BIOCHEMICAL CHARACTERIZATION OF DELTA FOSB

APPROVED BY SUPERVISORY COMMITTEE

MEG PHILLIPS

GEORGE DE MARTINO

LISA MONTEGGIA

ERIC NESTLER

DEDICATION

I dedicate this work to my husband, Bo, the seal on my heart.
His Faith strengthens and inspires me every day.

I also dedicate this work to my parents, Pat and Patti Carle, for their love and *endless* support: they pick me up when I stumble and never look back.

BIOCHEMICAL CHARACTERIZATION OF DELTA FOSB

by

TIFFANY CARLE

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

August 2006

BIOCHEMICAL CHARACTERIZATION OF DELTA FOSB

TIFFANY CARLE

ERIC J. NESTLER, M.D., Ph.D.

DeltaFosB, the truncated splice variant of FosB, is an important mediator of the long-term plasticity induced in brain by chronic exposure to many types of stimuli, such as repeated administration of drugs of abuse, stress, or compulsive running. Once induced, DeltaFosB persists in the brain for weeks or months following cessation of the chronic stimulus. In addition, DeltaFosB both activates and represses transcription. The biochemical basis of DeltaFosB's persistent expression and dual transcriptional regulation has remained unknown. Both the enhanced protein stability and transcriptional properties are unique to DeltaFosB, compared to FosB, and are critical for its role in neural plasticity. DeltaFosB lacks the C-terminal 101 amino acids of FosB as a result of alternative splicing. The purpose of this work is to biochemically characterize

DeltaFosB relative to FosB, to determine how truncation of the FosB C-terminus directs its function. Here, I show that the FosB C-terminus contains two destabilizing elements that promote the degradation of FosB by both proteasome dependent and independent mechanisms. Pulse chase experiments of FosB C-terminal truncation mutants indicate that removal of these C-terminal degrons increases the FosB half-life ~5 fold and prevents its proteasome-mediated degradation and ubiquitylation, properties similar to ΔFosB. These data indicate that alterative splicing specifically removes two destabilizing elements from FosB in order to generate a longer-lived transcription factor, DeltaFosB, in response to chronic perturbations to the brain. Truncation of the C-terminus from FosB also results in differing interaction partners for FosB and DeltaFosB that may contribute to the varying functions of each protein. Specifically, using co-immunoprecipitation assays both *in vitro* and *in vivo*, I determined that HDAC1 (histone deacetylase 1) is the preferential binding partner of ΔFosB compared to FosB. These data suggest an intriguing hypothesis that ΔFosB interactions with specific HATs and HDACs may be one mechanism by which ΔFosB mediates both activating and repressive transcriptional activities. DeltaFosB is a unique transcription factor compared to its Fos family members. Truncation of the FosB C-terminal domain liberates DeltaFosB, enabling long-term protein stability and promoting specific interactions with protein partners that are critical for gene regulation important for neural plasticity.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

PUBLICATIONS

Carle, T.L., Alibhai, I.N., Kumar, A., Nestler, E.J. (In revision) Proteasome dependent and independent mechanisms for FosB destabilization: identification of FosB degron domains and implications for ΔFosB stability.

DiNieri, J.A., **Carle, T.L.,** Nestler, E.J., Carlezon, W.A. (Submitted) Bias toward reward in mice with inducible disruption of CREB function within nucleus accumbens.

Berton O., Tsankova N.M., **Carle T.L**., Ulery P.G., Bhonsle A., Barrot M., Neve R.L., and Nestler E.J. (In revision) Induction of ΔFosB in the periaqueductal gray by stress promotes active coping responses.

Peakman M.C., Colby C., Perrotti L.I., Tekumalla P., **Carle T.L**., Ulery P., Chao J., Duman C., Steffen C., Monteggia L., Allen M.R., Stock J.L., Duman R.S., McNeish J.D., Barrot M., Self D.W., Nestler E.J., Schaeffer, E. (2003) Inducible, brain region-specific expression of a dominant negative mutant of c-Jun in transgenic mice decreases sensitivity to cocaine. *Brain Res.* 970, 73-86.

ABSTRACTS

Carle, T.L., Nestler, E.J. (2005) FosB C-terminal residues are necessary for ubiquitylation. *Soc. Neurosci. Abstr.* 451.16.

Carle, T.L., Nestler, E.J. (2004) FosB C-terminus is critical for proteasome-mediated degradation. *Soc. Neurosci. Abstr*. 692.2.

Carle, T.L., Ulery, P.G., Nestler, E.J. (2003) FosB and ΔFosB are differentially degraded. *Soc. Neurosci. Abstr*. 856.12.

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

α	anti
Δ	delta
AAV	Adeno-associted virus
AMC	Suc-Leu-Leu-Val-Tyr-7-amido-methylcoumarin
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AP-1	Activator-protein 1
ß-tubulin	Beta tubulin
BSA	Bovine Serum Albumin
Cdk5	Cyclin-dependent kinase 5
c-Fos	Cellular oncogene Fos
ChIP	Chromatin immunoprecipitation
CK2	Casein kinase 2
CNS	Central nervous system
CREB	Cyclic AMP Response Element Binding Protein
C-terminus	Carboxyl terminus
Cys	Cysteine
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
Dox	Doxycycline
DRB	5,6-dichloro-1-beta-D-ribofuranosyl-benzimidazole

ECS	Electroconvulsive seizure
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FosB	FBJ (Finkel-Biskis-Jinkins) osteosarcoma oncogene B
FRA-1	Fos-related antigen 1
FRA-2	Fos-related antigen 2
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
GluR2	AMPA glutamate receptor subunit 2
HA	Hemagglutinin
HAT	Histone acetyltransferase
HDAC1	Histone deacetylase 1
HS	Horse serum
HSL-RIPA	High salt solution RIPA
Hsc70	Heat shock cognate protein 70
Hsp40	Heat shock protein 40
Hsp70	Heat shock protein 70
Hsp90	Heat shock protein 90
HSV	Herpes simpex virus
IEG	Immediate Early Gene
IP	Immunoprecipitation
KNK437	*N*-Formyl-3,4-methylenedioxy-benzylidine-g-butyrolactam

LSL-RIPA	Low salt solution RIPA
Met	Methionine
MG132	Z-Leu-Leu-Leu-al
NF-κB	Nuclear factor-kappa B
NaB	Sodium butyrate
NAc	Nucleus accumbens
NaCl	Sodium chloride
NIP	Non-immune immunoprecipitation
NMDA	N-methyl-d-asparate
NMDAR1	NMDA repeceptor 1
NP-40	Igepal
NSE	Neuron-specific enolase
N-terminus	Amino terminus
OA	Okadaic acid
OFC	Orbitofrontal cortex
P1	Nuclear fraction (pellet 1)
PAGE	Polyacrylamide Gel Electrophoresis
PC12 cells	Rat adrenal pheochromocytoma cells
PCR	Polymerase Chain Reaction
PEST	Regions rich in proline, glutamate, serine, and threonine
PMA	Phorbol 12-myristate 13-acetate
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2a

PPT-A	Preprotachykinin-A
PTB	Polypyrimidine Tract Binding Protein
q-PCR	Quantitative Polymerase Chain Reaction
RIPA	Radio-immunoprecipitation assay
S3	Cytosolic fraction (supernatant 3)
SDS	Sodium Dodecyl Sulfate
siRNA	Small interfering RNA
SM	Starting material
SRE	Serum Response Element
SRF	Serum Response Factor
tTA	Tetracycline transactivator
TetOp	Tetracycline operon
UTR	Untranslated Region
VTA	Ventral tegmental area
WB	Western blot
WCE	Whole cell extract
YY1	Ying yang 1

CHAPTER 1

INTRODUCTION

In the brain, long-term adaptive responses to the environment require the conversion of extracellular stimuli to discrete intracellular signals. Many of these signals require regulation of gene expression. ΔFosB is a unique transcription factor that is important for the regulation of some long-term modifications in the brain in response to many diverse stimuli, such as drug addiction, stress, and depression.

Activator-Protein 1

ΔFosB is a member of the Fos family of transcription factors, which includes c-Fos, FosB, FRA1 (Fos-related antigen), and FRA2. All Fos proteins possess an N-terminal basic region, a central leucine zipper motif, and a C-terminal transactivation domain. The leucine zipper motif promotes heterodimerization to form the activator protein 1 (AP-1) transcription factor complex (Morgan *et al*, 1995; Chinenov & Kerppola, 2001). This complex binds the AP-1 consensus sequence, TGAC/GCTA, to regulate target genes that contain this sequence in their promoter region (Morgan & Curran, 1991). The AP-1 complex is composed Fos-Jun heterodimers, Jun homodimers, or Fos or Jun heterodimers with one of over 50 different interacting proteins, such as ATF2 (Chinenov & Kerppola, 2001). AP-1 expression is usually transient, with expression of both mRNA and protein levels degrading to basal levels within a few hours. Fos and Jun family genes are defined as immediate early genes (IEGs) because they are rapidly induced in response to stimuli. Rapidly induced genes, such as c-Fos,

were originally identified as cellular IEGs because they are analogous to the IEGs of viruses. These genes are transcribed in the presence of protein synthesis inhibitors, suggesting that the proteins required for their expression in unstimulated cells are activated by post-translational modifications (Curran & Morgan, 1987). Due to their rapid response to stimulation, IEGs and their protein products, such as AP-1, are important messengers that couple extracellular signals to alterations in gene expression and long-term changes in cellular functioning.

