between apoptotic and pro-survival programs. This interaction may be a critical factor in determining the balance between antagonistic inputs, and through positive feedback mechanisms allows rapid execution of the final outcome, be it survival or death.

Figure 28



B.



C.

Basal Activity

Activated Kinase

M

M

N

N

Closed (Inactive) Conformation

SH4

SH4

SH3

SH3

Caspase-8

$pY_{397}LEM$

SH2

SH2

SH1

Kinase

Y416

SH1

Kinase

C

P

Y527

C

Y527

Y416

P

Adapted from Yeatman 2004

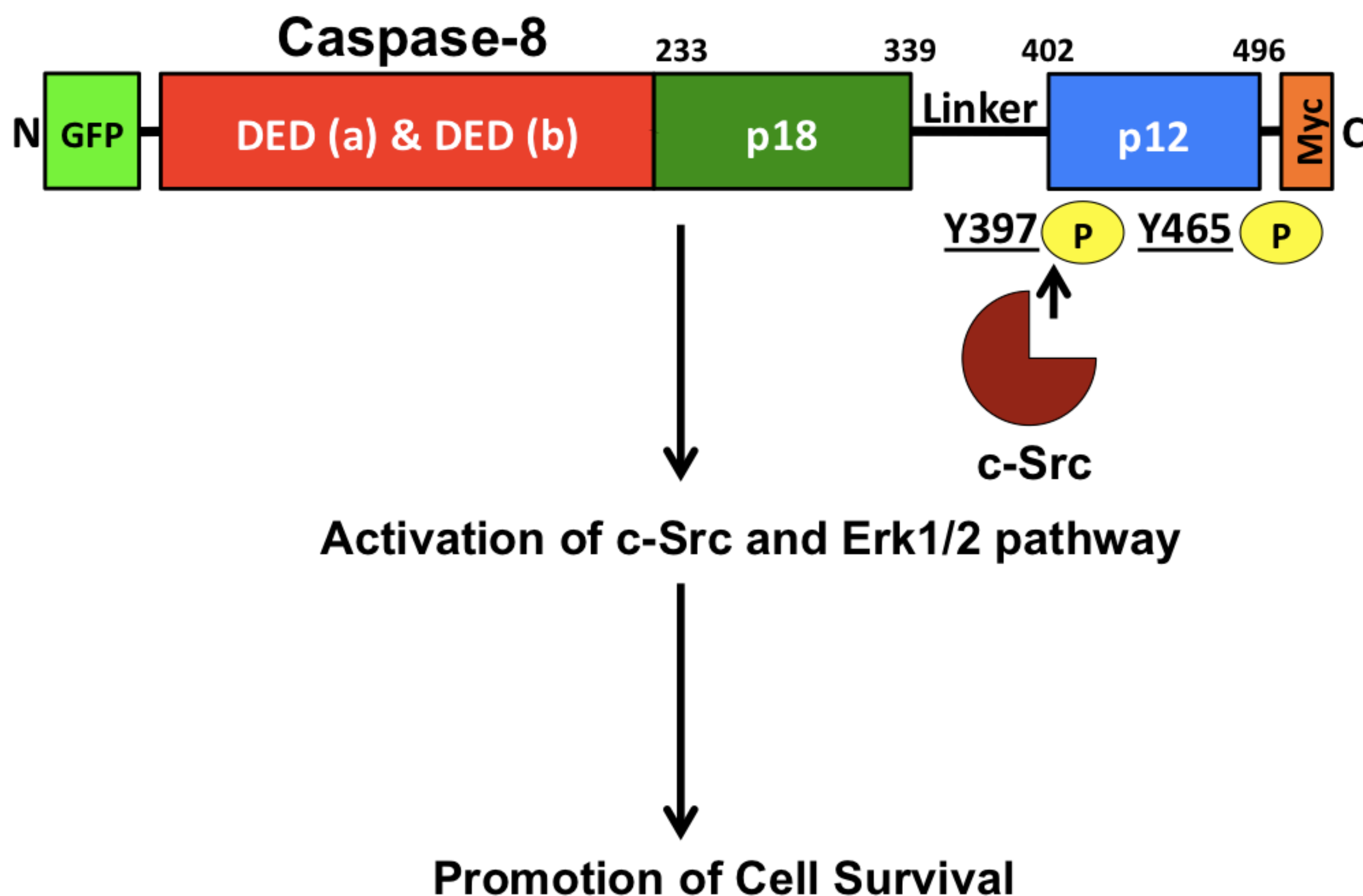


Figure 28. Phosphorylation of Y397 and Y465 of Caspase -8 Converts it into a Pro-Survival Protein

A) When SFK phosphorylates Y465 of caspase-8, it prevents it from cleavage and activation. Without caspase-8 enzyme activity, caspase-8 fails to inhibit c-Src activity. B) Without the inhibition of c-Src, c-Src then phosphorylates Y397 of caspase-8, C) allowing it to bind to SH2 domain of c-Src via its pY_{397}LEM phosphotyrosine peptide, keeping c-Src in an open (active) conformation. D) This further activates c-Src and Erk1/2 and promotes cell survival.

Chapter 6. OVERALL DISCUSSION AND FUTURE DIRECTION

In this final chapter, I will provide a general summary of the major findings of this thesis and will discuss the broader mechanisms underlying the reciprocal regulatory relationship between caspase-8 and c-Src. I will propose future experiments that will aid the elucidation of the mechanistic details of the proposed regulatory circuit. Finally, I will build a cancer biology model that involves several functional effects between caspase-8 and c-Src.

6.1 Summary of Major Findings

In this thesis, we discovered three major cell fate (apoptosis and survival) determining signaling regulatory mechanisms (Figure 29). Namely that

1. Active caspase-8 inhibits c-Src activity

2. Active c-Src tyrosine phosphorylates caspase-8 and inhibits its activity

3. Tyrosine phosphorylated caspase-8 stimulates c-Src activity

These three mechanisms serve to ensure cell fate-determining inputs are a) integrated and once the decision is made, b) rapidly and efficiently executed.

With the use of phosphomimetic and non-phosphorylatable caspase-8 mutants at tyrosine (Y) 397 and/or Y465 residues, we further characterized the mechanism of apoptosis inhibition and c-Src activation by tyrosine phosphorylated caspase-8. An overview of the major functional characteristics and effects of the various caspase-8 mutants is shown in table 6.

In aggregate, my studies have revealed a novel reciprocal regulatory mechanism between caspase-8 and c-Src and suggested an intricate regulatory circuit whereby a cell could use to commit to a cell fate-determining process, that is, apoptosis or cell survival.

However, as is commonly seen in research, these results opened up a plethora of intriguing questions to be answered in the future. Some of these are addressed below.

Figure 29

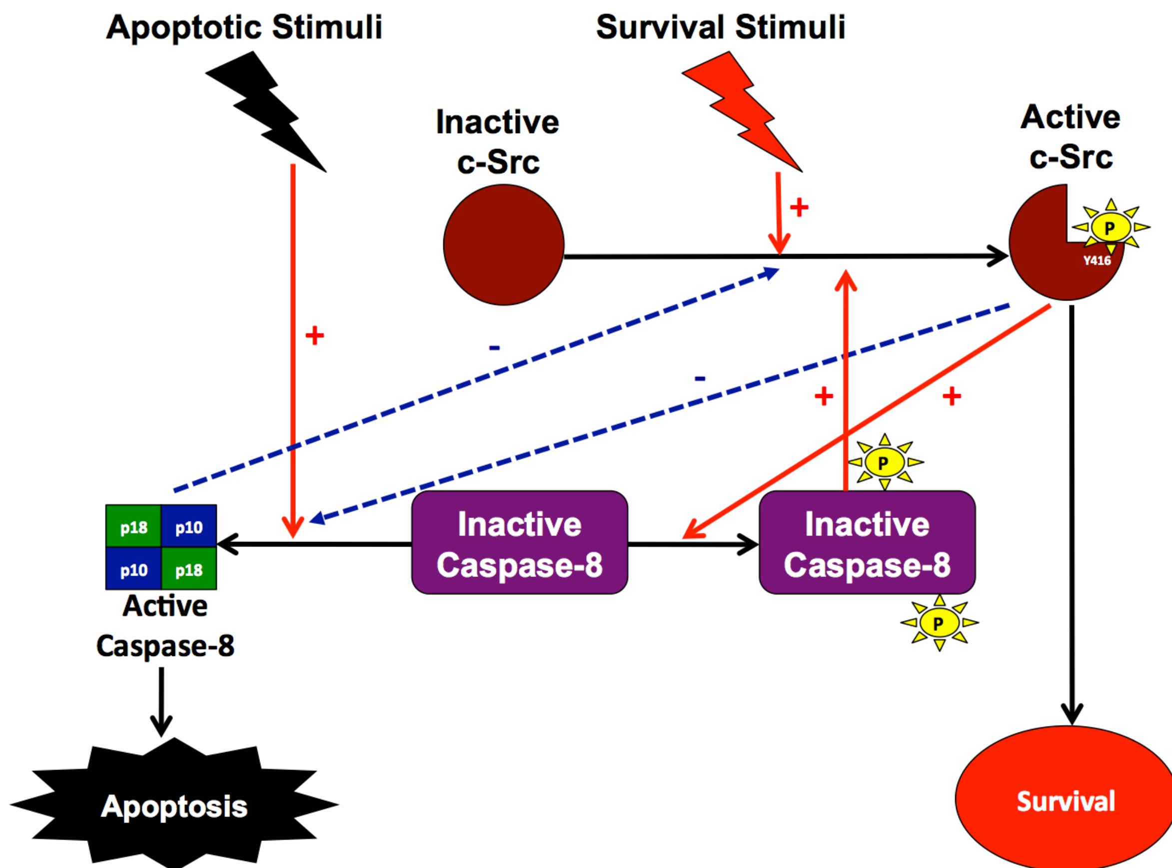


Figure 29. Apoptosis and Survival Signaling Involve Reciprocal Regulatory Mechanisms A schematic diagram of apoptosis and survival signaling reciprocal regulatory mechanisms is depicted here. Triggered by apoptotic stimuli, caspase-8 becomes activated and proceeds to activate the apoptotic program. Active caspase-8 also prevents the activation of c-Src, thereby preventing the activation of the cell survival program. Triggered by survival stimuli, c-Src becomes activated and proceeds to activate cell survival program. Active c-Src also tyrosine phosphorylates caspase-8 thereby preventing it from activation. Therefore, it preempts the activation of the apoptotic program. Moreover, tyrosine phosphorylated caspase-8 further promotes the activation of c-Src, allowing the propagation of the cell survival program.

Table 6

Caspase-8 Mutants	*Caspase-8 Dimerization	Caspase-8 Cleavage	Caspase-8 Activity	Phosphorylation of Caspase-8	Phosphorylation of Y416 of c-Src
Wild Type	+	+	+	-	↓
C377S	+	-	-	+	↑
Y397F	+?	+	+	-	↓
Y397E	+?	+	+	-	↓
Y465F	+?	+	+	+	↓
Y465E	-?	-	-	+	↑
Y397F/Y465E	-?	-	-	+	←→
Y397E/Y465E	-?	-	-	+	↑

* We did not examine dimerization in our studies. Those marked with a question mark are predicted outcomes

Table 6. Major Functional Characteristics and Effects of Various Caspase-8 Mutants

The major functional characteristics and effects of various caspase-8 mutants are shown. Cysteine (C) to serine (S) mutation at 377 residue resulted in an inactive mutant. A mutation from tyrosine (Y) to glutamic acid (E) represents a phosphomimetic mutant whereas a mutation from Y to phenylalanine (F) represents a non-phosphorylatable mutant.

In summary, a single mutation at residue 397, that is Y397F and Y397E mutants, behaved similarly to wild type caspase-8. These mutants could dimerize, were cleaved and harbored caspase-8 enzymatic activity. They also inhibited tyrosine phosphorylation of themselves and c-Src at Y416 residue. A non-phosphorylatable modification at Y465 behaved similarly to wild type caspase-8. However, unlike wild type caspase-8, it had the ability to be itself tyrosine phosphorylated. Phosphomimetic modification at Y465 rendered the mutant incapable of dimerization and cleavage. Therefore this mutant is functionally inactive. Similar to C377S inactive mutant, Y465E mutant could itself be tyrosine phosphorylated and promoted the phosphorylation of c-Src at Y416. Similar phenotype was seen in mutant with double phosphomimetic modification at Y397 and Y465. However, with non-phosphorylatable modification at Y397 as a background, the phosphomimetic modification at Y465 failed to promote phosphorylation of c-Src at Y416.

6.2 What is the Mechanism of Inhibition of Phosphorylation of c-Src at Y416 by Active Caspase-8?

Our work suggested that active caspase-8 inhibited the phosphorylation of c-Src at Y416. However, the exact mechanism of this inhibition is unclear. Here, we propose three potential mechanisms (Figure 30).

6.2.1 c-Src Cleavage by Caspase-8 or its Downstream Effectors

One potential explanation for the inhibition of Y416 phosphorylation of c-Src in the presence of active caspase-8 is the cleavage of c-Src resulting in its inactivation. There are precedents documented in the literature suggesting that SFKs can be cleaved during the process of apoptosis. For example, caspase-3 has been shown to cleave Fyn and caspases-3, -7 and -9 have been shown to cleave Lyn (403, 408). Moreover, c-Src has been shown to be cleaved during Fas-ligation induced apoptosis (415), although the responsible protease has not been identified. Thus, it is conceivable that c-Src could be cleaved either directly by caspase-8 or indirectly by caspase-8's downstream effectors.

To test this hypothesis, we will co-express differentially tagged caspase-8 (GFP-tagged) and Y527F c-Src (Flag-tagged) in cells and examine the steady state level of both cleaved and uncleaved Y527F c-Src. Nonetheless, this mechanism is not too likely since there is no obvious caspase consensus sequence for cleavage in c-Src.

6.2.2 Active Caspase-8-Induced Downregulation of a c-Src Adaptor Molecule Resulting in Inhibition of c-Src Phosphorylation at Y416

It has been shown that the binding of various adaptor molecules, which bind to c-Src usually via SH2 or SH3-interacting domains, keep c-Src in an open conformation, facilitating its autophosphorylation at Y416, and its consequent full activation. We propose that one of the mechanisms of active caspase-8 induced inhibition of c-Src phosphorylation at Y416 is through the cleavage of such (a) c-Src adaptor molecule(s). One of such candidate molecules is focal adhesion kinase (FAK), a known c-Src adaptor (413), that has been shown to be cleaved by caspases-8, -3, -6, -7 and -9 (220). We propose that active caspase-8 cleaves FAK directly or indirectly through the activation of effector caspases, preventing it from acting as a c-Src adaptor molecule, thereby, suppressing the activation of c-Src.

To investigate this possibility, we would look for cleavage product of FAK and compare the ratio of cleaved FAK to uncleaved FAK among GFP-empty plasmid or GFP-Caspase-8 (wild type or C377S) and Y527F c-Src constructs transfected cells using Western blotting. If our hypothesis were true, we would expect to see an increase in the ratio of cleaved FAK to uncleaved FAK in GFP-Caspase-8 (wild type) transfected cells.

6.2.3 Active Caspase-8 Activates a Protein Tyrosine Phosphatase Resulting in Dephosphorylation of c-Src at Y416

The regulation of c-Src activation is intricate. It requires the dephosphorylation of the inhibitory Y527 residue by a protein tyrosine phosphatase (PTP) such as receptor protein tyrosine phosphatase α (RPTPα) (416) and PTP1B (248), as well as the autophosphorylation of activation loop Y416 residue (289). After the activation of c-Src, its activity could be negatively regulated by low molecular protein tyrosine phosphatase (LMWPTP) through the dephosphorylation of Y416 (417). We propose that active caspase-8 could activate a protein tyrosine phosphatase, which in turn, dephosphorylates Y416 of c-Src.

To test this hypothesis we could first co-expressed GFP-empty plasmid or GFP-Caspase-8 (wild type or C377S mutant) with Y527F c-Src constructs in HEK293 cells followed by the downregulation of candidate protein tyrosine phosphatase. We could then examine the phosphorylation of c-Src at Y416. If our hypothesis were correct, we would expect to see an increase in phosphorylation of c-Src at Y416 in cells that were co-transfected with GFP-Caspase-8 (wild type) and Y527F c-Src constructs with the downregulation of candidate protein tyrosine phosphatase.