Persistent AP-1 complex

Nearly 15 years ago, a study from the Nestler laboratory found that acute treatment of rats with cocaine increased AP-1 binding in the nucleus accumbens (NAc), a brain region implicated in mediating the rewarding effects of drugs of abuse. This effect reverted to normal levels within 8 hrs. In contrast, chronic cocaine administration caused an increase in AP-1 binding that remained elevated for 18 following the last cocaine injection. Surprisingly, AP-1 binding remained elevated well after *c-fos* and *jun* protein and mRNA levels decreased to control levels (Hope, *et al,* 1992). This was the first study that described persistent AP-1 binding that appeared only following chronic drug treatment. In addition, chronic cocaine treatments induced a broad band of 33-37 kD Fos-like proteins, termed *chronic FRAs* (Hope, *et al,* 1994a) (Figure 1-1A).

Using antibodies specific to the amino or carboxy termini of FosB, subsequent studies were able to characterize the *chronic FRAs* and identified them to be isoforms of ΔFosB, the truncated splice variant of FosB (Hope, *et al,* 1994a,b). ΔFosB had been cloned a few years earlier from cultured fibroblasts. It was shown to lack the C-terminal

101 amino acids from full-length FosB as a result of alternative splicing (Nakabeppu & Nathans, 1991). The Nestler laboratory showed that following an acute stimulation, the Fos family of IEGs, including c-Fos, FosB, FRA1/2, and the 33kD ΔFosB isoform, is highly induced in brain, and promptly degraded (Figure 1-1B). In contrast, the 35-37 kD ΔFosB isoforms are only highly detected in brain following chronic treatments, such as repeated stress, repeated electroconvulsive seizure (ECS), or chronic drug treatments (Table 1-1), when the other Fos family members are induced to a much lesser extent. In addition, these isoforms persist in brain for at least several weeks following cessation of the chronic stimulus, unlike all other Fos proteins (Hope, *et al,* 1994a,b; Chen, *et al*, 1997; Hiroi, *et al,* 1997). The hypothesis has been that the 33kD protein isoform of ΔFosB is its native state, and that repeated perturbations to the brain signal post-translational modifications to ΔFosB that both increase its apparent molecular weight from 33kD to 35-37kD and stabilize the protein (Nestler, *et al,* 2001). Indeed, the 37kD isoform persists in brain for weeks, while the 33kD and 35kD isoforms become undetectable.

Because ΔFosB is induced in brain specifically by chronic treatments and remains in these brain regions for long periods of time, we have proposed that ΔFosB acts as a sustained ”molecular switch” that first initiates and then maintains some of the long-term adaptations of the brain in response to chronic perturbations (Nestler, *et al,* 2001; McClung, *et al*, 2004).

Figure 1-1

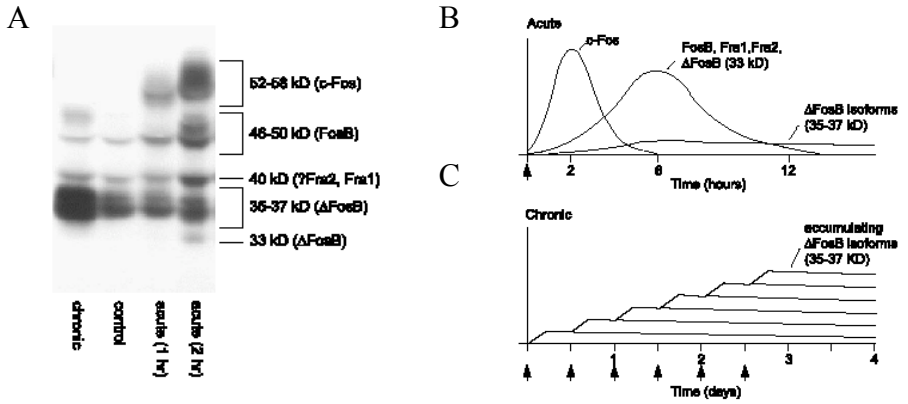


Figure 1-1. Scheme showing the gradual accumulation of ΔFosB versus the rapid and transient induction of other Fos family proteins. (A) The induction of c-Fos, FosB, Fra-1/2, and the 33-kDa isoform of ΔFosB in the nucleus accumbens after an acute cocaine exposure, and the switch to the predominant induction of 35– 37 kDa isoforms of ΔFosB after chronic cocaine administration. (B) Several waves of Fos family proteins are induced by acute administration of any of several stimuli (see Table 1-1). Also induced are biochemically modified isoforms of ΔFosB (35– 37 kDa); they, too, are induced (although at low levels) following an acute stimulus, but persist in brain for long periods due to their stability. (C) With repeated stimulation, each acute stimulus induces a low level of the stable ΔFosB isoforms. This is indicated by the lower set of overlapping lines, which indicate ΔFosB induced by each acute stimulus. The result is gradual increase in the total levels of ΔFosB with repeated stimuli during a course of chronic treatment. This is indicated by the increasing stepped line in the graph. (Adapted from McClung, *et al,* 2004).

The fosB *Gene and Splicing*

The *fosB* gene contains 4 exons that are expressed in FosB mRNA. Exon IV is divided into exons IVa and IVb, separated by a retained intron sequence found in the open reading frame of the final *fosb* transcript (Figure 1-2). Δ*fosB* mRNA is generated by the excision of this intronic sequence, which results in a one-nucleotide frameshift and the generation of a stop codon (TGA) that causes premature termination of ΔFosB translation. Accordingly, protein translated from ΔFosB mRNA lacks the C-terminal 101 amino acids present in full-length FosB (Yen, *et al*, 1991). Recent evidence from our laboratory has suggested that this splicing phenomenon is likely regulated by the polyprimidine tract binding protein (PTB1). Under basal conditions, PTB1 binds the

intronic sequence on the *fosB* pre-mRNA preventing access to the cellular splicing machinery. By this mechanism, full-length FosB mRNA is preferentially generated. Following a chronic stimulation and abundant transcription of *fosB* pre-mRNA, PTB1 is saturated, permitting the splicing machinery access to some proportion of *fosB* pre-mRNA. Unbound pre-mRNA will be spliced at the PTB1 binding site, excising the intronic sequence at exon IV. This excision will generate Δ*fosB* mRNA. By this mechanism, ΔFosB is specifically expressed only following chronic perturbations (Figure 1-2) (Alibhai, *et al,* in preparation).

Figure 1-2

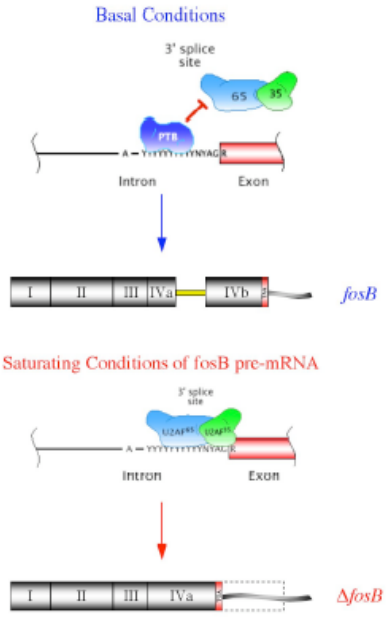


Figure 1-2. Model of *fosB* RNA Splicing. Under basal conditions PTB1 protein binds the majority of the *fosB* pre-mRNA, thereby inhibiting the generation of the Δ*fosB* transcript. When PTB1 protein is saturated with transcript, unbound pre-mRNA is spliced into Δ*fosB*. (Adapted from Alibhai, *et al,* in preparation).

ΔFosB in the Brain

The 35-37 kD isoforms of ΔFosB can be detected in specific brain regions under basal conditions, and a variety of chronic stimuli induce high levels of the proteins in region-specific patterns (Table 1-1). Generally, all drugs of abuse, stress, and natural rewards induce ΔFosB in brain reward regions. The nucleus accumbens (NAc), dorsal striatum, basal forebrain, and prefrontal cortex all receive rich dopaminergic projections from the ventral tegmental area (VTA) and related areas of the midbrain. These neural substrates are together often called the brain reward pathway (Nestler, *et al,* 2001b). ΔFosB is typically found in the NAc and dorsal striatum basally, barely detected in any other region of the CNS, and highly induced in a region-specific manner following chronic perturbations to the brain.

Virtually all drugs of abuse induce ΔFosB in the NAc and dorsal striatum. Chronic forms of stress, such as restraint stress or unpredictable stress, induce ΔFosB in variable regions depending on the type of stress, however, ΔFosB is mostly seen in the prefrontal cortex (Perrotti, *et al,* 2004). The same is true for treatment with chronic electroconvulsive seizure (ECS). Robust ΔFosB is induced in the prefrontal cortex and hippocampus following ECS (Hope, *et al,* 1994b; Hiroi, *et al,* 1998). Natural rewards, such as repeated sexual experience, sucrose drinking, and excessive wheel running, all induce ΔFosB specifically in the NAc (Werme, *et al,* 2002; unpublished observations). Interestingly, prolonged withdrawal from morphine induces ΔFosB in the VTA and locus coeruleus, where chronic treatment of the drug itself does not induce ΔFosB (Nye, *et al,* 1996). This could be a secondary effect due to the stress of opiate withdrawal, a possibility that must still be explored (McClung, *et al,* 2004). ΔFosB has also been

detected following anti-psychotic drug treatments. ΔFosB accumulates primarily in the NAc, dorsal striatum, and pre-cortical regions following treatment with first generation anti-psychotics, such as haloperidol; however, treatment with second generation anti-psychotics, such as clozipine and risperidone causes the induction in solely the frontal cortex (Atkins, *et al,* 1999; McClung, *et al,* 2004).