Figure 30

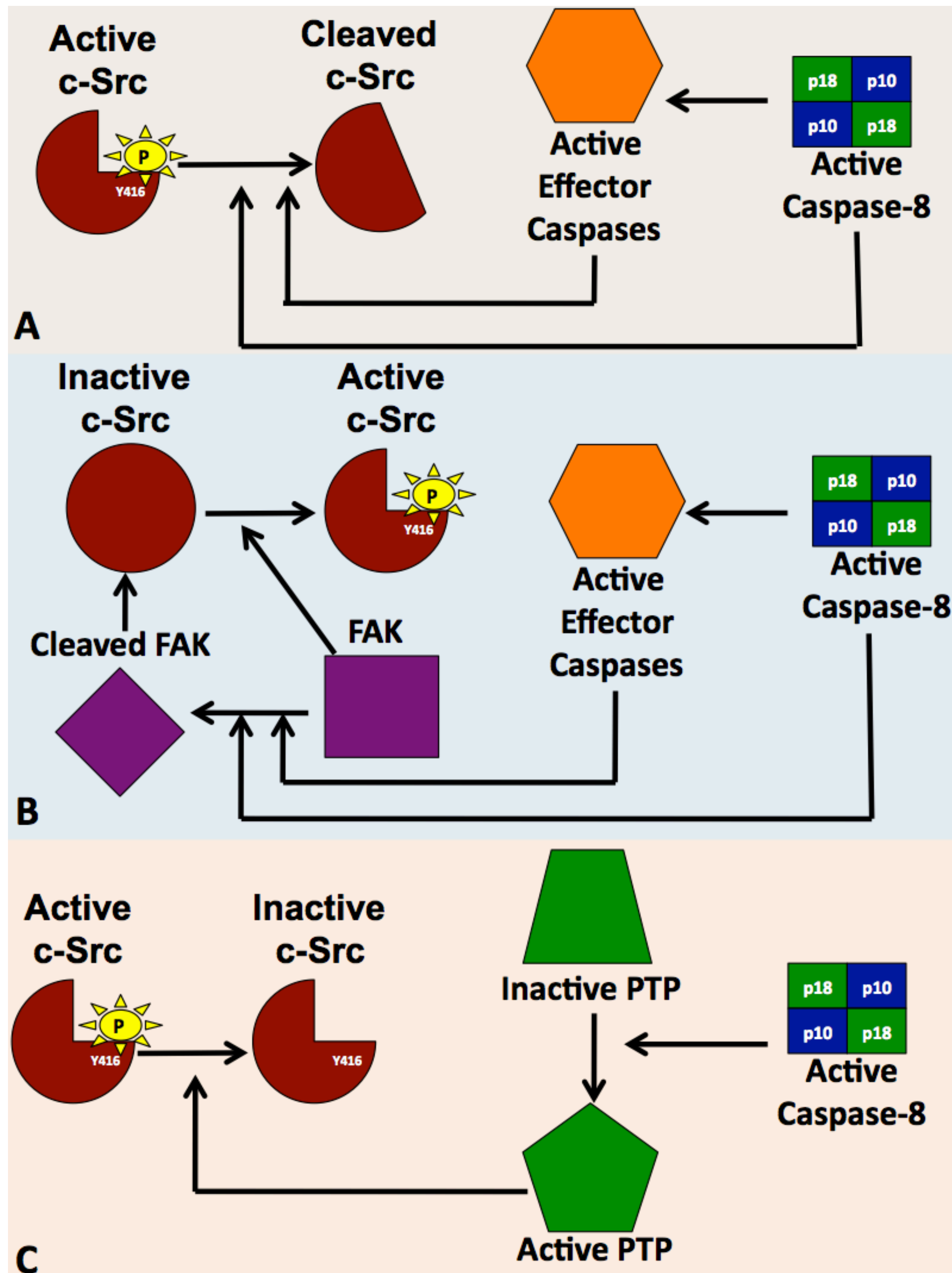


Figure 30. Potential Mechanisms of Active Caspase-8 Inhibition of c-Src Phosphorylation at Y416

The potential mechanisms of active caspase-8 inhibition of c-Src phosphorylation at Y416 are depicted here. A) Active caspase-8 cleaves c-Src directly or indirectly through active effector caspases, renders c-Src incapable of autophosphorylation at Y416. B) Active caspase-8 cleaves focal adhesion kinase (FAK) directly or indirectly through active effector caspases, renders FAK incapable of acting as a c-Src adaptor, preventing c-Src from autophosphorylation at Y416. C) Active caspase-8 activates protein tyrosine phosphatase (PTP), which then dephosphorylates c-Src at Y416.

6.3 Can Tyrosine 465 of Caspase-8 be Phosphorylated by c-Src?

It has been shown by mass spectrometry that c-Src could phosphorylate Y380 of caspase-8b isoform (177) and Y397 of caspase-8a isoform (chapter 4). However, based on our studies using various caspase-8 mutants, we conclude that phosphorylation at Y397 could not be the sole residue for caspase-8 regulation. Therefore, we sought to investigate whether Y465 could also be phosphorylated by c-Src. Unfortunately we were not yet successful in sequencing the region surrounding Y465. This is due to the fact that this residue lies in a very hydrophobic region of caspase-8, and thus the peptide fragments resulting from the digestion of this part of the molecule are retained in the SDS-PAGE gel. Hence, thus far, we were unable to confirm whether Y465 could indeed be phosphorylated by c-Src.

To overcome this hurdle, we are in the process of refining the method of peptide liberation from the gel or to use another method that would allow the sequencing of the protein without resolving it in SDS-PAGE. The confirmation of Y465 phosphorylation by c-Src will give further credence to our proposed model of a molecular switch based on tyrosine phosphorylation.

Since c-Src activity has been shown to be upregulated in colon carcinoma cells (367, 368) and caspase-8 in colon carcinoma cells have been shown to be phosphorylated, presumably at Y397 (177), it would be of paramount interest to determine whether Y465 of caspase-8 is also phosphorylated in human colon carcinoma cells. This would underline the physiological importance of Y465 phosphorylation of caspase-8 in cancer biology. Specifically, it would verify in this context the existence of a positive feedback loop between the tyrosine phosphorylation of caspase-8 by c-Src and

the further upregulation of c-Src activity by tyrosine phosphorylated caspase-8.

6.4 What Could be the Mechanism of Phosphorylation of Tyrosine 465 of Caspase-8 in Preventing its Cleavage?

We showed that phosphomimetic modification of Y465 abolished the cleavage of caspase-8 after aspartic acid (D) 391. However, the mechanism by which this modification prevents its cleavage is unknown. It has been shown that the dimerization of caspase-8 molecule is crucial for the activation of the enzyme (41, 42, 47) and its dimerization requires threonine (T) 484 and phenylalanine (F) 485 (31). Y465 is located in α-helix five, whereas T484 and F485 are located in β-sheet six. α-helix five and β-sheet six are in close proximity (Figure 31) in its three-dimensional structure, and we propose that a negative charge at residue 465 might distort the three-dimensional structure of the caspase-8 molecule near the dimerization interface, thereby interfering with the interaction of the monomers. Such block in dimerization would result in the loss of caspase-8 cleavage and activation. This would also explain the lack of apoptotic effects seen in phosphomimetic mutant of caspase-8 at Y465.

To test our hypothesis, we will perform co-immunoprecipitation of caspase-8 constructs with alternate protein tags to look for dimerization. We expect to see that C377S caspase-8 mutant dimerizes whereas Y465E caspase-8 mutant fails to dimerize.

Figure 31

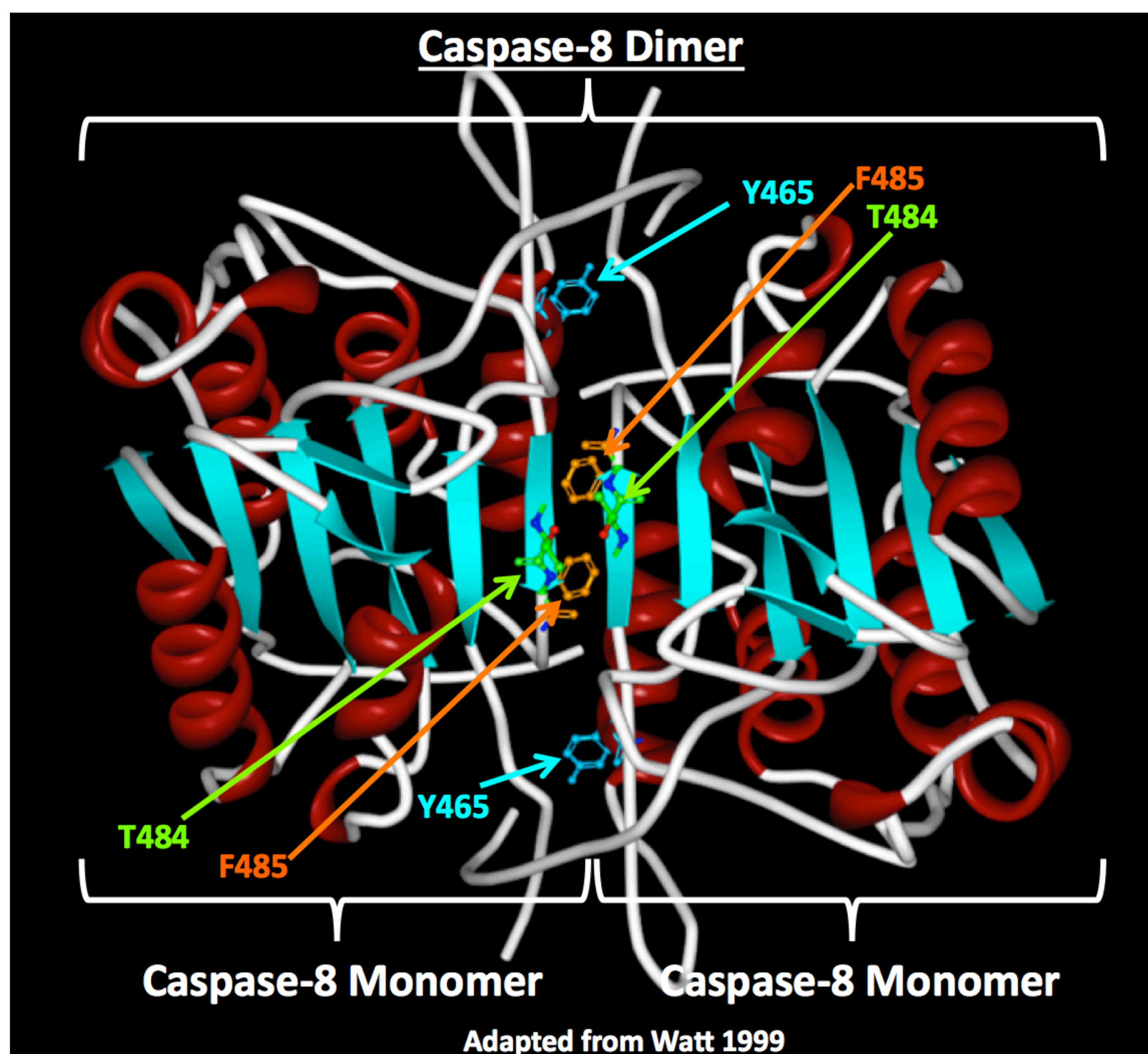


Figure 31. Three-Dimensional Structure of Caspase-8 Dimer

A three-dimensional structure of caspase-8 dimer is depicted. Individual caspase-8 monomers are labeled. As illustrated here, Y465 residue (in blue) lays in close proximity to T484 (in green) and F485 (in orange) residues, both of which are important for dimerization as they lie at the dimerization interface.

6.5 What is the Mechanism whereby Phosphorylation of Tyrosine 397 of Caspase-8 Promote c-Src Activation?

We have shown that phosphomimetic modification of Y465 (Y465E) or of both Y397 and Y465 (Y397E/Y465E) rendered caspase-8 non-cleavable and inactive. It also enabled caspase-8 to promote c-Src autophosphorylation at Y416. Moreover, we showed that a phosphomimetic mutation at Y397 alone (Y397E) failed to promote c-Src phosphorylation. However, non-phosphorylatable modification of Y397 residue rendered Y465E mutant (Y397F/Y465E) incapable of promoting c-Src autophosphorylation at Y416 (Figure 32). Together, these results suggest that phosphorylation of Y397 in a non-cleavable and inactive caspase-8 mutant (Y465E) played an important role in promoting c-Src autophosphorylation at Y416.

The question arises how phosphorylation of Y397 contributes to the autophosphorylation of c-Src at Y416. A potential answer stems from the fact that a number of c-Src substrates can also act as c-Src activators. Such mode of regulation has been amply documented with regards to cytoskeletal substrates of c-Src, including FAK (413), AFAP-110 (411) and XB-130 (418).

Here, we propose that phosphorylation of Y397 provides a phosphotyrosine peptide ($pY_{397}LEM$) that could bind to the SH2 domain of c-Src, maintaining the c-Src molecule in an open conformation, thereby allowing autophosphorylation at Y416 of c-Src, and its consequent increase in kinase activity towards other substrates. By reviewing the three-dimensional structure of caspase-8 by Keller *et al*, we observed that the $Y_{397}LEM$ peptide, located in the linker region of the molecule, is exposed to the surface of the molecule (Figure 33). This suggests that when phosphorylated, the phosphotyrosine

peptide pY_{397}LEM is free to interact with the SH2 domain of c-Src. Moreover, our lab has previously shown that the tyrosine phosphorylated form of caspase-8 can bind Lyn (178). Other labs have also demonstrated that in the presence of constitutively active (Y527F) c-Src, caspase-8b interacted with the c-Src SH2 domain. However, when caspase-8b harbored a non-phosphorylatable modification at Y380 (equivalent to Y397 in caspase-8a), it failed to interact with the SH2 domain of c-Src despite the presence of constitutively active (Y527F) c-Src (52), suggesting that phosphorylation at Y380 of caspase-8b was crucial for its interaction with the SH2 domain of c-Src. These data together support our hypothesis that phosphorylation of Y397 of caspase-8 gives rise to a phosphotyrosine-containing peptide sequence capable of binding to the c-Src SH2 domain and promoting c-Src autophosphorylation at Y416.

To test our hypothesis that caspase-8, when tyrosine phosphorylated, interacts with c-Src, we will perform co-immunoprecipitation study in cells transfected with both GFP-Caspase-8 (various mutants) and Y527F c-Src constructs. We expect to see that both Y465E and Y397E/Y465E mutants would interact with c-Src whereas Y397F/Y465E would not.

Taken together, we propose that caspase-8 is both a substrate and activator of c-Src by acting as an adaptor of c-Src after phosphorylation at Y397 and Y465 (Figure 34). It serves as a positive feedback loop to ensure the propagation of pro-survival process.

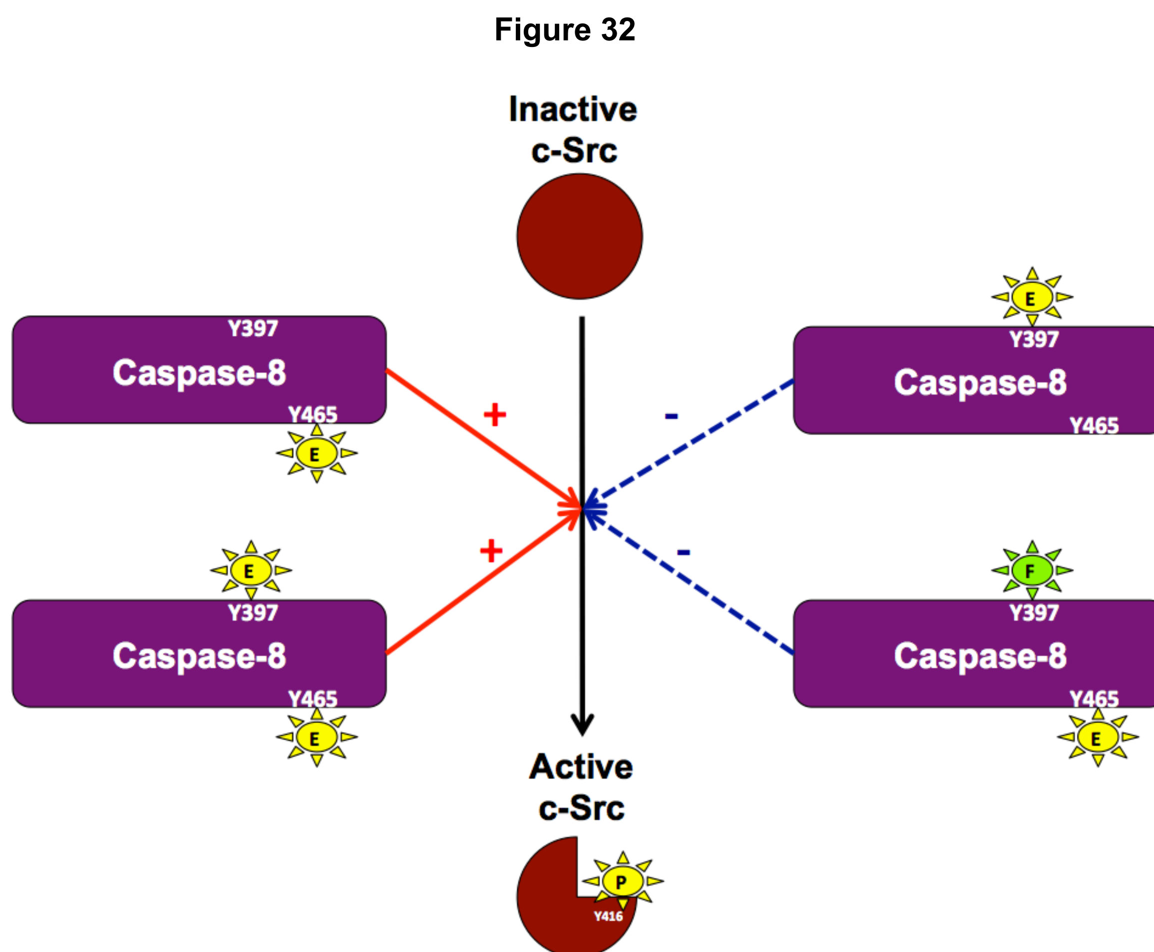


Figure 32. Phosphomimetic and Non-Phosphorylatable Caspase-8 Mutants and Their Ability to Promote c-Src Autophosphorylation at Y416

Caspase-8 mutants with phosphomimetic modification at Y465 alone or at both Y397 and Y465 were capable of stimulating c-Src autophosphorylation at Y416. However, caspase-8 mutants with phosphomimetic modification at Y397 alone or with both non-phosphorylatable modification at Y397 and phosphomimetic modification at Y465 were incapable of stimulating c-Src autophosphorylation at Y416.