Table 1-1

Examples of stimuli that induce ΔFosB in brain after chronic exposure

Stimulus	Brain regions[a]
Drugs of abuse	
Cocaine	Nucleus accumbens, dorsal striatum, prefrontal cortex, amygdala, ventral tegmental area
Amphetamine	Nucleus accumbens, dorsal striatum
Morphine	Nucleus accumbens, dorsal striatum
Nicotine	Nucleus accumbens, prefrontal cortex, dorsal striatum
Alcohol	Nucleus accumbens, prefrontal cortex
Phencyclidine	Nucleus accumbens, dorsal striatum
Morphine withdrawal	Locus coeruleus, ventral tegmental area, nucleus accumbens, dorsal striatum
Antidepressant treatments	
ECS	Hippocampus, frontal cortex, parietal cortex, striatum
Tranylcypromine	Hippocampus, frontal cortex
Antipsychotic drugs	
First generation	Nucleus accumbens, dorsal striatum, frontal cortex
Second generation	Frontal cortex
Stress	
Restraint	Prefrontal cortex, nucleus accumbens, amygdala, septum, locus coeruleus
Unpredictable	Prefrontal cortex, nucleus accumbens, amygdala, septum, locus coeruleus
Foot shock	Nucleus accumbens, dorsal raphe
Kainic acid	Hippocampus, striatum
Dopamine denervation	Dorsal striatum

Table 1-1. ΔFosB Mapping. In most cases, a comprehensive mapping of ΔFosB induction in brain has not been carried out. In addition, the Table does not list all brain regions where ΔFosB induction has been reported, but lists examples of the regions that show the most prominent induction. (adapted from McClung, *et al,* 2004).

The above discussion is not an exhaustive list of chronic stimulations that induce ΔFosB in particular brain regions. Another critical question, however, is in which types of cells is ΔFosB expressed in these particular brain regions after each stimulation. Growing evidence suggests that different stimulations selectively induce ΔFosB in different subsets of neurons in the NAc and dorsal striatum. Chronic administration of cocaine, opiates, nicotine, alcohol or excessive wheel running all selectively induce ΔFosB in the dynorphin/substance P-containing subset of medium spiny neurons in the NAc and dorsal striatum, while chronic exposure to anti-psychotic drugs induces ΔFosB preferentially in enkephalin-positive neurons (Moratella, *et al*, 1996; McClung, *et al,* 2004). In addition, very little ΔFosB is detected in interneurons or non-neuronal cells (McClung, *et al,* 2004). Even though many diverse stimuli induce ΔFosB in the same brain regions, the functional consequences may be very different because different cell types are involved and these cell types are known to regulate striatal function in very different ways.

Δ*FosB Model Systems*

To identify a functional role for ΔFosB in behavior, three separate models have been developed over the last decade. Early work examining *fosB* knockout mice revealed the first link between FosB-related proteins and deficient behavioral responses. In one study, *fosB* mutant mice showed increased inherent sensitivity to the locomotor-activating and rewarding effects of cocaine, meaning they appeared "pre-sensitized" to cocaine (Hiroi, *et al,* 1997). However, no further sensitization could be induced in the knockouts. In a second study, *fosB* mutants showed abnormal biochemical and

electrophysiological responses to electroconvulsive seizures. The *fosB* mutants showed delayed tolerance to chronic seizures and absence of regulation of specific NMDA subunits in the frontal cortex (Hiroi, *et al,* 1998). Limitations of these studies prevent further interpretations. As with any traditional knockout system, *fosB* gene products are absent throughout development, in all brain regions, such that their absence could lead to compensatory mechanisms that may complicate interpretations. In addition, since both FosB and ΔFosB are absent in this system, it is impossible to distinguish the precise role of each protein.

To overcome these limitations, transgenic mice were generated that overexpress ΔFosB in specific brain regions of adult mice. The tetracycline gene regulation system was used. This bitransgenic system involves two genes, one that encodes the tetracycline transactivator (tTA, which is inhibited by tetracycline), and a second that encodes ΔFosB downstream of the TetOp promoter. The TetOp promoter is bound by tTA to activate ΔFosB transcription in the absence of tetracycline. The transgene was placed downstream of the neuron-specific enolase (NSE) promoter to obtain region-specific expression in the CNS. Transgene expression is induced specifically in adulthood by raising transgenic pups with doxycyline (a tetracycline derivative) in their drinking water until 6-8 weeks of age. Transgene expression gradually increases following doxycyline removal for another 6-8 weeks. Multiple transgenic mouse lines were generated, and one line, Line A, specifically expressed ΔFosB in dynorphin/substance P-containing medium spiny neurons of the striatum, similar to expression following chronic cocaine administration or excessive wheel running, with much lower levels seen in hippocampus and frontal cortex. A second line, Line B, expressed ΔFosB in both dynorphin/substance

P and enkephalin-expressing striatal neurons, but preferentially in the latter (Kelz, *et al,* 1999).

Finally, one limitation of the transgenic mouse system is gradual accumulation of the transgene once doxycyline is removed. The developmental effects of ΔFosB in specific brain regions over time are not completely understood. In addition, although most ΔFosB is confined to the striatal regions of interest in Lines A and B, hippocampal and cortical regions also express ΔFosB, which may contribute unknown effects to experimental interpretations. Recent studies have utilized viral-mediated gene transfer to locally target ΔFosB expression to specific brain regions (Zachariou, *et al,* 2006; Winstanely, *et al,* in review; Olausson, *et al,* in review; Berton, *et al,* in review). Adeno-associated viral (AAV) or herpes simplex viral (HSV) vectors containing ΔFosB, or a control protein, are delivered bilaterally into NAc or other brain regions of interest to study the behavioral effects of the protein. Following behavioral testing, injection targeting is confirmed by immunohistochemistry. This method has recently become favorable for extremely localized targeting of gene transfer because viral expression is tightly confined to the region of interest following viral injection, while the inducible system is somewhat leaky, with gene expression in more than one brain region. Viral transfer also alleviates the developmental effects that may be caused by a transgene: gene expression is maximal after only 2-3 days for HSV, rather than the 6-8 weeks required gradual induction of a transgene. In addition, long-range experiments can be carried out, as AAV expression can last for months or possibly years, while transgene expression is optimal for only a few weeks. In contrast, the cell-type precision of the transgenic lines cannot be replicated by targeting with viral-mediated gene transfer. The *fosB* knockout

mice, the inducible transgenic system, and viral mediated gene transfer are critical tools for the characterization of ΔFosB neurobiology. Confirmation of findings in two or more of these systems is a powerful method to understanding the ΔFosB phenotype.

The ΔFosB Phenotype

The behavioral phenotype of mice overexpressing ΔFosB in many ways resembles animals after chronic drug exposure. These phenotypes are summarized in Table 1-2. Specifically, these mice show increased locomotor and rewarding responses to cocaine and morphine (Kelz, *et al,* 1999; Zachariou, *et al,* 2006). They show an increased preference for cocaine and morphine in conditioned-place preference paradigms and they will self-administer cocaine at lower doses than littermate controls that do not overexpress ΔFosB (Kelz, *et al,* 1999; Colby, *et al,* 2003). In addition, ΔFosB-expressing mice are less sensitive to the analgesic effects of morphine and develop increased physical dependence on the drug (Zachariou, *et al,* 2006). Most of these effects are specifically observed in the Line A transgenic animals, that express ΔFosB in the dynorphin/substance P positive medium spiny neurons of the NAc. These effects were not observed in Line B animals, with generally opposite effects seen in inducible Δc-Jun expressing transgenic mice. Δc-Jun is a dominant-negative form of c-Jun that antagonizes the transcriptional effects of ΔFosB and other AP-1 transcription factors (Peakman, *et al,* 2003; Zachariou, *et al*, 2006). These data suggest that induction of ΔFosB in dynorphin/substance P + striatal medium spiny neurons may "pre-sensitize" an animal to drugs of abuse and may be sufficient to make an individual more vulnerable to addiction.

Table 1-2

Stimulus	Phenotype
Cocaine	Increased locomotor responses to acute drug administration
	Increased locomotor sensitization to repeated drug administration
	Increased conditioned place preference at lower drug doses
	Increased acquisition of cocaine self-administration at lower drug doses
	Increased incentive for drug in progressive ratio procedure
Morphine	Increased conditioned place preference at lower drug doses
	Increased development of physical dependence and withdrawal
	Decreased analgesic responses, enhanced tolerance
Alcohol	Increased anxiolytic responses
Wheel	Increased wheel running
Sucrose	Increased incentive for food in progressive ratio procedure

Table 1-2. ΔFosB Behavioral Phenotype The phenotypes described in this Table are established upon inducible overexpression of ΔFosB in Line A bitransgenic NSE-tTA_TetOp-ΔFosB mice. (Adapted from McClung, *et al,* 2004).