Figure 33

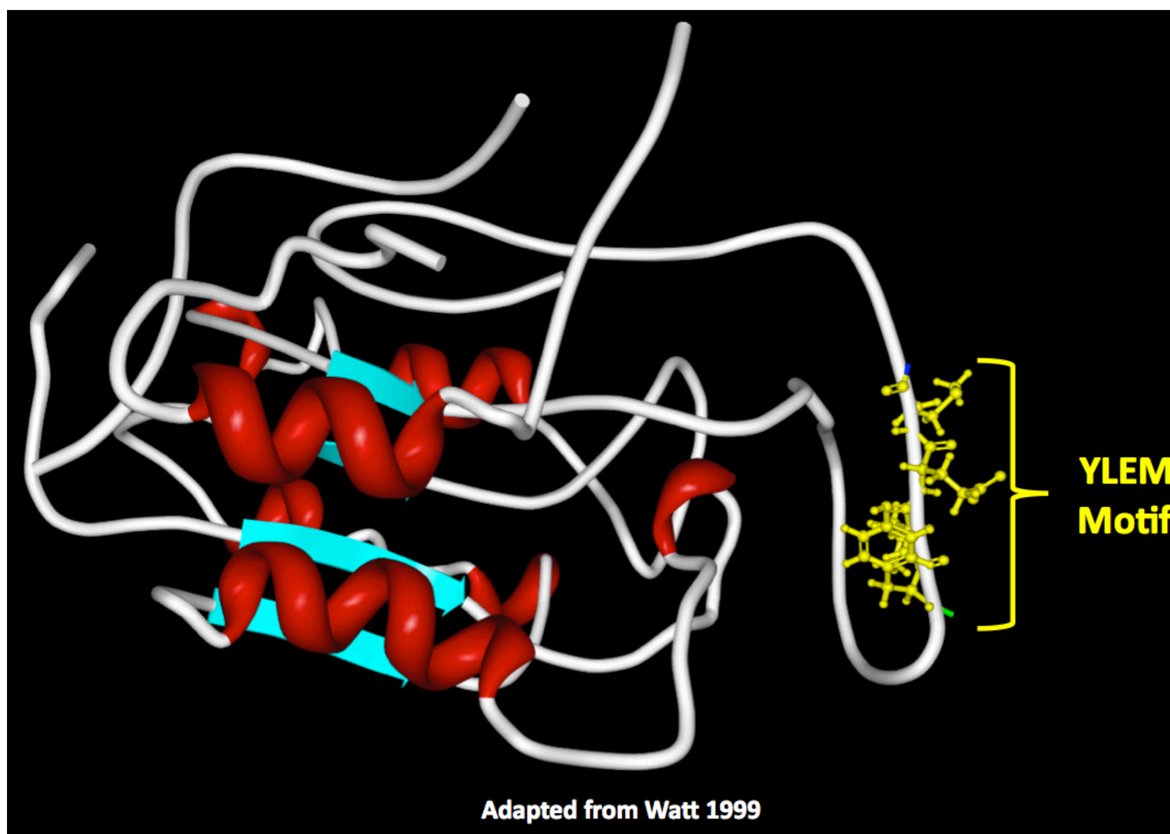


Figure 33. Three-Dimensional Structure of the Caspase-8 Monomer with the Linker Region This scheme illustrates the YLEM motif, which is situated on the surface of the caspase-8 monomer. This potentially allows the access of SH2 domain of c-Src for interaction.

Figure 34

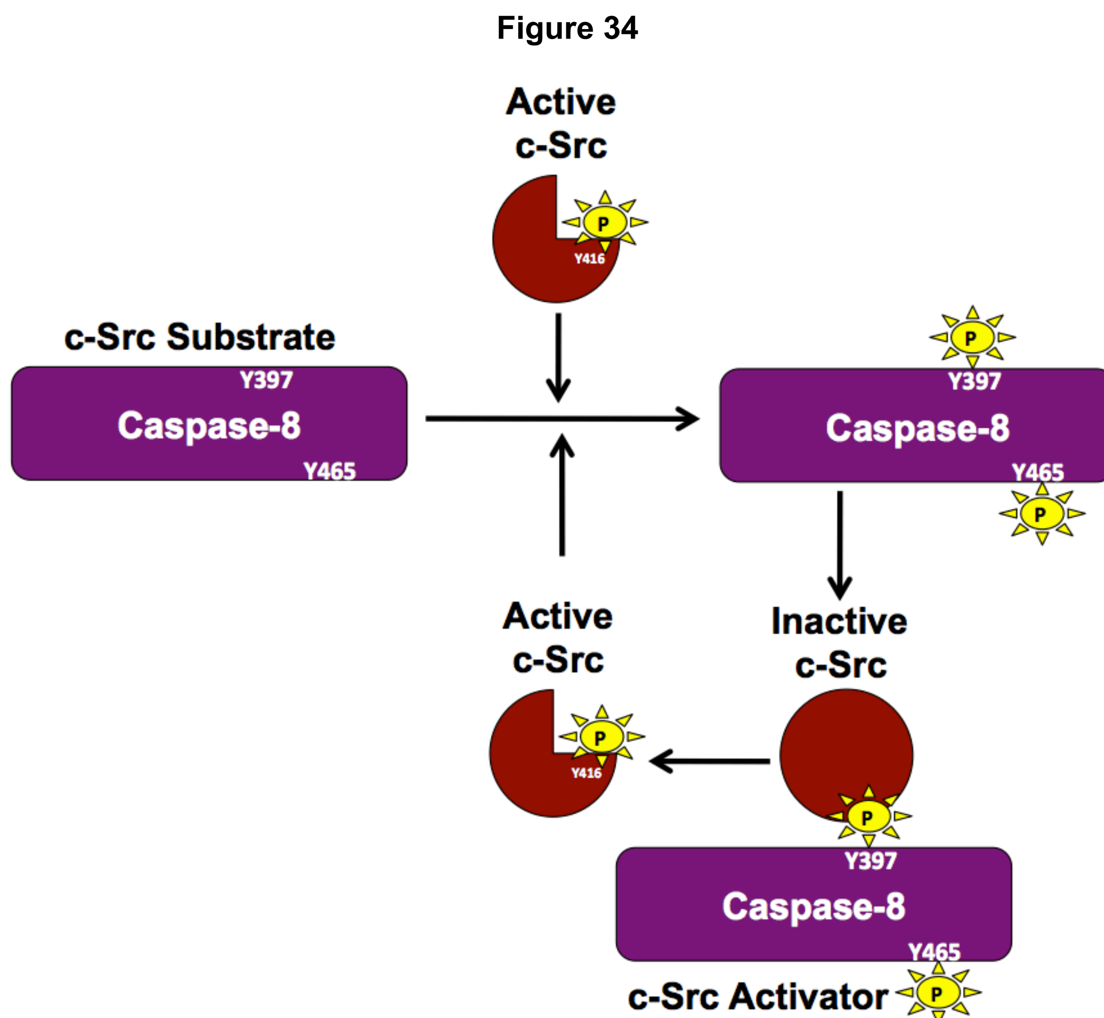


Figure 34. Caspase-8 as a Substrate and Activator of c-Src

A schematic diagram depicting the role of caspase-8 as a substrate and activator of c-Src is shown. As seen here, active c-Src phosphorylates caspase-8 at Y397 and Y465. This allows the binding of pY_{397}LEM phosphotyrosine peptide segment to SH2 domain of c-Src, maintaining c-Src in an open conformation, and allowing the autophosphorylation of Y416 and c-Src activation. This is an example of a positive feedback loop regulating c-Src activation.

6.6 What are the Implications of our Findings on Cancer Biology?

Published data suggest that: 1) c-Src activity is upregulated in colon cancer (367, 368); 2) some advanced human colon cancer cells harbor c-Src truncated mutation preventing inhibitory tyrosine residue from negatively regulating c-Src, resulting in increased c-Src activity (370); 3) caspase-8 is tyrosine phosphorylated in colon cancer (177); and 4) tyrosine phosphorylated caspase-8 could interact with c-Src (52). Moreover, our studies herein showed that tyrosine phosphorylated caspase-8 could stimulate c-Src activity. Together, these observations suggest that in colon cancer cells, tyrosine phosphorylation of caspase-8 by c-Src triggers a positive feedback loop that further activates c-Src via tyrosine phosphorylated caspase-8, which acts as an adaptor and activator (Figure 34). This promotes the survival, proliferation, migration and therefore metastasis-forming capacity of colon cancer cells.

We propose that this positive feedback loop can be broken if the interaction between tyrosine phosphorylated caspase-8 and c-Src is disrupted. It has been suggested that phosphorylation of Y380 of caspase-8b isoform (equivalent to Y397 of caspase-8a isoform) promoted interaction with the SH2 domain of c-Src. We propose using cell penetrating peptide technology (419) to deliver a peptide sequence corresponding to the interacting region of the c-Src SH2 domain into cancer cell. This is expected to disrupt the interaction between tyrosine phosphorylated caspase-8 and c-Src. Hence, tyrosine phosphorylated caspase-8 could no longer act as an adaptor and activator of c-Src, thus breaking the positive feedback loop, suppressing the further activation of c-Src. Without further activation of c-Src, caspase-8 could not be tyrosine phosphorylated. When caspase-8 is not tyrosine phosphorylated, it can be activated and induce apoptotic

program. Moreover, when caspase-8 is activated, it can also inhibit c-Src activity as it has been shown in chapter 4. This will switch the cell fate program from survival to apoptosis (Figure 35).

Figure 35

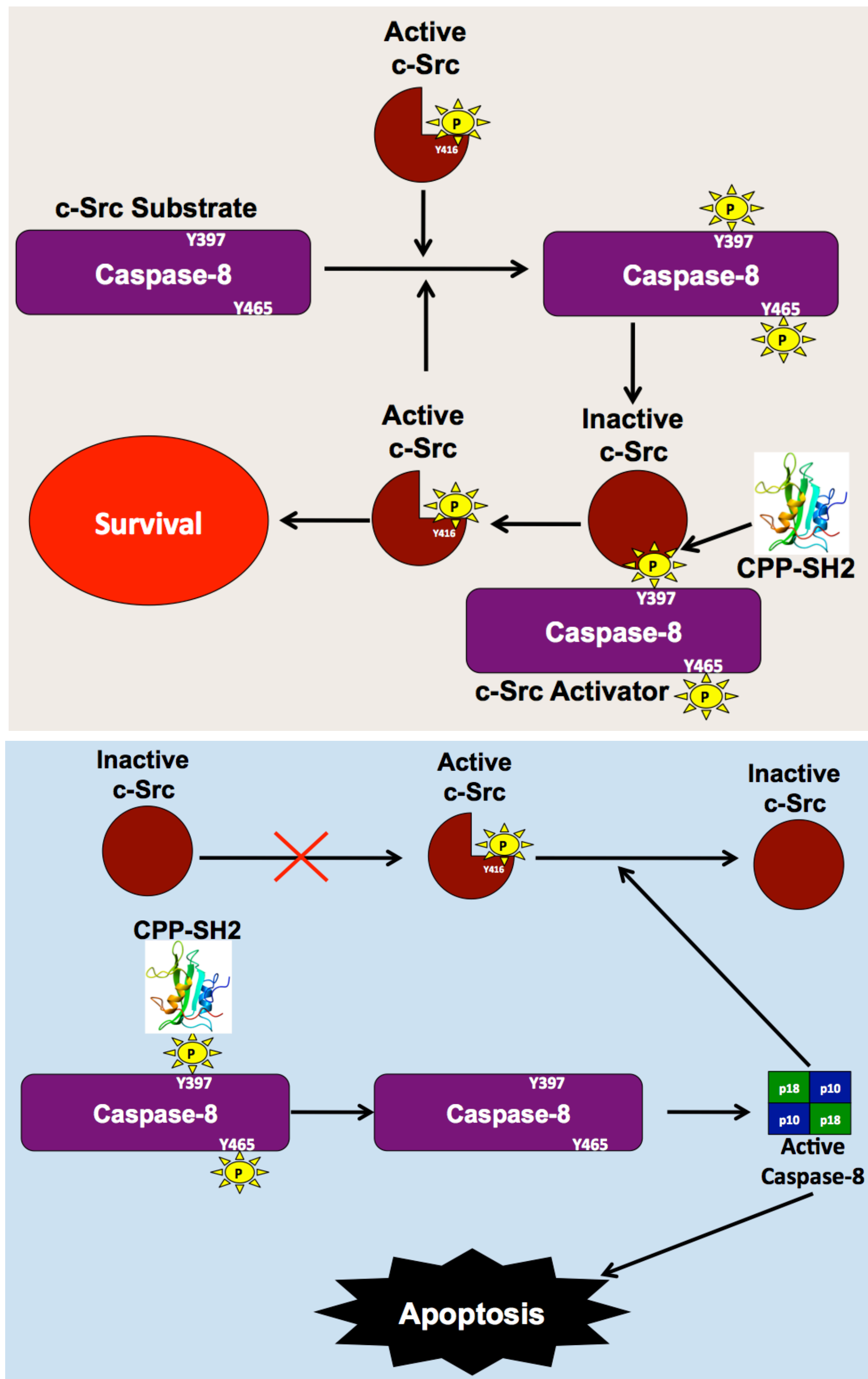


Figure 35. Cell Permeable Peptide as a Potential Therapeutic Option in Colon Cancer

A schematic diagram depicting the potential therapeutic role of cell penetrating peptide containing a portion of the c-Src SH2 domain for colon cancer is shown. Top panel: active c-Src phosphorylates caspase-8 at Y397 and Y465. This allows the binding of $pY_{397}LEM$ phosphotyrosine peptide to SH2 domain of c-Src, maintaining c-Src in an open conformation, thus allowing its autophosphorylation at Y416 and activation. These events promote cell survival. Bottom panel: With the use of a cell permeable peptide containing a portion of c-Src SH2 domain, we propose to disrupt the interaction between c-Src and tyrosine phosphorylated caspase-8. This will prevent further activation of c-Src. Without further activation of c-Src, caspase-8 cannot be tyrosine phosphorylated, therefore it can then become activated. Activated caspase-8 promotes apoptosis and in addition, it inhibits the activation of c-Src, further suppressing the survival pathway. The end result is apoptosis.

6.7 Overview and Concluding Remarks

In this thesis, I have presented a novel reciprocal regulatory relationship between caspase-8 (a pro-apoptotic protein) and c-Src (a pro-survival protein). My work also allowed insight into the potential mechanisms underlying this novel regulatory circuit. Further, I suggested a potential therapeutic approach to tackle the positive feedback loop in survival signaling, based on the regulatory relationship between caspase-8 and c-Src. Overall, the work in this thesis serves as a mechanistic basis for future studies aimed at deciphering the intricate relationship between apoptosis and cell survival and provides a foundation for future therapeutic approaches in the field of oncology.

REFERENCES

1. Fuentes-Prior, P., and Salvesen, G. S. (2004) The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochemical Journal* **384**, 201
2. Thornberry, N. A., and Lazebnik, Y. (1998) Caspases: enemies within. *Science* **281**, 1312–1316
3. Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem.* **68**, 383–424
4. Nicholson, D. W. (1999) Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ* **6**, 1028–1042
5. Chang, H. Y., and Yang, X. (2000) Proteases for cell suicide: functions and regulation of caspases. *Microbiol. Mol. Biol. Rev.* **64**, 821–846
6. Hengartner, M. O. (2000) The biochemistry of apoptosis. *Nature* **407**, 770–776
7. Riedl, S. J., and Salvesen, G. S. (2007) The apoptosome: signalling platform of cell death. *Nature Reviews Molecular Cell Biology* **8**, 405–413
8. Miura, M., Zhu, H., Rotello, R., Hartwieg, E. A., and Yuan, J. (1993) Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. *Cell* **75**, 653–660
9. Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., and Aunins, J. (1992) A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* **356**, 768–774
10. Cerretti, D. P., Kozlosky, C. J., Mosley, B., Nelson, N., Van Ness, K., Greenstreet, T. A., March, C. J., Kronheim, S. R., Druck, T., and Cannizzaro, L. A. (1992) Molecular cloning of the interleukin-1 beta converting enzyme. *Science* **256**, 97–100
11. van Raam, B. J., and Salvesen, G. S. (2012) Proliferative versus apoptotic functions of caspase-8 Hetero or homo: the caspase-8 dimer controls cell fate. *Biochim Biophys Acta* **1824**, 113–122
12. Pop, C., and Salvesen, G. S. (2009) Human Caspases: Activation, Specificity, and Regulation. *Journal of Biological Chemistry* **284**, 21777–21781
13. Shi, Y. (2002) Mechanisms of caspase activation and inhibition during apoptosis. *Molecular Cell* **9**, 459–470
14. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. *Cell* **85**, 817–827
15. Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* **85**, 803–815
16. Fernandes-Alnemri, T., Armstrong, R. C., Krebs, J., Srinivasula, S. M., Wang, L., Bullrich, F., Fritz, L. C., Trapani, J. A., Tomaselli, K. J., Litwack, G., and Alnemri, E. S. (1996) In vitro activation of CPP32 and Mch3 by Mch4, a novel

human apoptotic cysteine protease containing two FADD-like domains. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 7464–7469
17. Fulda, S. (2009) Caspase-8 in cancer biology and therapy. *Cancer Lett.* **281**, 128–133
18. Scaffidi, C., Medema, J. P., Krammer, P. H., and Peter, M. E. (1997) FLICE is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b. *J Biol Chem* **272**, 26953–26958
19. Himeji, D., Horiuchi, T., Tsukamoto, H., Hayashi, K., Watanabe, T., and Harada, M. (2002) Characterization of caspase-8L: a novel isoform of caspase-8 that behaves as an inhibitor of the caspase cascade. *Blood* **99**, 4070–4078
20. Horiuchi, T., Himeji, D., Tsukamoto, H., Harashima, S., Hashimura, C., and Hayashi, K. (2000) Dominant expression of a novel splice variant of caspase-8 in human peripheral blood lymphocytes. *Biochem Biophys Res Commun* **272**, 877–881
21. Fulda, S. (2009) Caspase-8 in cancer biology and therapy. *Cancer Lett.* **281**, 128–133
22. Xu, Z., Tang, K., Wang, M., Rao, Q., Liu, B., and Wang, J. (2009) A New Caspase-8 Isoform Caspase-8s Increased Sensitivity to Apoptosis in Jurkat Cells. *J. Biomed. Biotechnol.* **2009**, 1–11
23. Golks, A., Brenner, D., Schmitz, I., Watzl, C., Krueger, A., Krammer, P. H., and Lavrik, I. N. (2006) The role of CAP3 in CD95 signaling: new insights into the mechanism of procaspase-8 activation. *Cell Death Differ* **13**, 489–498
24. Vincenz, C., and Dixit, V. M. (1997) Fas-associated death domain protein interleukin-1beta-converting enzyme 2 (FLICE2), an ICE/Ced-3 homologue, is proximally involved in CD95- and p55-mediated death signaling. *J Biol Chem* **272**, 6578–6583
25. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1996) Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 14486–14491
26. Medema, J. P., Scaffidi, C., Kischkel, F. C., Shevchenko, A., Mann, M., Krammer, P. H., and Peter, M. E. (1997) FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J* **16**, 2794–2804
27. Lavrik, I., Krueger, A., Schmitz, I., Baumann, S., Weyd, H., Krammer, P. H., and Kirchhoff, S. (2003) The active caspase-8 heterotetramer is formed at the CD95 DISC. *Cell Death Differ* **10**, 144–145
28. Watt, W., Koeplinger, K. A., Mildner, A. M., Heinrikson, R. L., Tomasselli, A. G., and Watenpaugh, K. D. (1999) The atomic-resolution structure of human caspase-8, a key activator of apoptosis. *Structure* **7**, 1135–1143
29. Blanchard, H., Kodandapani, L., Mittl, P. R., Marco, S. D., Krebs, J. F., Wu, J. C., Tomaselli, K. J., and Grütter, M. G. (1999) The three-dimensional structure of caspase-8: an initiator enzyme in apoptosis. *Structure* **7**, 1125–1133
30. Blanchard, H., Donepudi, M., Tschopp, M., Kodandapani, L., Wu, J. C., and Grütter, M. G. (2000) Caspase-8 specificity probed at subsite S(4): crystal

structure of the caspase-8-Z-DEVD-cho complex. *J Mol Biol* **302**, 9–16
31. Keller, N., Mares, J., Zerbe, O., and Grütter, M. G. (2009) Structural and biochemical studies on procaspase-8: new insights on initiator caspase activation. *Structure* **17**, 438–448
32. Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholson, D. W. (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J Biol Chem* **272**, 17907–17911
33. Muzio, M., Salvesen, G., and Dixit, V. (1997) FLICE induced apoptosis in a cell-free system. *Journal of Biological Chemistry* **272**, 2952
34. Takahashi, A., Hirata, H., Yonehara, S., Imai, Y., Lee, K. K., Moyer, R. W., Turner, P. C., Mesner, P. W., Okazaki, T., Sawai, H., Kishi, S., Yamamoto, K., Okuma, M., and Sasada, M. (1997) Affinity labeling displays the stepwise activation of ICE-related proteases by Fas, staurosporine, and CrmA-sensitive caspase-8. *Oncogene* **14**, 2741–2752
35. Stennicke, H. R., Jürgensmeier, J. M., Shin, H., Deveraux, Q., Wolf, B. B., Yang, X., Zhou, Q., Ellerby, H. M., Ellerby, L. M., Bredesen, D., Green, D. R., Reed, J. C., Froelich, C. J., and Salvesen, G. S. (1998) Pro-caspase-3 is a major physiologic target of caspase-8. *J Biol Chem* **273**, 27084–27090
36. Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94**, 491–501
37. Lin, Y., Devin, A., Rodriguez, Y., and Liu, Z. G. (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev.* **13**, 2514–2526
38. Martinon, F., Holler, N., Richard, C., and Tschopp, J. (2000) Activation of a pro-apoptotic amplification loop through inhibition of NF-kappaB-dependent survival signals by caspase-mediated inactivation of RIP. *FEBS Letters* **468**, 134–136
39. Fischer, U., Stroh, C., and Schulze-Osthoff, K. (2006) Unique and overlapping substrate specificities of caspase-8 and caspase-10. *Oncogene* **25**, 152–159
40. O'Donnell, M. A., Perez-Jimenez, E., Oberst, A., Ng, A., Massoumi, R., Xavier, R., Green, D. R., and Ting, A. T. (2011) Caspase 8 inhibits programmed necrosis by processing CYLD. *Nat. Cell Biol.* **13**, 1437–1442
41. Boatright, K. M., Renatus, M., Scott, F. L., Sperandio, S., Shin, H., Pedersen, I. M., Ricci, J. E., Edris, W. A., Sutherlin, D. P., Green, D. R., and Salvesen, G. S. (2003) A unified model for apical caspase activation. *Molecular Cell* **11**, 529–541
42. Donepudi, M., Mac Sweeney, A., Briand, C., and Grütter, M. G. (2003) Insights into the regulatory mechanism for caspase-8 activation. *Molecular Cell* **11**, 543–549
43. Yang, X., Chang, H. Y., and Baltimore, D. (1998) Autoproteolytic activation of pro-caspases by oligomerization. *Molecular Cell* **1**, 319–325
44. Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M.

(1998) An induced proximity model for caspase-8 activation. *J Biol Chem* **273**, 2926–2930
45. Chen, M., Orozco, A., Spencer, D. M., and Wang, J. (2002) Activation of initiator caspases through a stable dimeric intermediate. *J Biol Chem* **277**, 50761–50767
46. Salvesen, G. S., and Dixit, V. M. (1999) Caspase activation: the induced-proximity model. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 10964–10967
47. Chang, D. W., Xing, Z., Capacio, V. L., Peter, M. E., and Yang, X. (2003) Interdimer processing mechanism of procaspase-8 activation. *EMBO J* **22**, 4132–4142
48. Pop, C., Fitzgerald, P., Green, D. R., and Salvesen, G. S. (2007) Role of proteolysis in caspase-8 activation and stabilization. *Biochemistry* **46**, 4398–4407
49. Keller, N., Grütter, M. G., and Zerbe, O. (2010) Studies of the molecular mechanism of caspase-8 activation by solution NMR. *Cell Death Differ* **17**, 710–718
50. Oberst, A., Pop, C., Tremblay, A. G., Blais, V., Denault, J.-B., Salvesen, G. S., and Green, D. R. (2010) Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation. *Journal of Biological Chemistry* **285**, 16632–16642
51. Mielgo, A., Torres, V. A., Schmid, M. C., Graf, R., Zeitlin, S. G., Lee, P., Shields, D. J., Barbero, S., Jamora, C., and Stupack, D. G. (2009) The death effector domains of caspase-8 induce terminal differentiation. *PLoS ONE* **4**, e7879
52. Barbero, S., Barilà, D., Mielgo, A., Stagni, V., Clair, K., and Stupack, D. (2008) Identification of a critical tyrosine residue in caspase 8 that promotes cell migration. *J Biol Chem* **283**, 13031–13034
53. Finlay, D., Howes, A., and Vuori, K. (2009) Critical role for caspase-8 in epidermal growth factor signaling. *Cancer Research* **69**, 5023–5029
54. Lee, K.-H., Feig, C., Tchikov, V., Schickel, R., Hallas, C., Schütze, S., Peter, M. E., and Chan, A. C. (2006) The role of receptor internalization in CD95 signaling. *EMBO J* **25**, 1009–1023
55. Torres, V. A., Mielgo, A., Barilà, D., Anderson, D. H., and Stupack, D. (2008) Caspase 8 promotes peripheral localization and activation of Rab5. *J Biol Chem* **283**, 36280–36289
56. Barbero, S., Mielgo, A., Torres, V., Teitz, T., Shields, D. J., Mikolon, D., Bogyo, M., Barila, D., Lahti, J. M., Schlaepfer, D., and Stupack, D. G. (2009) Caspase-8 Association with the Focal Adhesion Complex Promotes Tumor Cell Migration and Metastasis. *Cancer Research* **69**, 3755–3763
57. Mielgo, A., Torres, V. A., Clair, K., Barbero, S., and Stupack, D. G. (2009) Paclitaxel promotes a caspase 8-mediated apoptosis through death effector domain association with microtubules. *Oncogene* **28**, 3551–3562
58. Lagunoff, M., and Carroll, P. A. (2003) Inhibition of apoptosis by the gamma-herpesviruses. *Int. Rev. Immunol.* **22**, 373–399
59. Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J.-L., Schröter, M., Scaffidi, C., Krammer, P.

H., Peter, M. E., and Tschopp, J. (1997) Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* **386**, 517–521
60. McCormick, A. L. (2008) Control of apoptosis by human cytomegalovirus. *Curr. Top. Microbiol. Immunol.* **325**, 281–295
61. Skaletskaya, A., Bartle, L. M., Chittenden, T., McCormick, A. L., Mocarski, E. S., and Goldmacher, V. S. (2001) A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 7829–7834
62. Brune, W. (2011) Inhibition of programmed cell death by cytomegaloviruses. *Virus Res.* **157**, 144–150
63. Chen, P., Tian, J., Kovesdi, I., and Bruder, J. T. (1998) Interaction of the adenovirus 14.7-kDa protein with FLICE inhibits Fas ligand-induced apoptosis. *J Biol Chem* **273**, 5815–5820
64. Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V. M., and Salvesen, G. S. (1997) Target protease specificity of the viral serpin CrmA. Analysis of five caspases. *J Biol Chem* **272**, 7797–7800
65. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1996) Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 14486–14491
66. Xu, G., Cirilli, M., Huang, Y., Rich, R. L., Myszka, D. G., and Wu, H. (2001) Covalent inhibition revealed by the crystal structure of the caspase-8/p35 complex. *Nature* **410**, 494–497
67. Tschopp, J., Irmler, M., and Thome, M. (1998) Inhibition of fas death signals by FLIPs. *Curr. Opin. Immunol.* **10**, 552–558
68. Chun, H. J., Zheng, L., Ahmad, M., Wang, J., Speirs, C. K., Siegel, R. M., Dale, J. K., Puck, J., Davis, J., Hall, C. G., Skoda-Smith, S., Atkinson, T. P., Straus, S. E., and Lenardo, M. J. (2002) Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. *Nature* **419**, 395–399
69. Bertin, J., Armstrong, R. C., Ottilie, S., Martin, D. A., Wang, Y., Banks, S., Wang, G. H., Senkevich, T. G., Alnemri, E. S., Moss, B., Lenardo, M. J., Tomaselli, K. J., and Cohen, J. I. (1997) Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 1172–1176
70. Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schröter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E., and Tschopp, J. (1997) Inhibition of death receptor signals by cellular FLIP. *Nature* **388**, 190–195
71. Shu, H. B., Halpin, D. R., and Goeddel, D. V. (1997) Casper is a FADD- and caspase-related inducer of apoptosis. *Immunity* **6**, 751–763
72. Goltsev, Y. V., Kovalenko, A. V., Arnold, E., Varfolomeev, E. E., Brodianskii, V. M., and Wallach, D. (1997) CASH, a novel caspase homologue with death effector domains. *J Biol Chem* **272**, 19641–19644

73. Srinivasula, S. M., Ahmad, M., Ottilie, S., Bullrich, F., Banks, S., Wang, Y., Fernandes-Alnemri, T., Croce, C. M., Litwack, G., Tomaselli, K. J., Armstrong, R. C., and Alnemri, E. S. (1997) FLAME-1, a novel FADD-like anti-apoptotic molecule that regulates Fas/TNFR1-induced apoptosis. *J Biol Chem* **272**, 18542–18545
74. Inohara, N., Koseki, T., Hu, Y., Chen, S., and Nunez, G. (1997) CLARP, a death effector domain-containing protein interacts with caspase-8 and regulates apoptosis. *Proceedings of the National Academy of Sciences* **94**, 10717–10722
75. Rasper, D. M., Vaillancourt, J. P., Hadano, S., Houtzager, V. M., Seiden, I., Keen, S. L., Tawa, P., Xanthoudakis, S., Nasir, J., Martindale, D., Koop, B. F., Peterson, E. P., Thornberry, N. A., Huang, J., MacPherson, D. P., Black, S. C., Hornung, F., Lenardo, M. J., Hayden, M. R., Roy, S., and Nicholson, D. W. (1998) Cell death attenuation by "Usurpin," a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD-95 (Fas, APO-1) receptor complex. *Cell Death Differ* **5**, 271–288
76. Han, D. K., Chaudhary, P. M., Wright, M. E., Friedman, C., Trask, B. J., Riedel, R. T., Baskin, D. G., Schwartz, S. M., and Hood, L. (1997) MRIT, a novel death-effector domain-containing protein, interacts with caspases and BclXL and initiates cell death. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 11333–11338
77. Chang, D. W., Xing, Z., Pan, Y., Algeciras-Schimnich, A., Barnhart, B. C., Yaish-Ohad, S., Peter, M. E., and Yang, X. (2002) c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. *EMBO J* **21**, 3704–3714
78. Micheau, O., Thome, M., Schneider, P., Holler, N., Tschopp, J., Nicholson, D. W., Briand, C., and Grütter, M. G. (2002) The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. *J Biol Chem* **277**, 45162–45171
79. Pop, C., Oberst, A., Drag, M., van Raam, B. J., Riedl, S. J., Green, D. R., and Salvesen, G. S. (2011) FLIP(L) induces caspase 8 activity in the absence of interdomain caspase 8 cleavage and alters substrate specificity. *Biochem J* **433**, 447–457
80. Boatright, K. M., Deis, C., Denault, J.-B., Sutherlin, D. P., and Salvesen, G. S. (2004) Activation of caspases-8 and -10 by FLIP(L). *Biochem J* **382**, 651–657
81. McDonald, E. R., and El-Deiry, W. S. (2004) Suppression of caspase-8- and -10-associated RING proteins results in sensitization to death ligands and inhibition of tumor cell growth. *Proceedings of the National …*
82. Hitoshi, Y., Lorens, J., Kitada, S. I., Fisher, J., LaBarge, M., Ring, H. Z., Francke, U., Reed, J. C., Kinoshita, S., and Nolan, G. P. (1998) Toso, a cell surface, specific regulator of Fas-induced apoptosis in T cells. *Immunity* **8**, 461–471
83. Varfolomeev, E. E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Beckmann, J. S., Mett, I. L., Rebrikov, D., Brodianski, V. M., Kemper, O. C., Kollet, O., Lapidot, T., Soffer, D., Sobe, T., Avraham, K. B., Goncharov, T., Holtmann, H., Lonai, P., and Wallach, D. (1998) Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and

is lethal prenatally. *Immunity* **9**, 267–276

84. Sakamaki, K., Inoue, T., Asano, M., Sudo, K., Kazama, H., Sakagami, J., Sakata, S., Ozaki, M., Nakamura, S., Toyokuni, S., Osumi, N., Iwakura, Y., and Yonehara, S. (2002) Ex vivo whole-embryo culture of caspase-8-deficient embryos normalize their aberrant phenotypes in the developing neural tube and heart. *Cell Death Differ* **9**, 1196–1206
85. Kang, T.-B., Ben-Moshe, T., Varfolomeev, E. E., Pewzner-Jung, Y., Yogev, N., Jurewicz, A., Waisman, A., Brenner, O., Haffner, R., Gustafsson, E., Ramakrishnan, P., Lapidot, T., and Wallach, D. (2004) Caspase-8 serves both apoptotic and nonapoptotic roles. *J Immunol* **173**, 2976–2984
86. Krammer, P. H. (2000) CD95's deadly mission in the immune system. *Nature* **407**, 789–795
87. Siegel, R. M., Chan, F. K., Chun, H. J., and Lenardo, M. J. (2000) The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nature Publishing Group* **1**, 469–474
88. Wajant, H. (2002) The Fas signaling pathway: more than a paradigm. *Science* **296**, 1635–1636
89. Juo, P., Kuo, C. J., Yuan, J., and Blenis, J. (1998) Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade. *Curr Biol* **8**, 1001–1008
90. Ashkenazi, A. (1998) Death Receptors: Signaling and Modulation. *Science* **281**, 1305–1308
91. Nagata, S. (1997) Apoptosis by death factor. *Cell* **88**, 355–365
92. Nagata, S. (1999) Fas ligand-induced apoptosis. *Annu. Rev. Genet.* **33**, 29–55
93. Salmena, L., Lemmers, B., Hakem, A., Matysiak-Zablocki, E., Murakami, K., Au, P. Y. B., Berry, D. M., Tamblyn, L., Shehabeldin, A., Migon, E., Wakeham, A., Bouchard, D., Yeh, W. C., McGlade, J. C., Ohashi, P. S., and Hakem, R. (2003) Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity. *Genes Dev.* **17**, 883–895
94. Kischkel, F. C., Lawrence, D. A., Chuntharapai, A., Schow, P., Kim, K. J., and Ashkenazi, A. (2000) Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity* **12**, 611–620
95. Bodmer, J. L., Holler, N., Reynard, S., Vinciguerra, P., Schneider, P., Juo, P., Blenis, J., and Tschopp, J. (2000) TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nat. Cell Biol.* **2**, 241–243
96. Sprick, M. R., Weigand, M. A., Rieser, E., Rauch, C. T., Juo, P., Blenis, J., Krammer, P. H., and Walczak, H. (2000) FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity* **12**, 599–609
97. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* **94**, 481–490
98. Stupack, D. G., Teitz, T., Potter, M. D., Mikolon, D., Houghton, P. J., Kidd, V. J., Lahti, J. M., and Cheresh, D. A. (2006) Potentiation of neuroblastoma metastasis by loss of caspase-8. *Nature* **439**, 95–99
99. Stupack, D. G., Puente, X. S., Boutsaboualoy, S., Storgard, C. M., and Cheresh,