There is also evidence that ΔFosB may be important for the development of more complex behaviors, beyond sensitivity to reward and drug taking, that are critical for the actual addiction process. Specifically, ΔFosB influences the motivational properties of natural and drug reinforcers. Mice overexpressing ΔFosB exert more effort to maintain self-administration at high levels of cocaine in progressive ratio assays, an effect that could increase the risk of relapse, even following long periods of withdrawal (Colby, *et al,* 2003). ΔFosB also facilitates the addicted state by compensating for cognitive impairments when drug is on board. Chronic cocaine self-administration highly induces ΔFosB expression in the orbitofrontal cortex (OFC), a region implicated in cognitive changes associated with addiction. A recent study demonstrated that ΔFosB helps mediate tolerance to the detrimental cognitive effects caused by acute cocaine

administration (Winstanley, *et al,* in review), which could prevent deleterious effects to the user, promoting addiction. In addition, overexpression of ΔFosB specifically within the NAc enhances food-reinforced instrumental performance and increases motivation for food in the progressive ratio paradigm (Olausson, *et al,* in review). Overexpression ΔFosB also increases motivation for other natural rewards, such as sucrose drinking, wheel running, and possibly sex (Werme, *et al,* 2002; unpublished observations). Taken together, these observations extend the role of ΔFosB beyond a sensitizer towards the rewarding effects of abusive drugs. ΔFosB may act as a molecular switch associated with enhancing the motivational aspects of consistantly rewarding behavior. This switch may be a critical aspect for the formation of natural and pathological habits.

ΔFosB and Gene Regulation

The mechanism by which ΔFosB mediates these diverse behavioral phenotypes is likely through regulation of gene expression, given that ΔFosB is a transcription factor. The earliest analysis of ΔFosB gene regulation was in a reporter assay. ΔFosB repressed AP-1 activation when transiently transfected with various Fos and Jun family members. Initially, it was hypothesized that ΔFosB acted as a dominant-negative form of Fos that competed for Jun at the dimerization step to repress transactivation (Nakabeppu & Nathans, 1991). In contrast, a study later that same year demonstrated that ΔFosB could activate transcription of an AP-1 reporter in a stably transfected cell line, albeit to a lesser extent than FosB (Dobranzanski, *et al,* 1991). These observations indicated that ΔFosB could both activate and repress transcription depending on the conditions in which it was expressed.

To more clearly understand the transcriptional effects of ΔFosB *in vivo,* the overall pattern of ΔFosB gene expression was analyzed using DNA microarrays from Affymetrix. Using the ΔFosB Line A transgenic mice mentioned above, the pattern of gene expression from the NAc was characterized over a time course of ΔFosB induction and compared to that induced by the the dominant negative Δc-Jun transgenic mice. Interestingly, the pattern of gene expression did not simply rise and fall as ΔFosB levels increased over time. Instead, over half of the genes that were up-regulated when ΔFosB levels were at their lowest, down-regulated as ΔFosB levels increased, and vice-versa (Figure 1-3). In addition, short-term expression of ΔFosB largely mimicked the effects of Δc-Jun, meaning ΔFosB acted primarily as an AP-1 repressor. However, long-tem expression of ΔFosB had mostly opposing effects compared to Δc-Jun, meaning ΔFosB acted as an AP-1 activator. Interestingly, short- and long-term expression of ΔFosB has opposing effects on behavior. Short term-ΔFosB induction and Δc-Jun both reduce preference for cocaine, while long-term induction of ΔFosB increases preference for cocaine (McClung & Nestler, 2003). These data present a scheme in which ΔFosB acts as both an activator and a repressor of AP-1 transcription, and these actions mediate specific effects on drug reward.

ΔFosB Target Genes

Although ΔFosB plays a dominant role in the behavioral plasticity mediated by chronic treatment of abusive drugs, few individual gene targets responsible for these effects have been identified. However, using candidate approaches and microarray technology, some progress has been made and a few *bona fide* ΔFosB target genes are described below.

Figure 1-3

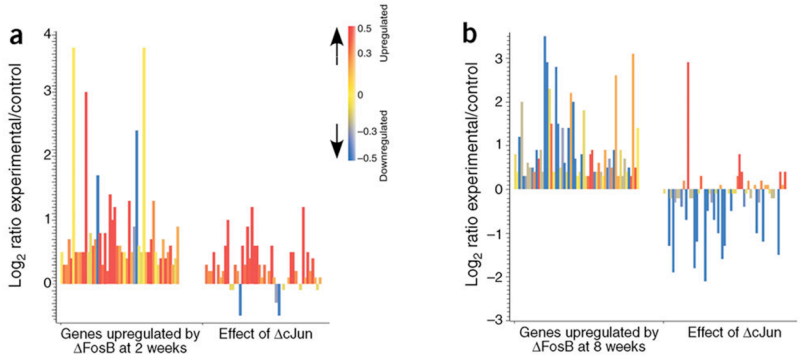


Figure 1-3. Comparison of the regulation of gene expression by ΔFosB and Δc-Jun.
RNA was extracted from the nucleus accumbens of mice overexpressing Δc-Jun for 8 weeks, ΔFosB for 2 weeks, ΔFosB for 8 weeks, and littermate controls, and was subjected to microarray analysis. Genes upregulated by ΔFosB at 2 weeks or 8 weeks and the effect of Δc-Jun are shown. The figure shows similar regulation by ΔFosB at 2 weeks compared to Δc-Jun, but reciprocal regulation by ΔFosB at 8 weeks. The effects shown in the figure were replicated at least twice on independent groups of animals ($P < 0.01$). (Adapted from McClung & Nestler, 2003).

ΔFosB regulates the NMDA receptor 1 glutamate receptor subunit (NMDAR1) in cerebral cortex in response to chronic ECS. This regulation may be related to the behavioral and biochemical effects of chronic seizures and the development of tolerance to repeated seizures in wild-type mice. As mentioned earlier, repeated ECS induces an increase in NMDAR1 and ΔFosB in the superficial layers of the neocortex. In addition, ΔFosB binds an AP-1 site in the *NMDAR1* promoter region. These effects were all absent in *fosB* knockout mice (Hiroi, *et al,* 1998).

The AMPA glutamate receptor subunit, GluR2, is also a ΔFosB target gene. Overexpression of ΔFosB in transgenic mice increases GluR2 expression by over 50% in the NAc, but no effect is seen on any other AMPA receptor subunit (Kelz, *et al,* 1999). GluR2 is also up-regulated by cocaine, an effect ablated by overexpression of Δc-Jun (Peakman, *et al,* 2003). ΔFosB binds the AP-1 consensus sequence at the GluR2

promoter region. In addition, viral-mediated overexpression of GluR2 increases the rewarding effects of cocaine, similar to ΔFosB (Kelz, *et al,* 1999).

Cyclin-dependent kinase 5 (Cdk5) and its activating cofactor, p35, were identified as a ΔFosB target gene in the hippocampus and striatum by use of DNA microarray analysis (Chen, *et al,* 2000; Bibb, *et al,* 2001). Cdk5 has been implicated in the regulation of neurite outgrowth, neuronal function and synaptic plasticity. Specifically, Cdk5 is involved in the regulation of cocaine-induced changes in dendritic spine density (Norrholm, *et al,* 2003). Cdk5 mRNA, protein, and activity are all up-regulated in response to ΔFosB overexpression or chronic cocaine treatment (Chen, *et al,* 2000; Bibb, *et al,* 2001). This effect is blocked by overexpression of Δc-Jun (Peakman, *et al,* 2003). In addition, chromatin immunoprecipitation (ChIP) assays demonstrate that ΔFosB is selectively associated with the *Cdk5* promoter following chronic, but not acute, cocaine administration (Kumar, *et al,* 2005). These data provide direct evidence that Cdk5 is a *bona fide* target gene of ΔFosB in the NAc in vivo and may be involved in the regulation of long-term adaptive changes in response to chronic cocaine (Kumar, *et al,* 2005; Bibb, *et al,* 2001; Nestler, *et al,* 2001).

Dynorphin appears to be another target for ΔFosB (Andersson, *et al,* 2003), and is an example of a gene repressed by the transcription factor (Zachariou, *et al*, 2006). ΔFosB represses dynorphin expression in the NAc and decreases activity of the dynorphin promoter in cell culture reporter assays. Additionally, administration of κ opioid receptor antagonists into the NAc, which would mimic decreased dynorphin levels, mimics the behavioral effects of ΔFosB induction in this brain region in several

assays of the behavioral effects of morphine, while κ opioid receptor agonists oppose these effects (Zachariou, *et al,* 2006).

The above discussion describes only a few ΔFosB target genes that have been identified to date. Other genes that have been characterized include the neuropeptide, substance P, and the transcription factor, NF-κB (Berton, *et al*, in submission; Ang, *et al,* 2001). ChIP is an invaluable technology that can functionally verify if ΔFosB actually binds to specific gene promoters in the brain in vivo. For example, recent ChIP data has indicated that the gene coding for the precursor for the neuropeptide, substance P (preprotachykinin or PPT-A) is also a direct target of ΔFosB in a subset of neurons (Berton, *et al,* in submission). Technological advances in ChIP and ChIP on chip (microarray) assays will rapidly improve our capacity to evaluate ΔFosB target genes and their effects on behavioral plasticity. Through these methods, a more comprehensive evaluation of ΔFosB target genes will be compiled to more completely understand and appreciate ΔFosB neurobiology.