D. A. (2001) Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *The Journal of Cell Biology* **155**, 459–470
100. Holler, N., Zaru, R., Micheau, O., Thome, M., Attinger, A., Valitutti, S., Bodmer, J. L., Schneider, P., Seed, B., and Tschopp, J. (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nature Publishing Group* **1**, 489–495
101. Zhang, D.-W., Shao, J., Lin, J., Zhang, N., Lu, B.-J., Lin, S.-C., Dong, M.-Q., and Han, J. (2009) RIP3, an Energy Metabolism Regulator That Switches TNF-Induced Cell Death from Apoptosis to Necrosis. *Science Signaling* **325**, 332
102. Micheau, O., and Tschopp, J. (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* **114**, 181–190
103. He, S., Wang, L., Miao, L., Wang, T., Du, F., Zhao, L., and Wang, X. (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell* **137**, 1100–1111
104. Vandenabeele, P., Galluzzi, L., Vanden Berghe, T., and Kroemer, G. (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nature Reviews Molecular Cell Biology* **11**, 700–714
105. Tenev, T., Bianchi, K., Darding, M., Broemer, M., Langlais, C., Wallberg, F., Zachariou, A., Lopez, J., MacFarlane, M., Cain, K., and Meier, P. (2011) The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. *Molecular Cell* **43**, 432–448
106. Feoktistova, M., Geserick, P., Kellert, B., Dimitrova, D. P., Langlais, C., Hupe, M., Cain, K., MacFarlane, M., Häcker, G., and Leverkus, M. (2011) cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Molecular Cell* **43**, 449–463
107. Vercammen, D., Beyaert, R., Denecker, G., Goossens, V., Van Loo, G., Declercq, W., Grooten, J., Fiers, W., and Vandenabeele, P. (1998) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *The Journal of experimental medicine* **187**, 1477–1485
108. Lüschen, S., Ussat, S., Scherer, G., Kabelitz, D., and Adam-Klages, S. (2000) Sensitization to death receptor cytotoxicity by inhibition of fas-associated death domain protein (FADD)/caspase signaling. Requirement of cell cycle progression. *J Biol Chem* **275**, 24670–24678
109. Launay, S., Hermine, O., Fontenay, M., Kroemer, G., Solary, E., and Garrido, C. (2005) Vital functions for lethal caspases. *Oncogene* **24**, 5137–5148
110. Kaiser, W. J., Upton, J. W., Long, A. B., Livingston-Rosanoff, D., Daley-Bauer, L. P., Hakem, R., Caspary, T., and Mocarski, E. S. (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature* **471**, 368–372
111. Oberst, A., Dillon, C. P., Weinlich, R., McCormick, L. L., Fitzgerald, P., Pop, C., Hakem, R., Salvesen, G. S., and Green, D. R. (2011) Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature* **471**, 363–367
112. Feng, S., Yang, Y., Mei, Y., Ma, L., Zhu, D.-E., Hoti, N., Castanares, M., and Wu, M. (2007) Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. *Cell Signal* **19**, 2056–2067
113. Günther, C., Martini, E., Wittkopf, N., Amann, K., Weigmann, B., Neumann, H.,

Waldner, M. J., Hedrick, S. M., Tenzer, S., Neurath, M. F., and Becker, C. (2011) Caspase-8 regulates TNF-α-induced epithelial necroptosis and terminal ileitis. *Nature* **477**, 335–339

114. Yu, L., Alva, A., Su, H., Dutt, P., Freundt, E., Welsh, S., Baehrecke, E. H., and Lenardo, M. J. (2004) Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science* **304**, 1500–1502
115. Bell, B. D., Leverrier, S., Weist, B. M., Newton, R. H., Arechiga, A. F., Luhrs, K. A., Morrissette, N. S., and Walsh, C. M. (2008) FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells. *Proceedings of the National Academy of Sciences* **105**, 16677–16682
116. Zhao, Y., Sui, X., and Ren, H. (2010) From procaspase-8 to caspase-8: revisiting structural functions of caspase-8. *J. Cell. Physiol.* **225**, 316–320
117. Ben-Moshe, T., Barash, H., Kang, T.-B., Kim, J.-C., Kovalenko, A., Gross, E., Schuchmann, M., Abramovitch, R., Galun, E., and Wallach, D. (2007) Role of caspase-8 in hepatocyte response to infection and injury in mice. *Hepatology* **45**, 1014–1024
118. Kovalenko, A., Kim, J.-C., Kang, T.-B., Rajput, A., Bogdanov, K., Dittrich-Breiholz, O., Kracht, M., Brenner, O., and Wallach, D. (2009) Caspase-8 deficiency in epidermal keratinocytes triggers an inflammatory skin disease. *Journal of Experimental Medicine* **206**, 2161–2177
119. Kang, T.-B., Yang, S.-H., Toth, B., Kovalenko, A., and Wallach, D. (2013) Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. *Immunity* **38**, 27–40
120. Schroder, K., and Tschopp, J. (2010) The inflammasomes. *Cell* **140**, 821–832
121. Maelfait, J., Vercammen, E., Janssens, S., Schotte, P., Haegman, M., Magez, S., and Beyaert, R. (2008) Stimulation of Toll-like receptor 3 and 4 induces interleukin-1 maturation by caspase-8. *Journal of Experimental Medicine* **205**, 1967–1973
122. Gringhuis, S. I., Kaptein, T. M., Wevers, B. A., Theelen, B., van der Vlist, M., Boekhout, T., and Geijtenbeek, T. B. H. (2012) Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1β via a noncanonical caspase-8 inflammasome. *Nat Immunol* **13**, 246–254
123. Abbas, A. K. (1996) Die and let live: eliminating dangerous lymphocytes. *Cell* **84**, 655–657
124. Salmena, L., and Hakem, R. (2005) Caspase-8 deficiency in T cells leads to a lethal lymphoinfiltrative immune disorder. *The Journal of experimental medicine* **202**, 727–732
125. Kennedy, N. J., Kataoka, T., Tschopp, J., and Budd, R. C. (1999) Caspase activation is required for T cell proliferation. *The Journal of experimental medicine* **190**, 1891–1896
126. Falk, M., Ussat, S., Reiling, N., Wesch, D., Kabelitz, D., and Adam-Klages, S. (2004) Caspase inhibition blocks human T cell proliferation by suppressing appropriate regulation of IL-2, CD25, and cell cycle-associated proteins. *J Immunol* **173**, 5077–5085
127. Boissonnas, A., Bonduelle, O., Lucas, B., Debré, P., Autran, B., and Combadière, B. (2002) Differential requirement of caspases during naive T cell

proliferation. *Eur. J. Immunol.* **32**, 3007–3015
128. Arechiga, A. F., Bell, B. D., Leverrier, S., Weist, B. M., Porter, M., Wu, Z., Kanno, Y., Ramos, S. J., Ong, S. T., Siegel, R., and Walsh, C. M. (2007) A Fas-associated death domain protein/caspase-8-signaling axis promotes S-phase entry and maintains S6 kinase activity in T cells responding to IL-2. *J Immunol* **179**, 5291–5300
129. Mack, A., and Häcker, G. (2002) Inhibition of caspase or FADD function blocks proliferation but not MAP kinase-activation and interleukin-2-production during primary stimulation of T cells - Mack - 2002 - European Journal of Immunology - Wiley Online Library. *Eur. J. Immunol.*
130. Olson, N. E., Graves, J. D., Shu, G. L., Ryan, E. J., and Clark, E. A. (2003) Caspase activity is required for stimulated B lymphocytes to enter the cell cycle. *J Immunol* **170**, 6065–6072
131. Kawadler, H., Gantz, M. A., Riley, J. L., and Yang, X. (2008) The paracaspase MALT1 controls caspase-8 activation during lymphocyte proliferation. *Molecular Cell* **31**, 415–421
132. Chau, H., Wong, V., Chen, N.-J., Huang, H.-L., Lin, W.-J., Mirtsos, C., Elford, A. R., Bonnard, M., Wakeham, A., You-Ten, A. I., Lemmers, B., Salmena, L., Pellegrini, M., Hakem, R., Mak, T. W., Ohashi, P., and Yeh, W. C. (2005) Cellular FLICE-inhibitory protein is required for T cell survival and cycling. *The Journal of experimental medicine* **202**, 405–413
133. Zhang, N., and He, Y.-W. (2005) An essential role for c-FLIP in the efficient development of mature T lymphocytes. *The Journal of experimental medicine* **202**, 395–404
134. Misra, R. S., Russell, J. Q., Koenig, A., Hinshaw-Makepeace, J. A., Wen, R., Wang, D., Huo, H., Littman, D. R., Ferch, U., Ruland, J., Thome, M., and Budd, R. C. (2007) Caspase-8 and c-FLIPL associate in lipid rafts with NF-kappaB adaptors during T cell activation. *J Biol Chem* **282**, 19365–19374
135. Ch'en, I. L., Beisner, D. R., Degterev, A., Lynch, C., Yuan, J., Hoffmann, A., and Hedrick, S. M. (2008) Antigen-mediated T cell expansion regulated by parallel pathways of death. *Proceedings of the National Academy of Sciences* **105**, 17463–17468
136. Rebe, C., Cathelin, S., Launay, S., Filomenko, R., Prevotat, L., L'Ollivier, C., Gyan, E., Micheau, O., Grant, S., Dubart-Kupperschmitt, A., Fontenay, M., and Solary, E. (2007) Caspase-8 prevents sustained activation of NF- B in monocytes undergoing macrophagic differentiation. *Blood* **109**, 1442–1450
137. Alam, A., Cohen, L. Y., Aouad, S., and Sékaly, R. P. (1999) Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells. *The Journal of experimental medicine* **190**, 1879–1890
138. Lemmers, B., Salmena, L., Bidère, N., Su, H., Matysiak-Zablocki, E., Murakami, K., Ohashi, P. S., Jurisicova, A., Lenardo, M., Hakem, R., and Hakem, A. (2007) Essential role for caspase-8 in Toll-like receptors and NFkappaB signaling. *J Biol Chem* **282**, 7416–7423
139. Beisner, D. R., Ch'en, I. L., Kolla, R. V., Hoffmann, A., and Hedrick, S. M. (2005) Cutting edge: innate immunity conferred by B cells is regulated by caspase-8. *J Immunol* **175**, 3469–3473

140. Senft, J., Helfer, B., and Frisch, S. M. (2007) Caspase-8 interacts with the p85 subunit of phosphatidylinositol 3-kinase to regulate cell adhesion and motility. *Cancer Research* **67**, 11505–11509
141. Finlay, D., and Vuori, K. (2007) Novel noncatalytic role for caspase-8 in promoting SRC-mediated adhesion and Erk signaling in neuroblastoma cells. *Cancer Research* **67**, 11704–11711
142. Helfer, B., Boswell, B. C., Finlay, D., Cipres, A., Vuori, K., Bong Kang, T., Wallach, D., Dorfleutner, A., Lahti, J. M., Flynn, D. C., and Frisch, S. M. (2006) Caspase-8 promotes cell motility and calpain activity under nonapoptotic conditions. *Cancer Research* **66**, 4273–4278
143. Torres, V. A., Mielgo, A., Barbero, S., Hsiao, R., Wilkins, J. A., and Stupack, D. G. (2010) Rab5 mediates caspase-8-promoted cell motility and metastasis. *Mol. Biol. Cell* **21**, 369–376
144. Hayden, M. S., and Ghosh, S. (2008) Shared Principles in NF-κB Signaling. *Cell* **132**, 344–362
145. Thome, M. (2004) CARMA1, BCL-10 and MALT1 in lymphocyte development and activation. *Nat Rev Immunol* **4**, 348–359
146. Su, H., Bidère, N., Zheng, L., Cubre, A., Sakai, K., Dale, J., Salmena, L., Hakem, R., Straus, S., and Lenardo, M. (2005) Requirement for caspase-8 in NF-kappaB activation by antigen receptor. *Science* **307**, 1465–1468
147. Chaudhary, P. M., Eby, M. T., Jasmin, A., Kumar, A., Liu, L., and Hood, L. (2000) Activation of the NF-kappaB pathway by caspase 8 and its homologs. *Oncogene* **19**, 4451–4460
148. Pellegrini, M., Bath, S., Marsden, V. S., Huang, D. C. S., Metcalf, D., Harris, A. W., and Strasser, A. (2005) FADD and caspase-8 are required for cytokine-induced proliferation of hemopoietic progenitor cells. *Blood* **106**, 1581–1589
149. Hu, W. H., Johnson, H., and Shu, H. B. (2000) Activation of NF-kappaB by FADD, Casper, and caspase-8. *J Biol Chem* **275**, 10838–10844
150. Kreuz, S., Siegmund, D., Rumpf, J.-J., Samel, D., Leverkus, M., Janssen, O., Häcker, G., Dittrich-Breiholz, O., Kracht, M., Scheurich, P., and Wajant, H. (2004) NFkappaB activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP. *The Journal of Cell Biology* **166**, 369–380
151. Hasegawa, M., Imamura, R., Kinoshita, T., Matsumoto, N., Masumoto, J., Inohara, N., and Suda, T. (2005) ASC-mediated NF-kappaB activation leading to interleukin-8 production requires caspase-8 and is inhibited by CLARP. *J Biol Chem* **280**, 15122–15130
152. Matsumoto, N., Imamura, R., and Suda, T. (2007) Caspase-8-and JNK-dependent AP-1 activation is required for Fas ligand-induced IL-8 production. *FEBS Journal* **274**, 2376–2384
153. Hu, W. H. (2000) Activation of NF-kappa B by FADD, Casper, and Caspase-8. *Journal of Biological Chemistry* **275**, 10838–10844
154. Roskoski, R. (2012) ERK1/2 MAP kinases: structure, function, and regulation. *Pharmacol. Res.* **66**, 105–143
155. Lüschen, S., Falk, M., Scherer, G., Ussat, S., Paulsen, M., and Adam-Klages, S. (2005) The Fas-associated death domain protein/caspase-8/c-FLIP signaling pathway is involved in TNF-induced activation of ERK. *Experimental cell*

research **310**, 33–42

156. Brunton, V. G., and Frame, M. C. (2008) Src and focal adhesion kinase as therapeutic targets in cancer. *Curr Opin Pharmacol* **8**, 427–432
157. Hanahan, D., and Weinberg, R. A. (2011) Hallmarks of Cancer: The Next Generation. *Cell* **144**, 646–674
158. Kim, H. S., Lee, J. W., Soung, Y. H., Park, W. S., Kim, S. Y., Lee, J. H., Park, J. Y., Cho, Y. G., Kim, C. J., and Jeong, S. W. (2003) Inactivating mutations of caspase-8 gene in colorectal carcinomas. *Gastroenterology* **125**, 708–715
159. Mandruzzato, S., Brasseur, F., Andry, G., Boon, T., and van der Bruggen, P. (1997) A CASP-8 mutation recognized by cytolytic T lymphocytes on a human head and neck carcinoma. *The Journal of experimental medicine* **186**, 785–793
160. Shivapurkar, N., Reddy, J., Matta, H., Sathyanarayana, U. G., Huang, C. X., Toyooka, S., Minna, J. D., Chaudhary, P. M., and Gazdar, A. F. (2002) Loss of expression of death-inducing signaling complex (DISC) components in lung cancer cell lines and the influence of MYC amplification. *Oncogene* **21**, 8510–8514
161. Joseph, B., Ekedahl, J., Sirzen, F., Lewensohn, R., and Zhivotovsky, B. (1999) Differences in expression of pro-caspases in small cell and non-small cell lung carcinoma. *Biochem Biophys Res Commun* **262**, 381–387
162. Soung, Y. H., Lee, J. W., Kim, S. Y., Jang, J., Park, Y. G., Park, W. S., Nam, S. W., Lee, J. Y., Yoo, N. J., and Lee, S. H. (2005) CASPASE-8 gene is inactivated by somatic mutations in gastric carcinomas. *Cancer Research* **65**, 815–821
163. Teitz, T., Wei, T., Valentine, M. B., Vanin, E. F., Grenet, J., Valentine, V. A., Behm, F. G., Look, A. T., Lahti, J. M., and Kidd, V. J. (2000) Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. *Nature Medicine* **6**, 529–535
164. Takita, J., Yang, H. W., Chen, Y. Y., Hanada, R., Yamamoto, K., Teitz, T., Kidd, V., and Hayashi, Y. (2001) Allelic imbalance on chromosome 2q and alterations of the caspase 8 gene in neuroblastoma. *Oncogene* **20**, 4424–4432
165. Harada, K., Toyooka, S., Shivapurkar, N., Maitra, A., Reddy, J. L., Matta, H., Miyajima, K., Timmons, C. F., Tomlinson, G. E., Mastrangelo, D., Hay, R. J., Chaudhary, P. M., and Gazdar, A. F. (2002) Deregulation of caspase 8 and 10 expression in pediatric tumors and cell lines. *Cancer Research* **62**, 5897–5901
166. Hopkins-Donaldson, S., Bodmer, J. L., Bourloud, K. B., Brognara, C. B., Tschopp, J., and Gross, N. (2000) Loss of caspase-8 expression in highly malignant human neuroblastoma cells correlates with resistance to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. *Cancer Research* **60**, 4315–4319
167. Takita, J., Yang, H. W., Bessho, F., Hanada, R., Yamamoto, K., Kidd, V., Teitz, T., Wei, T., and Hayashi, Y. (2000) Absent or reduced expression of the caspase 8 gene occurs frequently in neuroblastoma, but not commonly in Ewing sarcoma or rhabdomyosarcoma. *Med. Pediatr. Oncol.* **35**, 541–543
168. Grotzer, M. A., Eggert, A., Zuzak, T. J., Janss, A. J., Marwaha, S., Wiewrodt, B. R., Ikegaki, N., Brodeur, G. M., and Phillips, P. C. (2000) Resistance to TRAIL-induced apoptosis in primitive neuroectodermal brain tumor cells correlates with a loss of caspase-8 expression. *Oncogene* **19**, 4604–4610