Directions

Once induced, ΔFosB protein persists in the brain for relatively long periods of time in the absence of further stimulation. ΔFosB mediates changes in gene expression by both transcriptional activation and repression. While this long-term expression is the cornerstone of ΔFosB's unique function in neural plasticity and addiction, the biochemical mechanism of this persistence has never been directly studied. The major objective of my thesis is to better understand the basis of ΔFosB's persistent expression. Chapter 2 describes a study that critically examines the degradation of ΔFosB and full-

length FosB in a cell culture system that recapitulates their kinetics of protein induction and stabilization observed in brain. Using this system, we identify specific FosB destablizers, whose absence in ΔFosB are critical to its long-term expression in cells. This is the first analysis of the effect of alternative splicing on the stability of *fosB* protein isoforms. In addition, even though more and more ΔFosB target genes are being identified, the biochemical mechanism by which ΔFosB both activates and represses different genes has not been examined. We hypothesize that ΔFosB mediates these effects through interaction with different binding partners. Chapter 3 details two separate studies that identify novel interacting partners for ΔFosB and FosB by two different approaches. The identification of ΔFosB- and FosB-specific binding partners offers new insight into how each protein may be capable of activating its own genetic profile. Finally, I offer a general discussion about my overall findings, their significance and limitations, and my overall conclusions about ΔFosB neurobiology.

FCHAPTER 2

Proteasome Dependent and Independent Mechanisms for FosB Destabilization: Identification of FosB Degron Domains and Implications for ΔFosB Stability.

Abstract

The transcription factor ΔFosB accumulates in brain during chronic exposure to stress, drugs of abuse, and other chronic stimuli. Once induced, ΔFosB persists in brain for at least several weeks following cessation of the chronic stimulus. The biochemical basis of ΔFosB's persistent expression has remained unknown. Here, we show that the FosB C-terminus, absent in ΔFosB as a result of alternative splicing, contains two degron domains. Pulse chase experiments of C-terminal truncation mutants of full-length FosB indicate that removal of its most C-terminal degron increases its half-life ~4 fold and prevents its proteasome-mediated degradation and ubiquitylation, properties similar to ΔFosB. In addition, removal of a second degron domain, which generates ΔFosB, further stabilizes FosB ~2 fold, but in a proteasome-independent manner. These data indicate that alterative splicing specifically removes two destabilizing elements from FosB in order to generate a longer-lived transcription factor, ΔFosB, in response to chronic perturbations to the brain.

Introduction

The transcription factor ΔFosB is induced in specific brain regions important for reward by chronic exposure to a range of stimuli, including drugs of abuse, stress, antipsychotic and antidepressant treatments, and certain lesions. Distinctly, ΔFosB persists in brain for long periods of time following the termination of these stimulations, unlike all other Fos family proteins that are transiently expressed and quickly degraded (Andersson *et al,* 2003; Hope *et al,* 1994a,b; Hiroi & Graybiel, 1996; Hiroi *et al*, 1997; Mandelzys *et al,* 1997; McClung *et al*, 2004; Perrotti *et al*, 2004). Work using transgenic animals and viral vectors has demonstrated that overexpression of ΔFosB in brain reward regions, such as the NAc, induces a behavioral phenotype resembling animals after chronic drug exposure. Specifically, overexpression of ΔFosB increases the rewarding effects of abused drugs, such as cocaine and morphine. In addition, ΔFosB heightens an animal's sensitivity to these drugs and promotes drug-seeking behavior after long periods of drug withdrawal. The mechanism by which ΔFosB mediates these conditions is still unclear; however, the long-lasting accumulation of ΔFosB in brain is one crucial element for causing the long-lasting neural and behavioral plasticity associated with addiction (Hiroi *et al*, 1997; Kelz *et al,* 1999; Nestler *et al,* 2001; Colby *et al*, 2003; Zachariou *et al*, 2006).

ΔFosB is the truncated splice variant of full-length FosB and is generated by the excision of a 140-nucleotide sequence from exon 4 of the *fosB* primary transcript. This excision results in a one-nucleotide frameshift and forms a stop codon (TGA) that causes premature termination of ΔFosB translation (Fig. 2-1A). Accordingly, protein translated from ΔFosB mRNA lacks the C-terminal 101 amino acids present in full-length FosB

(Yen *et al*, 1991). All Fos proteins possess an N-terminal basic region, a central leucine zipper motif, and a C-terminal transactivation domain (Fig. 2-1B). The leucine zipper motif promotes heterodimerization to form the activator protein 1 (AP-1) transcription factor complex (Morgan *et al*, 1995; Chinenov & Kerppola, 2001). Fos proteins bind to Jun family proteins, however, more than 50 different Fos-Jun interacting proteins have been reported (Chinenov & Kerppola, 2001). The AP-1 complex regulates numerous cellular processes by binding to its AP-1 site regulatory element in the promoter region of a wide range of mammalian genes (Morgan *et al,* 1995; Chinenov & Keppola, 2001; Acquaviva *et al*, 2002). c-Fos and FosB are generally expressed at low to undetectable levels under basal conditions, but are rapidly and transiently induced by diverse types of stimuli in a cell-type specific manner. Their expression is transient because these proteins and their mRNA's are extremely unstable: in cell culture, c-Fos and FosB exhibit half-lives of approximately 60 and 90 minutes, respectively (Stancovski *et al*, 1995; Acquaviva *et al*, 2001).

In contrast, following chronic administration of several types of stimuli, ΔFosB accumulates in particular brain regions and persists long after other Fos proteins become undetectable (Hope *et al,* 1994a,b; Chen *et al*, 1997; Hiroi *et al,* 1997). Accordingly, ΔFosB, through heterodimerization predominately with JunD, forms a long-lasting AP-1 complex that persists in brain for at least several weeks following cessation of the chronic stimulus (Hope *et al,* 1992; Chen *et al*, 1995; Chen *et al*, 1997; Hiroi *et al,* 1998). However, the biochemical basis of ΔFosB's persistent expression has remained unknown. In the present study, we demonstrate directly that this persistence is due to enhanced stability of the ΔFosB protein, rather than increased mRNA translation. In addition, we

identify two C-terminal destabilization domains of full-length FosB that promote FosB's rapid degradation. The excision of these domains from ΔFosB by alternative splicing is a critical cellular mechanism that underlies ΔFosB's enhanced protein stability.

Figure 2-1

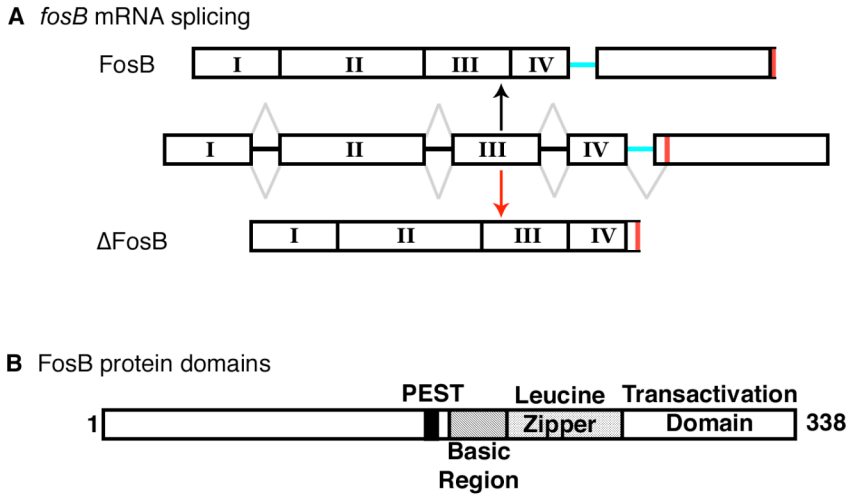


Figure 2-1. FosB mRNA splicing and protein domain structure.

(A) Schematic of *fosB* splicing. ΔFosB is generated by a 140 nucleotide excision of "intronic" sequence found in the open reading frame of exon 4 of full length *fosB*. The splice event results in a one nucleotide frameshift in which a stop codon (TGA) is recognized (colored red). Primers used in this study amplify the "intronic" region (colored blue) specific to full-length *fosB* and the unique exon-exon junction specific to Δ*fosB*.
(B) Schematic of FosB protein domain structure.

Results

Endogenous ΔFosB protein is relatively stable and proteasome-insensitive.

To identify the molecular mechanism of ΔFosB's enhanced protein stability, we first established a cell culture system that recapitulated the kinetics of FosB and ΔFosB mRNA and protein induction and degradation observed in brain following injection of drugs of abuse like cocaine (Hope *et al,* 1992; Hope *et al,* 1994a). We used cultured

pheochromocytoma (PC12) cells because they are known to produce dopamine and dopamine transporters (Greene and Tischler, 1982; Kadota *et al*, 1995), and are used extensively for studies that necessitate a neuronal phenotype. In addition, they mimic the ratio of FosB to ΔFosB mRNA isoforms in neurons (data not shown). Other cell types, such as Hela or HEK-293 cells, produce several fold more ΔFosB mRNA, compared to FosB. Using PC12 cells, we tested a variety of stimuli to induce endogenous FosB and ΔFosB mRNA production, such as nicotine, PMA, and serum. All of these stimuli resulted in FosB and ΔFosB mRNA and protein induction to a greater or lesser extent (data not shown). We considered the possibility that a specific stimulus could affect the stability of FosB/ΔFosB mRNAs or proteins; however, we did not observe an obvious difference in the accumulation and disappearance of FosB/ΔFosB mRNA or protein with the different stimuli tested. Therefore, we chose to use serum stimulation for these experiments as it provided the highest levels of FosB and ΔFosB.