169. Eggert, A., Grotzer, M. A., Zuzak, T. J., Wiewrodt, B. R., Ho, R., Ikegaki, N., and Brodeur, G. M. (2001) Resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression. *Cancer Research* **61**, 1314–1319
170. Lázcoz, P., Muñoz, J., Nistal, M., Pestaña, A., Encío, I., and Castresana, J. S. (2006) Frequent promoter hypermethylation of RASSF1A and CASP8 in neuroblastoma. *BMC Cancer* **6**, 254
171. Pingoud-Meier, C., Lang, D., Janss, A. J., Rorke, L. B., Phillips, P. C., Shalaby, T., and Grotzer, M. A. (2003) Loss of caspase-8 protein expression correlates with unfavorable survival outcome in childhood medulloblastoma. *Clin. Cancer Res.* **9**, 6401–6409
172. Hopkins-Donaldson, S., Ziegler, A., Kurtz, S., Bigosch, C., Kandioler, D., Ludwig, C., Zangemeister-Wittke, U., and Stahel, R. (2003) Silencing of death receptor and caspase-8 expression in small cell lung carcinoma cell lines and tumors by DNA methylation. *Cell Death Differ* **10**, 356–364
173. Shivapurkar, N., Toyooka, S., Eby, M. T., Huang, C. X., Sathyanarayana, U. G., Cunningham, H. T., Reddy, J. L., Brambilla, E., Takahashi, T., Minna, J. D., Chaudhary, P. M., and Gazdar, A. F. (2002) Differential inactivation of caspase-8 in lung cancers. *Cancer Biol. Ther.* **1**, 65–69
174. Liedtke, C., Zschemisch, N.-H., Cohrs, A., Roskams, T., Borlak, J., Manns, M. P., and Trautwein, C. (2005) Silencing of caspase-8 in murine hepatocellular carcinomas is mediated via methylation of an essential promoter element. *Gastroenterology* **129**, 1602–1615
175. Mohr, A., Zwacka, R. M., Jarmy, G., Büneker, C., Schrezenmeier, H., Döhner, K., Beltinger, C., Wiesneth, M., Debatin, K.-M., and Stahnke, K. (2005) Caspase-8L expression protects CD34+ hematopoietic progenitor cells and leukemic cells from CD95-mediated apoptosis. *Oncogene* **24**, 2421–2429
176. Miller, M. A., Karacay, B., Zhu, X., O'Dorisio, M. S., and Sandler, A. D. (2006) Caspase 8L, a novel inhibitory isoform of caspase 8, is associated with undifferentiated neuroblastoma. *Apoptosis* **11**, 15–24
177. Cursi, S., Rufini, A., Stagni, V., Condò, I., Matafora, V., Bachi, A., Bonifazi, A. P., Coppola, L., Superti-Furga, G., Testi, R., and Barilà, D. (2006) Src kinase phosphorylates Caspase-8 on Tyr380: a novel mechanism of apoptosis suppression. *EMBO J* **25**, 1895–1905
178. Jia, S. H., Parodo, J., Kapus, A., Rotstein, O. D., and Marshall, J. C. (2008) Dynamic regulation of neutrophil survival through tyrosine phosphorylation or dephosphorylation of caspase-8. *J Biol Chem* **283**, 5402–5413
179. Kim, H. S., Lee, J. W., Soung, Y. H., Park, W. S., Kim, S. Y., Lee, J. H., Park, J. Y., Cho, Y. G., Kim, C. J., Jeong, S. W., Nam, S. W., Kim, S. H., Lee, J. Y., Yoo, N. J., and Lee, S. H. (2003) Inactivating mutations of caspase-8 gene in colorectal carcinomas. *Gastroenterology* **125**, 708–715
180. Son, J.-W., Kang, H.-K., Chae, M. H., Choi, J. E., Park, J. M., Lee, W. K., Kim, C. H., Kim, D. S., Kam, S., Kang, Y. M., and Park, J. Y. (2006) Polymorphisms in the caspase-8 gene and the risk of lung cancer. *Cancer Genet. Cytogenet.* **169**, 121–127
181. Finlay, D., Howes, A., and Vuori, K. (2009) Caspase-8 as a potential mediator of

pro-tumorigenic signals. *Cell cycle (Georgetown, Tex.)* **8**, 3441–3442

182. Shen, J., Behrens, C., Wistuba, I. I., Feng, L., Lee, J. J., Hong, W. K., and Lotan, R. (2006) Identification and validation of differences in protein levels in normal, premalignant, and malignant lung cells and tissues using high-throughput Western Array and immunohistochemistry. *Cancer Research* **66**, 11194–11206
183. Yao, Z., Duan, S., Hou, D., Heese, K., and Wu, M. (2007) Death effector domain DEDa, a self-cleaved product of caspase-8/Mch5, translocates to the nucleus by binding to ERK1/2 and upregulates procaspase-8 expression via a p53-dependent mechanism. *EMBO J* **26**, 1068–1080
184. Jin, Z., Li, Y., Pitti, R., Lawrence, D., Pham, V. C., Lill, J. R., and Ashkenazi, A. (2009) Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. *Cell* **137**, 721–735
185. Kang, T.-B., Oh, G.-S., Scandella, E., Bolinger, B., Ludewig, B., Kovalenko, A., and Wallach, D. (2008) Mutation of a self-processing site in caspase-8 compromises its apoptotic but not its nonapoptotic functions in bacterial artificial chromosome-transgenic mice. *J Immunol* **181**, 2522–2532
186. Alvarado-Kristensson, M., Melander, F., Leandersson, K., Rönnstrand, L., Wernstedt, C., and Andersson, T. (2004) p38-MAPK signals survival by phosphorylation of caspase-8 and caspase-3 in human neutrophils. *The Journal of experimental medicine* **199**, 449–458
187. Peng, C., Cho, Y.-Y., Zhu, F., Zhang, J., Wen, W., Xu, Y., Yao, K., Ma, W.-Y., Bode, A. M., and Dong, Z. (2011) Phosphorylation of caspase-8 (Thr-263) by ribosomal S6 kinase 2 (RSK2) mediates caspase-8 ubiquitination and stability. *Journal of Biological Chemistry* **286**, 6946–6954
188. Lawen, A. (2003) Apoptosis-an introduction. *Bioessays* **25**, 888–896
189. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**, 239–257
190. Eguchi, Y., Shimizu, S., and Tsujimoto, Y. (1997) Intracellular ATP levels determine cell death fate by apoptosis or necrosis. *Cancer Research* **57**, 1835–1840
191. Snyder, C. M., and Chandel, N. S. (2009) Mitochondrial regulation of cell survival and death during low-oxygen conditions. *Antioxid. Redox Signal.* **11**, 2673–2683
192. Cory, S., and Adams, J. M. (2002) The Bcl2 family: regulators of the cellular life-or-death switch. *Nat. Rev. Cancer* **2**, 647–656
193. Green, D. R., and Kroemer, G. (2004) The pathophysiology of mitochondrial cell death. *Science* **305**, 626–629
194. Labbé, K., and Saleh, M. (2008) Cell death in the host response to infection. *Cell Death Differ* **15**, 1339–1349
195. Acehan, D., Jiang, X., Morgan, D. G., Heuser, J. E., Wang, X., and Akey, C. W. (2002) Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Molecular Cell* **9**, 423–432
196. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479–489

197. Rodriguez, J., and Lazebnik, Y. (1999) Caspase-9 and APAF-1 form an active holoenzyme. *Genes Dev.* **13**, 3179–3184
198. Zou, H., Li, Y., Liu, X., and Wang, X. (1999) An APAF-1· cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* **274**, 11549–11556
199. Saleh, A., Srinivasula, S. M., Acharya, S., Fishel, R., and Alnemri, E. S. (1999) Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. *J Biol Chem* **274**, 17941–17945
200. Suda, T., Takahashi, T., Golstein, P., and Nagata, S. (1993) Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* **75**, 1169–1178
201. Vandenabeele, P., Declercq, W., Beyaert, R., and Fiers, W. (1995) Two tumour necrosis factor receptors: structure and function. *Trends in cell biology* **5**, 392
202. Nagata, S., and Golstein, P. (1995) The Fas death factor. *Science* **267**, 1449–1456
203. Tartaglia, L. A., and Goeddel, D. V. (1992) Two TNF receptors. *Immunol. Today* **13**, 151–153
204. Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* **66**, 233–243
205. Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H., and Peter, M. E. (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J* **14**, 5579–5588
206. Scott, F. L., Stec, B., Pop, C., Dobaczewska, M. K., Lee, J. J., Monosov, E., Robinson, H., Salvesen, G. S., Schwarzenbacher, R., and Riedl, S. J. (2009) The Fas-FADD death domain complex structure unravels signalling by receptor clustering. *Nature* **457**, 1019–1022
207. Chinnaiyan, A. M., Tepper, C. G., Seldin, M. F., O'Rourke, K., Kischkel, F. C., Hellbardt, S., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. *J Biol Chem* **271**, 4961–4965
208. Peter, M. E., and Krammer, P. H. (2003) The CD95 (APO-1/Fas) DISC and beyond. *Cell Death Differ* **10**, 26–35
209. Martin, D. A., Siegel, R. M., Zheng, L., and Lenardo, M. J. (1998) Membrane oligomerization and cleavage activates the caspase-8 (FLICE/MACHalpha1) death signal. *J Biol Chem* **273**, 4345–4349
210. Hirata, H., Takahashi, A., Kobayashi, S., Yonehara, S., Sawai, H., Okazaki, T., Yamamoto, K., and Sasada, M. (1998) Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. *The Journal of experimental medicine* **187**, 587–600
211. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998) Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* **17**, 1675–1687
212. Gonzalvez, F., Schug, Z. T., Houtkooper, R. H., MacKenzie, E. D., Brooks, D. G., Wanders, R. J. A., Petit, P. X., Vaz, F. M., and Gottlieb, E. (2008)

Cardiolipin provides an essential activating platform for caspase-8 on mitochondria. *The Journal of Cell Biology* **183**, 681–696
213. Schug, Z. T., Gonzalvez, F., Houtkooper, R. H., Vaz, F. M., and Gottlieb, E. (2011) BID is cleaved by caspase-8 within a native complex on the mitochondrial membrane. *Cell Death Differ* **18**, 538–548
214. Kantari, C., and Walczak, H. (2011) Caspase-8 and bid: caught in the act between death receptors and mitochondria. *Biochim Biophys Acta* **1813**, 558–563
215. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391**, 43–50
216. Wyllie, A. H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284**, 555–556
217. Nagata, S. (2000) Apoptotic DNA fragmentation. *Experimental cell research* **256**, 12–18
218. Rao, L., Perez, D., and White, E. (1996) Lamin proteolysis facilitates nuclear events during apoptosis. *The Journal of Cell Biology* **135**, 1441–1455
219. Buendia, B., Santa-Maria, A., and Courvalin, J. C. (1999) Caspase-dependent proteolysis of integral and peripheral proteins of nuclear membranes and nuclear pore complex proteins during apoptosis. *J Cell Sci* **112 (Pt 11)**, 1743–1753
220. Wen, L. P., Fahrni, J. A., Troie, S., Guan, J. L., Orth, K., and Rosen, G. D. (1997) Cleavage of focal adhesion kinase by caspases during apoptosis. *J Biol Chem* **272**, 26056–26061
221. Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K., McGarry, T. J., Kirschner, M. W., Koths, K., Kwiatkowski, D. J., and Williams, L. T. (1997) Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* **278**, 294–298
222. Hengartner, M. O. (2000) The biochemistry of apoptosis. *Nature* **407**, 770–776
223. Rudel, T., and Bokoch, G. M. (1997) Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. *Science* **276**, 1571–1574
224. Coleman, M. L., Sahai, E. A., Yeo, M., Bosch, M., Dewar, A., and Olson, M. F. (2001) Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nat. Cell Biol.* **3**, 339–345
225. Sebbagh, M., Renvoizé, C., Hamelin, J., Riché, N., Bertoglio, J., and Bréard, J. (2001) Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. *Nat. Cell Biol.* **3**, 346–352
226. Leverrier, Y., and Ridley, A. J. (2001) Apoptosis: caspases orchestrate the ROCK "n" bleb. *Nat. Cell Biol.* **3**, E91–3
227. Messmer, U. K., and Pfeilschifter, J. (2000) New insights into the mechanism for clearance of apoptotic cells. *Bioessays* **22**, 878–881
228. Daleke, D. L., and Lyles, J. V. (2000) Identification and purification of aminophospholipid flippases. *Biochim Biophys Acta* **1486**, 108–127
229. Savill, J., and Fadok, V. (2000) Corpse clearance defines the meaning of cell death. *Nature* **407**, 784–788
230. Green, D. R., and Beere, H. M. (2000) Apoptosis. Gone but not forgotten. *Nature* **405**, 28–29

231. Roche, S., Koegl, M., Barone, M. V., Roussel, M. F., and Courtneidge, S. A. (1995) DNA synthesis induced by some but not all growth factors requires Src family protein tyrosine kinases. *Mol Cell Biol* **15**, 1102–1109
232. Summy, J. M., and Gallick, G. E. (2006) Treatment for advanced tumors: SRC reclaims center stage. *Clin. Cancer Res.* **12**, 1398–1401
233. Roche, S., Fumagalli, S., and Courtneidge, S. A. (1995) Requirement for Src family protein tyrosine kinases in G2 for fibroblast cell division. *Science* **269**, 1567–1569
234. Thomas, S. M., and BRUGGE, J. S. (1997) Cellular functions regulated by Src family kinases. *Annual review of cell and developmental biology* **13**, 513–609
235. Cooke, M. P., and Perlmutter, R. M. (1989) Expression of a novel form of the fyn proto-oncogene in hematopoietic cells. *New Biol.* **1**, 66–74
236. Corey, S. J., Dombrosky-Ferlan, P. M., Zuo, S., Krohn, E., Donnenberg, A. D., Zorich, P., Romero, G., Takata, M., and Kurosaki, T. (1998) Requirement of Src kinase Lyn for induction of DNA synthesis by granulocyte colony-stimulating factor. *J Biol Chem* **273**, 3230–3235
237. STEHELIN, D., VARMUS, H. E., BISHOP, J. M., and VOGT, P. K. (1976) DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature* **260**, 170–173
238. Sicheri, F., Moarefi, I., and Kuriyan, J. (1997) Crystal structure of the Src family tyrosine kinase Hck. *Nature* **385**, 602–609
239. Parker, R. C., Varmus, H. E., and Michael Bishop, J. (1984) Expression of v-src and chicken c-src in rat cells demonstrates qualitative differences between pp60v-src and pp60c-src. *Cell* **37**, 131–139
240. Shalloway, D., Coussens, P. M., and Yaciuk, P. (1984) Overexpression of the c-src protein does not induce transformation of NIH 3T3 cells. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 7071–7075
241. Williams, J. C., Weijland, A., Gonfloni, S., Thompson, A., Courtneidge, S. A., Superti-Furga, G., and Wierenga, R. K. (1997) The 2.35 A crystal structure of the inactivated form of chicken Src: a dynamic molecule with multiple regulatory interactions. *J Mol Biol* **274**, 757–775
242. Iba, H., Takeya, T., Cross, F. R., Hanafusa, T., and Hanafusa, H. (1984) Rous sarcoma virus variants that carry the cellular src gene instead of the viral src gene cannot transform chicken embryo fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 4424–4428
243. Xu, W., Harrison, S. C., and Eck, M. J. (1997) Three-dimensional structure of the tyrosine kinase c-Src. *Nature* **385**, 595–602
244. Johnson, P. J., Coussens, P. M., Danko, A. V., and Shalloway, D. (1985) Overexpressed pp60c-src can induce focus formation without complete transformation of NIH 3T3 cells. *Mol Cell Biol* **5**, 1073–1083
245. Xu, W., Doshi, A., Lei, M., Eck, M. J., and Harrison, S. C. (1999) Crystal structures of c-Src reveal features of its autoinhibitory mechanism. *Molecular Cell* **3**, 629–638
246. Schindler, T., Sicheri, F., Pico, A., Gazit, A., Levitzki, A., and Kuriyan, J. (1999) Crystal structure of Hck in complex with a Src family-selective tyrosine kinase inhibitor. *Molecular Cell* **3**, 639–648

247. Luttrell, D. K., Luttrell, L. M., and Parsons, S. J. (1988) Augmented mitogenic responsiveness to epidermal growth factor in murine fibroblasts that overexpress pp60c-src. *Mol Cell Biol* **8**, 497–501
248. BRUGGE, J. S., and ERIKSON, R. L. (1977) Identification of a transformation-specific antigen induced by an avian sarcoma virus. *Nature* **269**, 346–348
249. Bjorge, J. D., Pang, A., and Fujita, D. J. (2000) Identification of protein-tyrosine phosphatase 1B as the major tyrosine phosphatase activity capable of dephosphorylating and activating c-Src in several human breast cancer cell lines. *J Biol Chem* **275**, 41439–41446
250. Collett, M. S., and ERIKSON, R. L. (1978) Protein kinase activity associated with the avian sarcoma virus src gene product. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 2021–2024
251. Wilson, L. K., Luttrell, D. K., Parsons, J. T., and Parsons, S. J. (1989) pp60c-src tyrosine kinase, myristylation, and modulatory domains are required for enhanced mitogenic responsiveness to epidermal growth factor seen in cells overexpressing c-src. *Mol Cell Biol* **9**, 1536–1544
252. Levinson, A. D., Oppermann, H., Levintow, L., VARMUS, H. E., and BISHOP, J. M. (1978) Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. *Cell* **15**, 561–572
253. Eckhart, W., Hutchinson, M. A., and Hunter, T. (1979) An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. *Cell* **18**, 925–933
254. Hunter, T., and Sefton, B. M. (1980) Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 1311–1315
255. Collett, M. S., Brugge, J. S., and ERIKSON, R. L. (1978) Characterization of a normal avian cell protein related to the avian sarcoma virus transforming gene product. *Cell* **15**, 1363–1369
256. Oppermann, H., Levinson, A. D., VARMUS, H. E., Levintow, L., and BISHOP, J. M. (1979) Uninfected vertebrate cells contain a protein that is closely related to the product of the avian sarcoma virus transforming gene (src). *Proceedings of the National Academy of Sciences of the United States of America* **76**, 1804–1808
257. Burnham, M. R., Bruce-Staskal, P. J., Harte, M. T., Weidow, C. L., Ma, A., Weed, S. A., and Bouton, A. H. (2000) Regulation of c-SRC activity and function by the adapter protein CAS. *Mol Cell Biol* **20**, 5865–5878
258. Boggon, T. J., and Eck, M. J. (2004) Structure and regulation of Src family kinases. *Oncogene* **23**, 7918–7927
259. Parsons, S. J., and Parsons, J. T. (2004) Src family kinases, key regulators of signal transduction. *Oncogene* **23**, 7906–7909
260. Geahlen, R. L., Handley, M. D., and Harrison, M. L. (2004) Molecular interdiction of Src-family kinase signaling in hematopoietic cells. *Oncogene* **23**, 8024–8032
261. Yeatman, T. J. (2004) A renaissance for SRC. *Nat. Rev. Cancer* **4**, 470–480
262. Koegl, M., Zlatkine, P., Ley, S. C., Courtneidge, S. A., and Magee, A. I. (1994) Palmitoylation of multiple Src-family kinases at a homologous N-terminal motif. *Biochem J* **303 (Pt 3)**, 749–753