PC12 cells were starved with 0.5% FBS for at least 20 hr to generate a quiescent cell population, then treated with 20% FBS for 2 hr. Following this stimulation, cells were incubated in serum-free DMEM and collected at various time points for Western blotting. FosB protein peaked at 2 hr, then disappeared rapidly, consistent with previous studies (Fig. 2-2A) (Acquaviva *et al,* 2001). ΔFosB also appeared early, but did not show appreciable signs of disappearance until 24 hr (Fig. 2-2C). Western blots identified the two isoforms of ΔFosB (35 and 37 kD) previously recognized in brain (Hope *et a.,* 1994a,b) and in cultured cells expressing exogenous ΔFosB (Chen *et al*, 1997). Consistent with these earlier studies, the 37 kD isoform of ΔFosB was found to be more stable than the 35 kD protein.

We next considered the possibility that the long-lived expression of ΔFosB was due to prolonged expression or stability of its mRNA. To test this idea, we performed quantitative RT-PCR analysis of FosB and ΔFosB mRNA levels at various times after serum stimulation. Interestingly, we observed that both mRNAs peaked around 2 hours, then decayed to basal levels within 12 hours (Fig. 2-2D). In contrast, the ΔFosB protein remained present for long after this time, indicating that the differential stability of ΔFosB compared with FosB is not due to differences in mRNA transcription or stability.

One major cellular mechanism for regulated degradation of soluble proteins is the 26S proteasome (reviewed by Glickman & Ciechanover, 2001). To test whether the proteasome might contribute to the rapid degradation of FosB compared to ΔFosB, we incubated PC12 cells in the presence or absence of a specific inhibitor of the 26S proteasome, epoxomicin (2.5 μM), then stimulated the cells with serum to induce FosB and ΔFosB expression. Proteasome inhibition blocked the degradation of FosB and resulted in its accumulation in the cell, indicating that the rapid turnover of endogenous FosB protein involves proteasomal degradation (Fig.2-2B). In contrast, proteasome inhibition did not result in ΔFosB accumulation, suggesting that ΔFosB is not appreciably degraded by the proteasome during the time course of these experiments. These studies establish for the first time a difference in stability between endogenously produced FosB and ΔFosB and implicate proteasomal degradation of FosB as one of the cellular mechanisms that promotes the more rapid degradation of FosB.

Figure 2-2

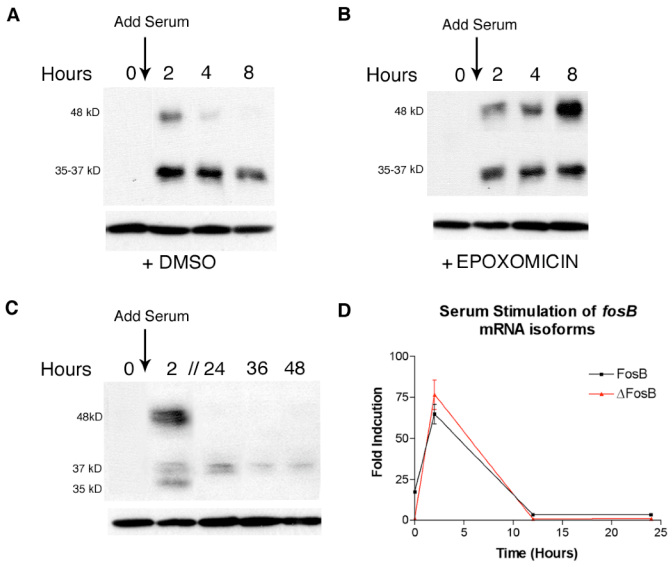


Figure 2-2. Time course of endogenous FosB/ΔFosB protein and mRNA disappearance. (A-C) PC12 cells were serum starved (0.5% FBS in DMEM) for approximately 20 hr, followed by serum stimulation (20% FBS in DMEM) for 2 hr. Cells were then incubated in serum-free DMEM (+/- 2.5 μM epoxomicin or vehicle) for indicated time points and western blotted. Membranes were probed for FosB/ΔFosB, then stripped and re-blotted for GAPDH as a protein loading control. Representative blots are shown. The two ΔFosB isoforms (35kD and 37kD) are resolved by loading less protein and by running the gel for a longer period of time (C). (D) FosB and ΔFosB mRNA's are produced rapidly following serum stimulation, then quickly degrade back to basal levels within 12 hr. Data are expressed as fold difference from ΔFosB mRNA levels at time 0.

To determine the molecular mechanisms that regulate FosB protein stability, we sought to establish an ectopic expression system that recapitulated the differential protein stability of FosB and ΔFosB. As such, we transiently transfected into PC12 cells either wild-type FosB or ΔFosB, then performed standard ^{35}S-methionine pulse-chase experiments followed by immunoprecipitation to measure directly the protein degradation rates. We attempted to also measure the precise half-life of endogenous FosB and ΔFosB after serum induction, but we were unable to reliably accomplish this due to the low levels of the endogenous proteins and limits in detection. We were able, however, to reliably measure the half-life of transfected FosB and ΔFosB proteins. Wild-type FosB protein degraded rapidly with a measured half-life of 1.65 hours (Fig. 2-3A).

In contrast, and consistent with the differences observed by western blotting for endogenous proteins, ΔFosB protein was dramatically more stable ($t_{1/2}$ = 13.3 hrs) (Fig. 2-3B). To determine if the rapid degradation of FosB is due to proteasomal degradation, we incubated the transfected PC12 cells with or without epoxomicin (5 μM) or MG-132 (10 μM), another proteasome inhibitor, during the pulse-chase period. Similar to our observations of endogenous FosB, proteasome inhibition significantly increased the half-life of transfected FosB by several fold ($t_{1/2}$ = 7.5 hours) (Fig. 2-3A). These data suggest that a significant portion of FosB instability is due to proteasome degradation. In contrast, proteasome inhibition did not increase the ΔFosB half-life (Fig. 2-3B). We confirmed effective proteasome inhibition by the proteasome inhibitors, particularly for the longer time points, by measuring proteasome activity in the presence or absence of the inhibitors as indicated by the hydrolysis of a flourogenic peptide (Suc-LLVY-AMC) (Figure 2-4A) and by monitoring the disappearance of an endogenous proteasome substrate, c-Fos (Fig. 2-4B). Both techniques verified effective inhibition.

Figure 2-3

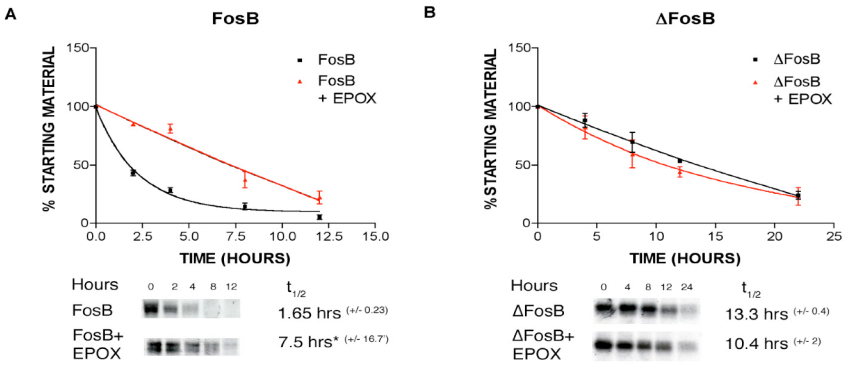


Figure 2-3. Stability analysis of overexpressed FosB and ΔFosB.

(A-B) Plasmids expressing full-length FosB (A) or ΔFosB (B) were transfected into PC12 cells and then analyzed by pulse chase in the presence or absence of proteasomal inhibition. The degradation profile, representative autoradiograms, and the estimated half-life of each protein are shown.

Figure 2-4

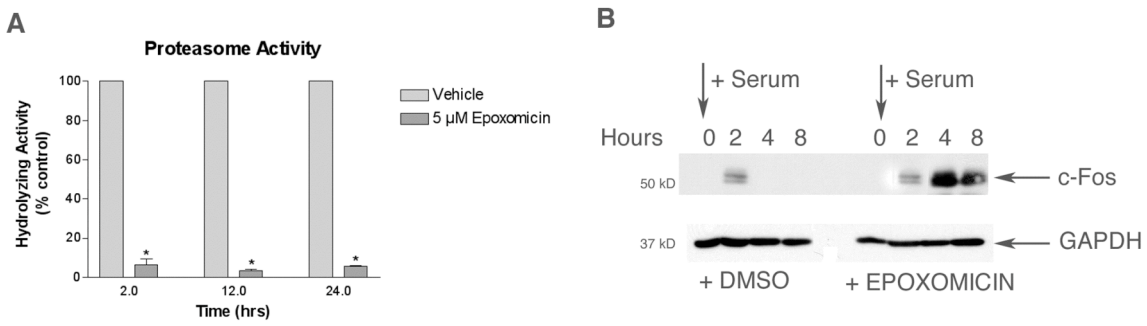


Figure 2-4. Proteasome inhibition control
PC12 cells were incubated in the presence of 5µM epoxomicin or DMSO, re-added every 6 hrs. Proteasomal activity was measured by the hydrolysis of Suc-Leu-Leu-Val-Tyr-7-amido-methylcoumarin (AMC).

PEST sequence and co-expression with JunD do not contribute to FosB stability.

We hypothesized that FosB destabilization motifs may be absent or obscured in ΔFosB, thus facilitating its enhanced stability. To identify these motifs, we began by analyzing the FosB protein sequence and detected an extremely high scoring PEST sequence (a region rich in proline, glutamate, serine, and threonine) directly upstream of the leucine zipper domain (Fig. 2-1B). Hydrophilic PEST sequences have been shown to mediate degradation of some proteins, including the transcription factors papillomavirus E2, NPDC-1 (neural differentiation and control protein 1) and CPEB (cytoplasmic polyadenylation-element-binding protein) (Reichsteiner & Rogers, 1996; Garcia-Alai *et al,* 2006; Penrose & McBride, 2000; Spencer *et al*, 2004; Thom *et al,* 2003); therefore, we tested the importance of the PEST domain for FosB protein stability by analyzing the half-life of a specific PEST domain mutant (FosB P152A). Our pulse-chase assay measurements revealed that the FosB PEST domain mutant has a similar half-life as wild-type FosB (Fig. 2-5A), which indicates that the PEST domain does not promote

FosB degradation. Although previous literature has demonstrated that specific point mutations are sufficient to ablate PEST destabilization (Penrose & McBride, 2000), it is possible that deletion of the entire PEST domain may be required to determine its effect on FosB stability. Of note, the stability of c-Fos has also been reported to be independent of its central PEST domain (Acquaviva *et al*, 2001).

Previous studies have shown that association with c-Jun and different kinases can accelerate c-Fos degradation (Tsurumi *et al,* 1995; Salvat *et al,* 1999). Therefore, we considered the possibility that Jun-family binding to FosB or ΔFosB might affect their protein stability. Earlier work has shown that JunD is the preferred Jun family binding partner for ΔFosB and FosB (Chen *et al*, 1995; Hiroi *et al,* 1998). Therefore, we co-transfected JunD or control vector along with FosB or ΔFosB and western blotted for JunD to confirm increased co-immunoprecipitation with each protein. We found JunD co-immunoprecipitated with both FosB and ΔFosB following co-overexpression (Fig. 2-5D). In addition, co-overexpression of JunD with FosB or ΔFosB produced an additive effect on AP-1 luciferase reporter activity, as compared to FosB/ΔFosB overexpressed with a vector control (Fig. 2-5E). Satisfied co-transfection of JunD with FosB or ΔFosB increased AP-1 dimer formation, we analyzed the stability of the proteins with our pulse-chase method. We found no significant change in stability for either protein upon JunD co-expression (Fig. 2-5B,C), indicating that overexpression of JunD does not affect FosB's protein stability. As PC12 cells are known to express JunD and other Jun family members (Zentrich, *et al,* 2002), we cannot rule out the possibility that endogenous Jun proteins regulate the stability of overexpressed FosB in our experiments. In the future, it

will be interesting to study the stability of FosB and ΔFosB under conditions where endogenous Jun proteins are ablated.

Figure 2-5

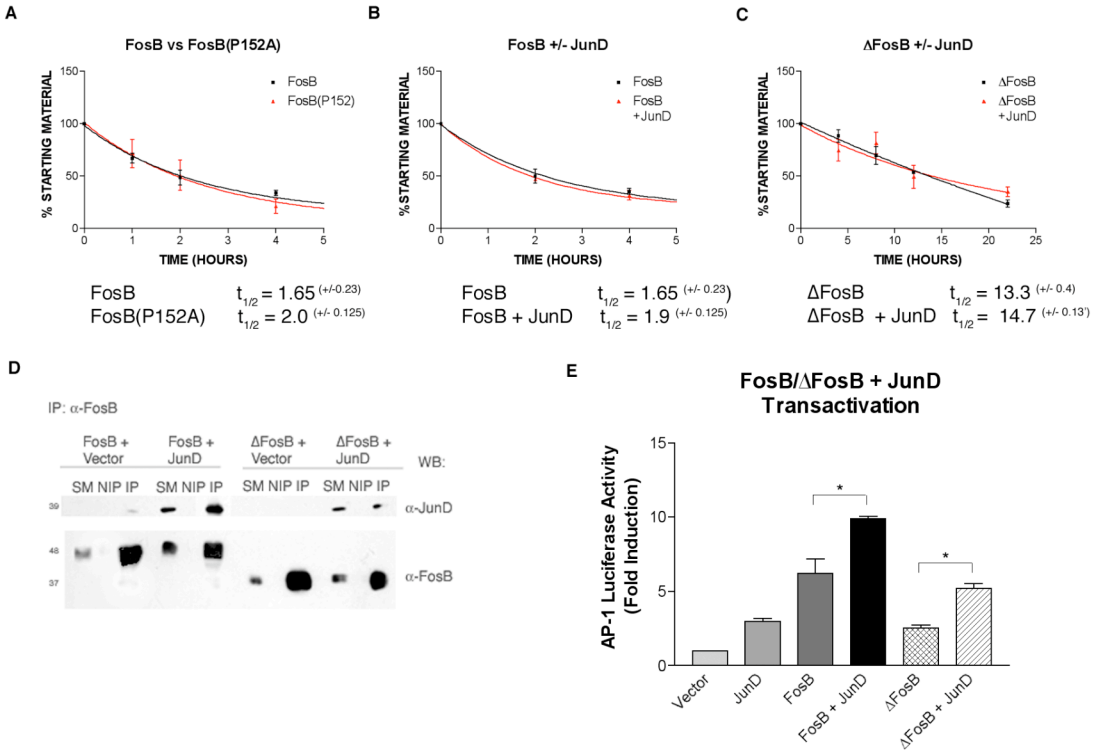


Figure 2-5. Stability analysis of FosB PEST mutant, and FosB or ΔFosB co-overexpression with JunD.(A) Pulse chase degradation profiles and half-life of FosB(P152A) compared to FosB.(B) FosB or (C) ΔFosB was co-transfected with JunD or empty vector control and analyzed by pulse chase. Degradation profiles and each protein half-life shown. (E) Co-immunoprecipitation of FosB/ΔFosB with vector control or JunD. (E) Luciferase reporter activity for FosB and ΔFosB following JunD co-transfection.

FosB C-terminus contributes to its instability and proteasomal degradation.

As the PEST domain did not appear to account for the enhanced destabilization of FosB compared with ΔFosB, we hypothesized that the C-terminus of FosB that is absent in ΔFosB possesses regions that promote its degradation. To test this idea, we generated C-terminal truncation mutants and measured their protein stability. We deleted the C-terminal 21 and 61 amino acids from FosB to generate two new mutants, FosB(1-317)

and FosB(1-277), respectively. We verified these mutants were transciptionally functional with a luciferase reporter assay (Figure 2-6), then compared their stabilities to full-length FosB(1-338) and ΔFosB(1-237).

Figure 2-6

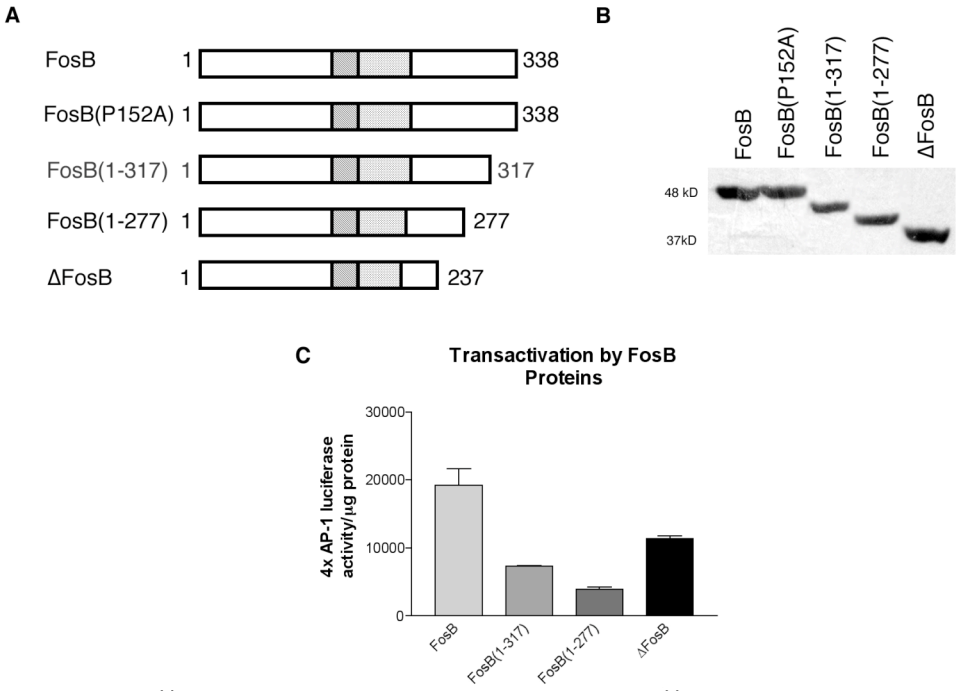


Figure 2-6. Design, expression, and transcriptional activity of FosB proteins (A) Design of FosB mutants. (B) Expression of FosB deletion mutants. Plasmids for full-length FosB, FosB mutants, or ΔFosB were transiently transfected into PC12 cells. 24 hr post-transfection, cell lysates were harvested for Western blotting. (C) PC12 cells were co-transfected with FosB, FosB(1-317), FosB(1-277), or ΔFosB, and an 4 x AP-1 luciferase reporter plasmid. Luciferase activity was measured and calculated as enzyme activity per microgram of total protein.