263. Resh, M. D. (1999) Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim Biophys Acta* **1451**, 1–16
264. Wolven, A., Okamura, H., Rosenblatt, Y., and Resh, M. D. (1997) Palmitoylation of p59fyn is reversible and sufficient for plasma membrane association. *Mol. Biol. Cell* **8**, 1159–1173
265. Tice, D. A., Biscardi, J. S., Nickles, A. L., and Parsons, S. J. (1999) Mechanism of biological synergy between cellular Src and epidermal growth factor receptor. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 1415–1420
266. Johnson, D. R., Bhatnagar, R. S., Knoll, L. J., and Gordon, J. I. (1994) Genetic and biochemical studies of protein N-myristoylation. *Annu. Rev. Biochem.* **63**, 869–914
267. DeMali, K. A., Godwin, S. L., Soltoff, S. P., and Kazlauskas, A. (1999) Multiple roles for Src in a PDGF-stimulated cell. *Experimental cell research* **253**, 271–279
268. Paige, L. A., Nadler, M. J., Harrison, M. L., Cassady, J. M., and Geahlen, R. L. (1993) Reversible palmitoylation of the protein-tyrosine kinase p56lck. *J Biol Chem* **268**, 8669–8674
269. Bowman, T., Broome, M. A., Sinibaldi, D., Wharton, W., Pledger, W. J., Sedivy, J. M., Irby, R., Yeatman, T., Courtneidge, S. A., and Jove, R. (2001) Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 7319–7324
270. Kypta, R. M., Goldberg, Y., Ulug, E. T., and Courtneidge, S. A. (1990) Association between the PDGF receptor and members of the src family of tyrosine kinases. *Cell* **62**, 481–492
271. Muthuswamy, S. K., Siegel, P. M., Dankort, D. L., Webster, M. A., and Muller, W. J. (1994) Mammary tumors expressing the neu proto-oncogene possess elevated c-Src tyrosine kinase activity. *Mol Cell Biol* **14**, 735–743
272. Landgren, E., Blume-Jensen, P., Courtneidge, S. A., and Claesson-Welsh, L. (1995) Fibroblast growth factor receptor-1 regulation of Src family kinases. *Oncogene* **10**, 2027–2035
273. Courtneidge, S. A., Dhand, R., Pilat, D., Twamley, G. M., Waterfield, M. D., and Roussel, M. F. (1993) Activation of Src family kinases by colony stimulating factor-1, and their association with its receptor. *EMBO J* **12**, 943–950
274. Mao, W., Irby, R., Coppola, D., Fu, L., Wloch, M., Turner, J., Yu, H., Garcia, R., Jove, R., and Yeatman, T. J. (1997) Activation of c-Src by receptor tyrosine kinases in human colon cancer cells with high metastatic potential. *Oncogene* **15**, 3083–3090
275. Gauld, S. B., and Cambier, J. C. (2004) Src-family kinases in B-cell development and signaling. *Oncogene* **23**, 8001–8006
276. Ren, R., Mayer, B. J., Cicchetti, P., and Baltimore, D. (1993) Identification of a ten-amino acid proline-rich SH3 binding site. *Science* **259**, 1157–1161
277. Martin, G. S. (2004) The road to Src. *Oncogene* **23**, 7910–7917
278. Waksman, G., Kominos, D., Robertson, S. C., Pant, N., Baltimore, D., Birge, R.

B., Cowburn, D., Hanafusa, H., Mayer, B. J., Overduin, M., Resh, M. D., Rios, C. B., Silverman, L., and Kuriyan, J. (1992) Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides. *Nature* **358**, 646–653

279. Tatosyan, A. G., and Mizenina, O. A. (2000) Kinases of the Src family: structure and functions. *Biochemistry Mosc.* **65**, 49–58
280. Moran, M. F., Koch, C. A., Anderson, D., Ellis, C., England, L., Martin, G. S., and Pawson, T. (1990) Src homology region 2 domains direct protein-protein interactions in signal transduction. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 8622–8626
281. Matsuda, M., Mayer, B. J., Fukui, Y., and Hanafusa, H. (1990) Binding of transforming protein, P47gag-crk, to a broad range of phosphotyrosine-containing proteins. *Science* **248**, 1537–1539
282. Roussel, R. R., Brodeur, S. R., Shalloway, D., and Laudano, A. P. (1991) Selective binding of activated pp60c-src by an immobilized synthetic phosphopeptide modeled on the carboxyl terminus of pp60c-src. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 10696–10700
283. Liu, X., Brodeur, S. R., Gish, G., Songyang, Z., Cantley, L. C., Laudano, A. P., and Pawson, T. (1993) Regulation of c-Src tyrosine kinase activity by the Src SH2 domainv.
284. Murphy, S. M., Bergman, M., and Morgan, D. O. (1993) Suppression of c-Src activity by C-terminal Src kinase involves the c-Src SH2 and SH3 domains: analysis with Saccharomyces cerevisiae. *Mol Cell Biol* **13**, 5290–5300
285. Superti-Furga, G., Fumagalli, S., Koegl, M., Courtneidge, S. A., and Draetta, G. (1993) Csk inhibition of c-Src activity requires both the SH2 and SH3 domains of Src. *EMBO J* **12**, 2625–2634
286. Cooper, J. A., Gould, K. L., Cartwright, C. A., and Hunter, T. (1986) Tyr527 is phosphorylated in pp60c-src: implications for regulation. *Science* **231**, 1431–1434
287. Takeya, T., and Hanafusa, H. (1983) Structure and sequence of the cellular gene homologous to the RSV src gene and the mechanism for generating the transforming virus. *Cell* **32**, 881–890
288. Brown, M. T., and Cooper, J. A. (1996) Regulation, substrates and functions of src. *Biochim Biophys Acta* **1287**, 121
289. Martin, G. S. (2001) The hunting of the Src. *Nature Reviews Molecular Cell Biology* **2**, 467–475
290. Luttrell, L. M., Ferguson, S. S., Daaka, Y., Miller, W. E., Maudsley, S., Rocca, Della, G. J., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G., and Lefkowitz, R. J. (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* **283**, 655–661
291. Wong, B. R., Besser, D., Kim, N., Arron, J. R., Vologodskaia, M., Hanafusa, H., and Choi, Y. (1999) TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. *Molecular Cell* **4**, 1041–1049
292. Maller, J. L. (2001) The elusive progesterone receptor in Xenopus oocytes.

Proceedings of the National Academy of Sciences of the United States of America **98**, 8–10
293. Okada, M., and Nakagawa, H. (1989) A protein tyrosine kinase involved in regulation of pp60c-src function. *J Biol Chem* **264**, 20886–20893
294. Nada, S., Okada, M., MacAuley, A., Cooper, J. A., and Nakagawa, H. (1991) Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60c-src. *Nature* **351**, 69–72
295. Veillette, A., Latour, S., and Davidson, D. (2002) Negative regulation of immunoreceptor signaling. *Annu. Rev. Immunol.* **20**, 669–707
296. Goi, T., Shipitsin, M., Lu, Z., Foster, D. A., Klinz, S. G., and Feig, L. A. (2000) An EGF receptor/Ral-GTPase signaling cascade regulates c-Src activity and substrate specificity. *EMBO J* **19**, 623–630
297. Zhang, S. Q., Yang, W., Kontaridis, M. I., Bivona, T. G., Wen, G., Araki, T., Luo, J., Thompson, J. A., Schraven, B. L., Philips, M. R., and Neel, B. G. (2004) Shp2 regulates SRC family kinase activity and Ras/Erk activation by controlling Csk recruitment. *Molecular Cell* **13**, 341–355
298. Bromann, P. A., Korkaya, H., and Courtneidge, S. A. (2004) The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene* **23**, 7957–7968
299. Kim, M., Tezuka, T., Tanaka, K., and Yamamoto, T. (2004) Cbl-c suppresses v-Src-induced transformation through ubiquitin-dependent protein degradation. *Oncogene* **23**, 1645–1655
300. Kamei, T., Machida, K., Nimura, Y., Senga, T., Yamada, I., Yoshii, S., Matsuda, S., and Hamaguchi, M. (2000) C-Cbl protein in human cancer tissues is frequently tyrosine phosphorylated in a tumor-specific manner. *Int. J. Oncol.* **17**, 335–339
301. Irby, R. B., Mao, W., Coppola, D., Kang, J., Loubeau, J. M., Trudeau, W., Karl, R., Fujita, D. J., Jove, R., and Yeatman, T. J. (1999) Activating SRC mutation in a subset of advanced human colon cancers. *Nat Genet* **21**, 187–190
302. Sugimura, M., Kobayashi, K., Sagae, S., Nishioka, Y., Ishioka, S., Terasawa, K., Tokino, T., and Kudo, R. (2000) Mutation of the SRC gene in endometrial carcinoma. *Jpn. J. Cancer Res.* **91**, 395–398
303. Johnson, D., Frame, M. C., and Wyke, J. A. (1998) Expression of the v-Src oncoprotein in fibroblasts disrupts normal regulation of the CDK inhibitor p27 and inhibits quiescence. *Oncogene* **16**, 2017–2028
304. Riley, D., Carragher, N. O., Frame, M. C., and Wyke, J. A. (2001) The mechanism of cell cycle regulation by v-Src. *Oncogene* **20**, 5941–5950
305. Wyke, A. W., Frame, M. C., Gillespie, D. A., Chudleigh, A., and Wyke, J. A. (1995) Mitogenesis by v-Src: fluctuations throughout G1 of classical immediate early AP-1 and mitogen-activated protein kinase responses that parallel the need for the oncoprotein. *Cell Growth Differ.* **6**, 1225–1234
306. Frame, M. C. (2002) Src in cancer: deregulation and consequences for cell behaviour. *Biochim Biophys Acta* **1602**, 114–130
307. Chackalaparampil, I., and Shalloway, D. (1988) Altered phosphorylation and activation of PP60c-src during fibroblast mitosis. *Cell* **52**, 801–810
308. Shenoy, S., Choi, J. K., Bagrodia, S., Copeland, T. D., Maller, J. L., and

Shalloway, D. (1989) Purified maturation promoting factor phosphorylates pp60c-src at the sites phosphorylated during fibroblast mitosis. *Cell* **57**, 763–774
309. Morgan, D. O., Kaplan, J. M., BISHOP, J. M., and VARMUS, H. E. (1989) Mitosis-specific phosphorylation of p60c-src by p34cdc2-associated protein kinase. *Cell* **57**, 775–786
310. Bagrodia, S., Taylor, S. J., and Shalloway, D. (1993) Myristylation is required for Tyr-527 dephosphorylation and activation of pp60c-src in mitosis. *Mol Cell Biol* **13**, 1464–1470
311. Twamley-Stein, G. M., Pepperkok, R., Ansorge, W., and Courtneidge, S. A. (1993) The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH 3T3 cells. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 7696–7700
312. Broome, M. A., and Hunter, T. (1996) Requirement for c-Src catalytic activity and the SH3 domain in platelet-derived growth factor BB and epidermal growth factor mitogenic signaling. *J Biol Chem* **271**, 16798–16806
313. Blake, R. A., Broome, M. A., Liu, X., Wu, J., Gishizky, M., Sun, L., and Courtneidge, S. A. (2000) SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. *Mol Cell Biol* **20**, 9018–9027
314. Erpel, T., Alonso, G., Roche, S., and Courtneidge, S. A. (1996) The Src SH3 domain is required for DNA synthesis induced by platelet-derived growth factor and epidermal growth factor. *J Biol Chem* **271**, 16807–16812
315. Maa, M. C., Leu, T. H., McCarley, D. J., Schatzman, R. C., and Parsons, S. J. (1995) Potentiation of epidermal growth factor receptor-mediated oncogenesis by c-Src: implications for the etiology of multiple human cancers. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 6981–6985
316. Lowell, C. A., and Soriano, P. (1996) Knockouts of Src-family kinases: stiff bones, wimpy T cells, and bad memories. *Genes Dev.* **10**, 1845–1857
317. Kilkenny, D. M., Rocheleau, J. V., Price, J., Reich, M. B., and Miller, G. G. (2003) c-Src regulation of fibroblast growth factor-induced proliferation in murine embryonic fibroblasts. *J Biol Chem* **278**, 17448–17454
318. Krystal, G. W., DeBerry, C. S., Linnekin, D., and Litz, J. (1998) Lck associates with and is activated by Kit in a small cell lung cancer cell line: inhibition of SCF-mediated growth by the Src family kinase inhibitor PP1. *Cancer Research* **58**, 4660–4666
319. Hong, L., Munugalavadla, V., and Kapur, R. (2004) c-Kit-mediated overlapping and unique functional and biochemical outcomes via diverse signaling pathways. *Mol Cell Biol* **24**, 1401–1410
320. Boney, C. M., Sekimoto, H., Gruppuso, P. A., and Frackelton, A. R. (2001) Src family tyrosine kinases participate in insulin-like growth factor I mitogenic signaling in 3T3-L1 cells. *Cell Growth Differ.* **12**, 379–386
321. Barone, M. V., and Courtneidge, S. A. (1995) Myc but not Fos rescue of PDGF signalling block caused by kinase-inactive Src. *Nature* **378**, 509–512
322. Bouchard, C., Staller, P., and Eilers, M. (1998) Control of cell proliferation by Myc. *Trends in cell biology* **8**, 202–206
323. Biscardi, J. S., Maa, M. C., Tice, D. A., Cox, M. E., Leu, T. H., and Parsons, S. J. (1999) c-Src-mediated phosphorylation of the epidermal growth factor receptor

on Tyr845 and Tyr1101 is associated with modulation of receptor function. *J Biol Chem* **274**, 8335–8343

324. Olayioye, M. A., Beuvink, I., Horsch, K., Daly, J. M., and Hynes, N. E. (1999) ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. *J Biol Chem* **274**, 17209–17218
325. Chiariello, M., Marinissen, M. J., and Gutkind, J. S. (2001) Regulation of c-myc expression by PDGF through Rho GTPases. *Nat. Cell Biol.* **3**, 580–586
326. Servitja, J.-M., Marinissen, M. J., Sodhi, A., Bustelo, X. R., and Gutkind, J. S. (2003) Rac1 function is required for Src-induced transformation. Evidence of a role for Tiam1 and Vav2 in Rac activation by Src. *J Biol Chem* **278**, 34339–34346
327. Chiu, V. K., Bivona, T., Hach, A., Sajous, J. B., Silletti, J., Wiener, H., Johnson, R. L., Cox, A. D., and Philips, M. R. (2002) Ras signalling on the endoplasmic reticulum and the Golgi. *Nat. Cell Biol.* **4**, 343–350
328. Bivona, T. G., Pérez De Castro, I., Ahearn, I. M., Grana, T. M., Chiu, V. K., Lockyer, P. J., Cullen, P. J., Pellicer, A., Cox, A. D., and Philips, M. R. (2003) Phospholipase Cgamma activates Ras on the Golgi apparatus by means of RasGRP1. *Nature* **424**, 694–698
329. Li, W., Yu, J. C., Michieli, P., Beeler, J. F., Ellmore, N., Heidaran, M. A., and Pierce, J. H. (1994) Stimulation of the platelet-derived growth factor beta receptor signaling pathway activates protein kinase C-delta. *Mol Cell Biol* **14**, 6727–6735
330. Denning, M. F., Dlugosz, A. A., Threadgill, D. W., Magnuson, T., and Yuspa, S. H. (1996) Activation of the epidermal growth factor receptor signal transduction pathway stimulates tyrosine phosphorylation of protein kinase C delta. *J Biol Chem* **271**, 5325–5331
331. Blake, R. A., Garcia-Paramio, P., Parker, P. J., and Courtneidge, S. A. (1999) Src promotes PKCdelta degradation. *Cell Growth Differ.* **10**, 231–241
332. Kronfeld, I., Kazimirsky, G., Lorenzo, P. S., Garfield, S. H., Blumberg, P. M., and Brodie, C. (2000) Phosphorylation of protein kinase Cdelta on distinct tyrosine residues regulates specific cellular functions. *J Biol Chem* **275**, 35491–35498
333. Joseloff, E., Cataisson, C., Aamodt, H., Ocheni, H., Blumberg, P., Kraker, A. J., and Yuspa, S. H. (2002) Src family kinases phosphorylate protein kinase C delta on tyrosine residues and modify the neoplastic phenotype of skin keratinocytes. *J Biol Chem* **277**, 12318–12323
334. Johnson, D., Agochiya, M., Samejima, K., Earnshaw, W., Frame, M., and Wyke, J. (2000) Regulation of both apoptosis and cell survival by the v-Src oncoprotein. *Cell Death Differ* **7**, 685–696
335. Frisch, S. M., and Francis, H. (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. *The Journal of Cell Biology* **124**, 619–626
336. Kulik, G., Klippel, A., and Weber, M. J. (1997) Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. *Mol Cell Biol* **17**, 1595–1606
337. Khwaja, A., Rodriguez-Viciana, P., Wennström, S., Warne, P. H., and Downward, J. (1997) Matrix adhesion and Ras transformation both activate a

phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J* **16**, 2783–2793

338. Coll, M. L., Rosen, K., Ladeda, V., and Filmus, J. (2002) Increased Bcl-xL expression mediates v-Src-induced resistance to anoikis in intestinal epithelial cells. *Oncogene* **21**, 2908–2913
339. Bromberg, J. F., Horvath, C. M., Besser, D., Lathem, W. W., and Darnell, J. E. (1998) Stat3 activation is required for cellular transformation by v-src. *Mol Cell Biol* **18**, 2553–2558
340. Frame, M. C. (2004) Newest findings on the oldest oncogene; how activated src does it. *J Cell Sci* **117**, 989–998
341. Windham, T. C., Parikh, N. U., Siwak, D. R., Summy, J. M., McConkey, D. J., Kraker, A. J., and Gallick, G. E. (2002) Src activation regulates anoikis in human colon tumor cell lines. *Oncogene* **21**, 7797–7807
342. Bouchard, V., Demers, M. J., Thibodeau, S., Laquerre, V., Fujita, N., Tsuruo, T., Beaulieu, J. F., Gauthier, R., Vézina, A., and Villeneuve, L. (2007) Fak/Src signaling in human intestinal epithelial cell survival and anoikis: Differentiation state-specific uncoupling with the PI3-K/Akt-1 and MEK/Erk pathways. *J. Cell. Physiol.* **212**, 717–728
343. Encinas, M., Tansey, M. G., Tsui-Pierchala, B. A., Comella, J. X., Milbrandt, J., and Johnson, E. M. (2001) c-Src is required for glial cell line-derived neurotrophic factor (GDNF) family ligand-mediated neuronal survival via a phosphatidylinositol-3 kinase (PI-3K)-dependent pathway. *J. Neurosci.* **21**, 1464–1472
344. Iravani, S., Mao, W., Fu, L., Karl, R., Yeatman, T., Jove, R., and Coppola, D. (1998) Elevated c-Src protein expression is an early event in colonic neoplasia. *Lab. Invest.* **78**, 365–371
345. Talamonti, M. S., Roh, M. S., Curley, S. A., and Gallick, G. E. (1993) Increase in activity and level of pp60c-src in progressive stages of human colorectal cancer. *Journal of Clinical Investigation* **91**, 53–60
346. Termuhlen, P. M., Curley, S. A., Talamonti, M. S., Saboorian, M. H., and Gallick, G. E. (1993) Site-specific differences in pp60c-src activity in human colorectal metastases. *J. Surg. Res.* **54**, 293–298
347. Brunton, V. G., Ozanne, B. W., Paraskeva, C., and Frame, M. C. (1997) A role for epidermal growth factor receptor, c-Src and focal adhesion kinase in an in vitro model for the progression of colon cancer. *Oncogene* **14**, 283–293
348. Carragher, N. O., Westhoff, M. A., Fincham, V. J., Schaller, M. D., and Frame, M. C. (2003) A novel role for FAK as a protease-targeting adaptor protein: regulation by p42 ERK and Src. *Curr Biol* **13**, 1442–1450
349. Carragher, N. O., and Frame, M. C. (2002) Calpain: a role in cell transformation and migration. *Int J Biochem Cell Biol* **34**, 1539–1543
350. Avizienyte, E., Wyke, A. W., Jones, R. J., McLean, G. W., Westhoff, M. A., Brunton, V. G., and Frame, M. C. (2002) Src-induced de-regulation of E-cadherin in colon cancer cells requires integrin signalling. *Nat. Cell Biol.* **4**, 632–638
351. Fincham, V. J., and Frame, M. C. (1998) The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these

structures during cell motility. *EMBO J* **17**, 81–92
352. Zou, J. X., Liu, Y., Pasquale, E. B., and Ruoslahti, E. (2002) Activated SRC oncogene phosphorylates R-ras and suppresses integrin activity. *J Biol Chem* **277**, 1824–1827
353. Fujita, Y., Krause, G., Scheffner, M., Zechner, D., Leddy, H. E. M., Behrens, J., Sommer, T., and Birchmeier, W. (2002) Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. *Nat. Cell Biol.* **4**, 222–231
354. Schaller, M. D. (2001) Biochemical signals and biological responses elicited by the focal adhesion kinase. *Biochim Biophys Acta* **1540**, 1–21
355. Webb, D. J., Donais, K., Whitmore, L. A., Thomas, S. M., Turner, C. E., Parsons, J. T., and Horwitz, A. F. (2004) FAK–Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat. Cell Biol.* **6**, 154–161
356. Schaller, M. D., and Parsons, J. T. (1995) pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. *Mol Cell Biol* **15**, 2635–2645
357. Owens, L. V., Xu, L., Craven, R. J., Dent, G. A., Weiner, T. M., Kornberg, L., Liu, E. T., and Cance, W. G. (1995) Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. *Cancer Research* **55**, 2752–2755
358. Lin, P. H., Shenoy, S., Galitski, T., and Shalloway, D. (1995) Transformation of mouse cells by wild-type mouse c-Src. *Oncogene* **10**, 401–405
359. Tanaka, A., and Fujita, D. J. (1986) Expression of a molecularly cloned human c-src oncogene by using a replication-competent retroviral vector. *Mol Cell Biol* **6**, 3900–3909
360. Levy, J. B., Iba, H., and Hanafusa, H. (1986) Activation of the transforming potential of p60c-src by a single amino acid change. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 4228–4232
361. Tanaka, A., Salem, M., Eckroade, R. J., and Fujita, D. J. (1990) Characterization of avian retroviruses carrying activated transforming human c-src genes and of steps involved in expression of activated src-PKases in vitro. *Oncogene Res.* **5**, 305–322
362. IRBY, R. B., and Yeatman, T. J. (2000) Role of Src expression and activation in human cancer. *Oncogene* **19**, 5636–5642
363. Hynes, N. E. (2000) Tyrosine kinase signalling in breast cancer. *Breast Cancer Research* **2**, 154
364. Wiener, J. R., Windham, T. C., Estrella, V. C., Parikh, N. U., Thall, P. F., Deavers, M. T., Bast, R. C., Mills, G. B., and Gallick, G. E. (2003) Activated SRC protein tyrosine kinase is overexpressed in late-stage human ovarian cancers. *Gynecol. Oncol.* **88**, 73–79
365. Masaki, T., Okada, M., Shiratori, Y., Rengifo, W., Matsumoto, K., Maeda, S., Kato, N., Kanai, F., Komatsu, Y., Nishioka, M., and Omata, M. (1998) pp60c-src activation in hepatocellular carcinoma of humans and LEC rats. *Hepatology* **27**, 1257–1264
366. Masaki, T., Okada, M., Tokuda, M., Shiratori, Y., Hatase, O., Shirai, M., Nishioka, M., and Omata, M. (1999) Reduced C-terminal Src kinase (Csk) activities in hepatocellular carcinoma. *Hepatology* **29**, 379–384

367. Cam, W. R., Masaki, T., Shiratori, Y., Kato, N., Ikenoue, T., Okamoto, M., Igarashi, K., Sano, T., and Omata, M. (2001) Reduced C-terminal Src kinase activity is correlated inversely with pp60(c-src) activity in colorectal carcinoma. *Cancer* **92**, 61–70
368. Park, J., and Cartwright, C. A. (1995) Src activity increases and Yes activity decreases during mitosis of human colon carcinoma cells. *Mol Cell Biol* **15**, 2374–2382
369. Jones, R. J., Avizienyte, E., Wyke, A. W., Owens, D. W., Brunton, V. G., and Frame, M. C. (2002) Elevated c-Src is linked to altered cell-matrix adhesion rather than proliferation in KM12C human colorectal cancer cells. *Br. J. Cancer* **87**, 1128–1135
370. Yeatman, T. J., IRBY, R. B., Mao, W., Coppola, D., Kang, J., Loubeau, J. M., Trudeau, W., Karl, R., Fujita, D. J., and Jove, R. (1999) Activating SRC mutation in a subset of advanced human colon cancers - Nature Genetics. *Nat Genet* **21**, 187–190
371. Vaux, D. L., Cory, S., and Adams, J. M. (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* **335**, 440–442
372. Tsujimoto, Y., and Shimizu, S. (2000) Bcl-2 family: life-or-death switch. *FEBS Letters* **466**, 6–10
373. Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**, 609–619
374. Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995) Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* **80**, 285–291
375. Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon, H. S., Shuker, S. B., Chang, B. S., Minn, A. J., Thompson, C. B., and Fesik, S. W. (1997) Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. *Science* **275**, 983–986
376. Huang, D. C., Adams, J. M., and Cory, S. (1998) The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4. *EMBO J* **17**, 1029–1039
377. Chittenden, T., Flemington, C., Houghton, A. B., Ebb, R. G., Gallo, G. J., Elangovan, B., Chinnadurai, G., and Lutz, R. J. (1995) A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. *EMBO J* **14**, 5589–5596
378. Tsujimoto, Y., and Shimizu, S. (2000) VDAC regulation by the Bcl-2 family of proteins. *Cell Death Differ* **7**, 1174–1181
379. Zong, W. X., Lindsten, T., Ross, A. J., MacGregor, G. R., and Thompson, C. B. (2001) BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev.* **15**, 1481–1486
380. Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., and Korsmeyer, S. J. (2001) BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Molecular Cell* **8**, 705–711

381. Puthalakath, H., Villunger, A., O'Reilly, L. A., Beaumont, J. G., Coultas, L., Cheney, R. E., Huang, D. C., and Strasser, A. (2001) Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science* **293**, 1829–1832
382. Puthalakath, H., Huang, D. C., O'Reilly, L. A., King, S. M., and Strasser, A. (1999) The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Molecular Cell* **3**, 287–296
383. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Serine Phosphorylation of Death Agonist BAD in Response to Survival Factor Results in Binding to 14-3-3 Not BCL-X L. *Cell* **87**, 619–628
384. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Serine Phosphorylation of Death Agonist BAD in Response to Survival Factor Results in Binding to 14-3-3 Not BCL-X L. *Cell* **87**, 619–628
385. Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000) Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288**, 1053–1058
386. Yu, J., Zhang, L., Hwang, P. M., Kinzler, K. W., and Vogelstein, B. (2001) PUMA induces the rapid apoptosis of colorectal cancer cells. *Molecular Cell* **7**, 673–682
387. Nakano, K., and Vousden, K. H. (2001) PUMA, a novel proapoptotic gene, is induced by p53. *Molecular Cell* **7**, 683–694
388. Imaizumi, K., Tsuda, M., Imai, Y., Wanaka, A., Takagi, T., and Tohyama, M. (1997) Molecular cloning of a novel polypeptide, DP5, induced during programmed neuronal death. *J Biol Chem* **272**, 18842–18848
389. Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J. C. (1999) Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *The Journal of Cell Biology* **144**, 891–901
390. Wang, K., Yin, X. M., Chao, D. T., Milliman, C. L., and Korsmeyer, S. J. (1996) BID: a novel BH3 domain-only death agonist. *Genes Dev.* **10**, 2859–2869
391. Wolter, K. G., Hsu, Y. T., Smith, C. L., Nechushtan, A., Xi, X. G., and Youle, R. J. (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. *The Journal of Cell Biology* **139**, 1281–1292
392. Nomura, M., Shimizu, S., Ito, T., Narita, M., Matsuda, H., and Tsujimoto, Y. (1999) Apoptotic cytosol facilitates Bax translocation to mitochondria that involves cytosolic factor regulated by Bcl-2. *Cancer Research* **59**, 5542–5548
393. Gross, A., Jockel, J., Wei, M. C., and Korsmeyer, S. J. (1998) Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *EMBO J* **17**, 3878–3885
394. Martinou, J. C., and Green, D. R. (2001) Breaking the mitochondrial barrier. *Nature Reviews Molecular Cell Biology* **2**, 63–67
395. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999) BCL-2 family members and the mitochondria in apoptosis. *Genes Dev.* **13**, 1899–1911
396. Hsu, Y. T., and Youle, R. J. (1998) Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations. *J*

Biol Chem **273**, 10777–10783
397. Nechushtan, A., Smith, C. L., Lamensdorf, I., Yoon, S. H., and Youle, R. J. (2001) Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. *The Journal of Cell Biology* **153**, 1265–1276
398. Antonsson, B., Montessuit, S., Sanchez, B., and Martinou, J. C. (2001) Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. *J Biol Chem* **276**, 11615–11623
399. Mikhailov, V., Mikhailova, M., Pulkrabek, D. J., Dong, Z., Venkatachalam, M. A., and Saikumar, P. (2001) Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane. *J Biol Chem* **276**, 18361–18374
400. Haldar, S., Jena, N., and Croce, C. M. (1995) Inactivation of Bcl-2 by phosphorylation. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 4507–4511
401. Cheng, E. H., Kirsch, D. G., Clem, R. J., Ravi, R., Kastan, M. B., Bedi, A., Ueno, K., and Hardwick, J. M. (1997) Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science* **278**, 1966–1968
402. Tsujimoto, Y. (1998) Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? *Genes to Cells* **3**, 697–707
403. Luciano, F., Ricci, J. E., and Auberger, P. (2001) Cleavage of Fyn and Lyn in their N-terminal unique regions during induction of apoptosis: a new mechanism for Src kinase regulation. *Oncogene* **20**, 4935–4941
404. Xue, Y., Ren, J., Gao, X., Jin, C., Wen, L., and Yao, X. (2008) GPS 2.0, a tool to predict kinase-specific phosphorylation sites in hierarchy. *Mol. Cell Proteomics* **7**, 1598–1608
405. Zheng, X. M., Wang, Y., and Pallen, C. J. (1992) Cell transformation and activation of pp60c-src by overexpression of a protein tyrosine phosphatase. *Nature* **359**, 336–339
406. Jung, E. J., and Kim, C. W. (2002) Interaction between chicken protein tyrosine phosphatase 1 (CPTP1)-like rat protein phosphatase 1 (PTP1) and p60(v-src) in v-src-transformed Rat-1 fibroblasts. *Exp. Mol. Med.* **34**, 476–480
407. Smart, J. E., and Oppermann, H. (1981)
408. Luciano, F., Herrant, M., Jacquel, A., Ricci, J. E., and Auberger, P. (2003) The p54 cleaved form of the tyrosine kinase Lyn generated by caspases during BCR-induced cell death in B lymphoma acts as a negative regulator of apoptosis. *FASEB J.* **17**, 711–713
409. Marchetti, S., Gamas, P., Belhacène, N., Grosso, S., Pradelli, L. A., Colosetti, P., Johansen, C., Iversen, L., Deckert, M., Luciano, F., Hofman, P., Ortonne, N., Khemis, A., Mari, B., Ortonne, J.-P., Ricci, J. E., and Auberger, P. (2009) The caspase-cleaved form of LYN mediates a psoriasis-like inflammatory syndrome in mice. *EMBO J* **28**, 2449–2460
410. Kmiecik, T. E., and Shalloway, D. (1987) Activation and suppression of pp60c-src transforming ability by mutation of its primary sites of tyrosine phosphorylation. *Cell* **49**, 65–73
411. Lodyga, M., Bai, X.-H., Mourgeon, E., Han, B., Keshavjee, S., and Liu, M. (2002) Molecular cloning of actin filament-associated protein: a putative adaptor in stretch-induced Src activation. *Am. J. Physiol. Lung Cell Mol. Physiol.* **283**,

L265–74
412. Xu, J., Bai, X.-H., Lodyga, M., Han, B., Xiao, H., Keshavjee, S., Hu, J., Zhang, H., Yang, B. B., and Liu, M. (2007) XB130, a novel adaptor protein for signal transduction. *J Biol Chem* **282**, 16401–16412
413. Hanks, S. K., and Polte, T. R. (1997) Signaling through focal adhesion kinase. *Bioessays* **19**, 137–145
414. Hughes, M. A., Harper, N., Butterworth, M., Cain, K., Cohen, G. M., and MacFarlane, M. (2009) Reconstitution of the death-inducing signaling complex reveals a substrate switch that determines CD95-mediated death or survival. *Molecular Cell* **35**, 265–279
415. Widmann, C. (1998) Caspase-dependent Cleavage of Signaling Proteins during Apoptosis. A TURN-OFF MECHANISM FOR ANTI-APOPTOTIC SIGNALS. *Journal of Biological Chemistry* **273**, 7141–7147
416. Hertog, den, J., Pals, C. E., Peppelenbosch, M. P., Tertoolen, L. G., de Laat, S. W., and Kruijer, W. (1993) Receptor protein tyrosine phosphatase alpha activates pp60c-src and is involved in neuronal differentiation. *EMBO J* **12**, 3789–3798
417. Zambuzzi, W. F., Granjeiro, J. M., Parikh, K., Yuvaraj, S., Peppelenbosch, M. P., and Ferreira, C. V. (2008) Modulation of Src activity by low molecular weight protein tyrosine phosphatase during osteoblast differentiation. *Cell. Physiol. Biochem.* **22**, 497–506
418. Xu, J., Bai, X.-H., Lodyga, M., Han, B., Xiao, H., Keshavjee, S., Hu, J., Zhang, H., Yang, B. B., and Liu, M. (2007) XB130, a novel adaptor protein for signal transduction. *J Biol Chem* **282**, 16401–16412
419. Koren, E., and Torchilin, V. P. (2012) Cell-penetrating peptides: breaking through to the other side. *Trends Mol Med* **18**, 385–393