Using the pulse-chase assay, the measured half-life of the FosB(1-317) mutant was nearly twice that of full-length FosB, with a half-life of 3.3 hr (Fig. 2-8A). In the presence of a proteasome inhibitor, FosB(1-317) was further stabilized to a half-life of 7.3 hours, similar to that seen for full-length FosB under these conditions (Fig. 2-8A). FosB(1-277) was more stable still than FosB(1-317), with a half-life of about 7.0 hr in the absence of proteasomal inhibition (Fig. 2-8B), and the stability of this mutant was not

enhanced in the presence of a proteasome inhibitor (Fig. 2-8B). These data are summarized in Fig. 2-7. The FosB(1-277) mutant was still ~2-fold less stable that ΔFosB, suggesting that the C-terminal region of FosB contains at least two regions that regulate FosB instability: one region (amino acids 278-337) that contributes to proteasome-dependent FosB degradation, and another that is independent of proteasome-mediated degradation (amino acids 238-277) (see Discussion).

Figure 2-7

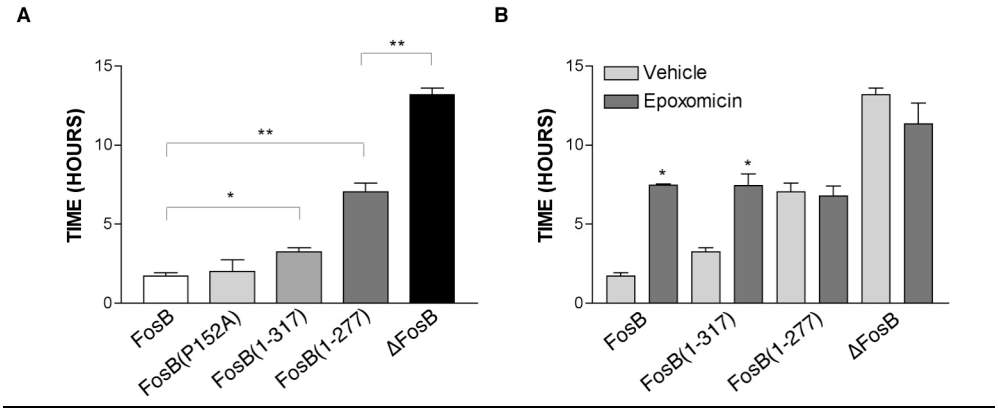


Figure 2-7. Stability of FosB and ΔFosB.

(A) Summary of stability analysis of FosB, ΔFosB, and the several FosB mutants studied.
(B) Stability comparison of FosB, ΔFosB, and FosB C-terminal truncation mutants in the presence or absence of proteasome inhibition (vehicle vs 5 μM epoxomicin).

Figure 2-8

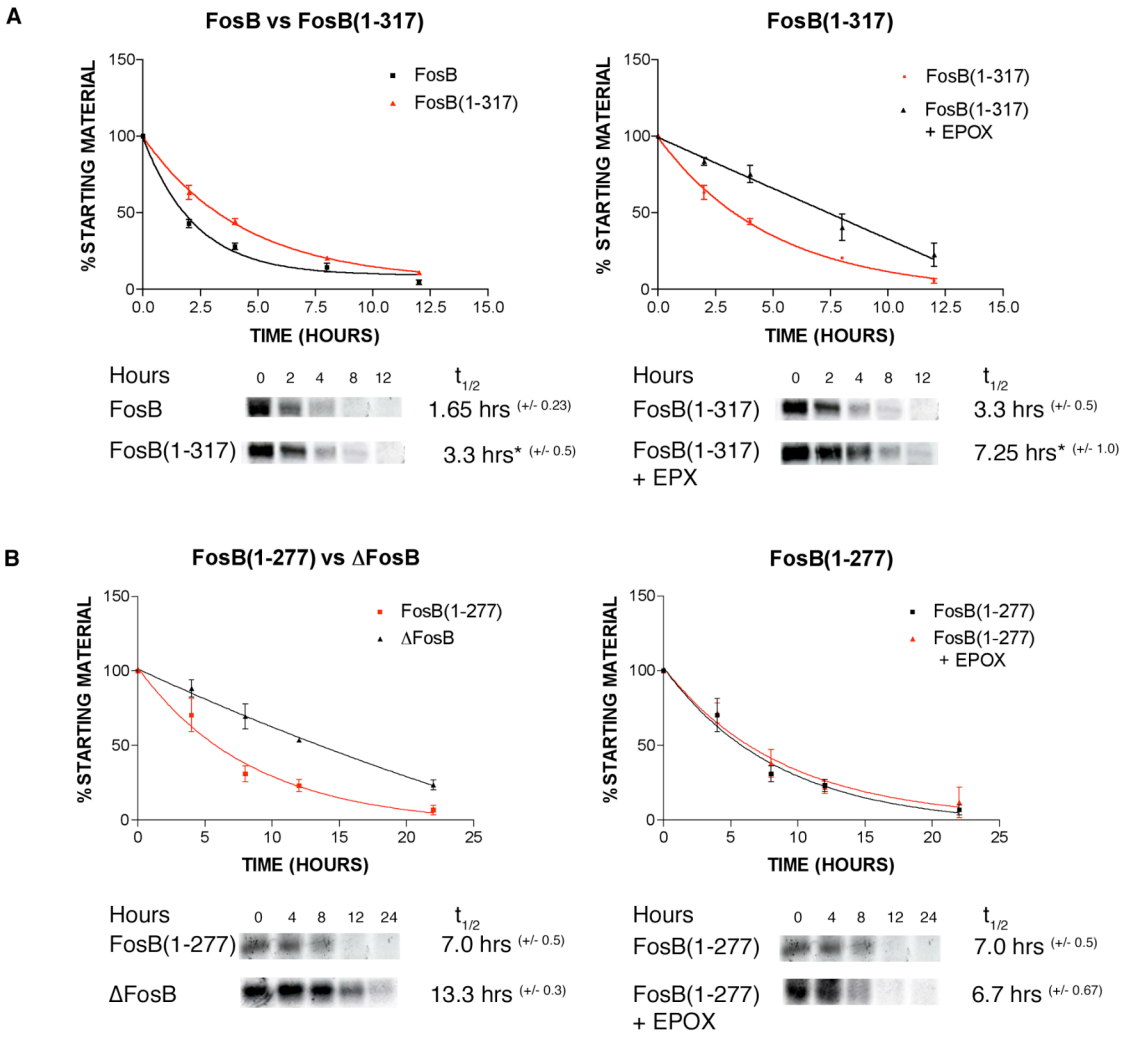


Figure 2-8. Stability analysis of FosB truncation mutants.
Pulse chase analysis of FosB(1-317) (A) and FosB(1-277) (B) in the presence or absence of proteasomal inhibition (5 µM epoxomicin). The degradation profile, representative autoradiograms, and estimated half-life of each protein are shown.

FosB, but not ΔFosB, is poly-ubiquitylated.

Proteins that are degraded by the 26S proteasome are typically targeted for degradation by poly-ubiquitylation. Since a significant portion of the FosB protein's instability is proteasome dependent, we tested whether full-length FosB or ΔFosB is ubiquitylated. To this end, we co-expressed FosB, ΔFosB or vector control along with HA-tagged ubiquitin, then performed immunoprecipitation followed by Western blotting to detect incorporation of HA-ubiquitin into the FosB proteins. To preserve the potentially transient ubiquitylated FosB species, we performed the experiments in the presence of a proteasome inhibitor (2.5 μM epoxomicin) for 24 hr before harvesting. Lysates were immunoprecipiated with nonimmune goat IgG or with anti-FosB antibody and Western blotted for ubiquitin. We detected HA-ubiquitin in FosB immunoprecipitations, but not with ΔFosB (Fig. 2-9), consistent with the fact that FosB, but not ΔFosB, contains proteasome-sensitive domains. The membrane was then stripped and Western blotted for FosB/ΔFosB, which confirmed the efficacy of the transfections and immunoprecipitations.

To determine if the ubiquitylation of FosB correlates with the proteasome-sensitive domains of FosB identified above [i.e., FosB(1-317) and FosB(1-277)], we co-transfected HA-ubiquitin with the FosB deletion constructs. Interestingly, deletion of the C-terminal 21 amino acids [FosB(1-317)], which increased FosB protein stability ~2 fold, did not alter the incorporation of HA-ubiquitin. In contrast, deletion of the C-terminal 61 residues [FosB(1-277)], which increased FosB stability ~4-fold, eliminated the incorporation of HA-ubiquitin (Fig. 2-9). These data suggest that the residues 278-317

mediate the poly-ubiquitylation of full-length FosB and underlie its ubiquitin-dependent proteasomal degradation.

Figure 2-9

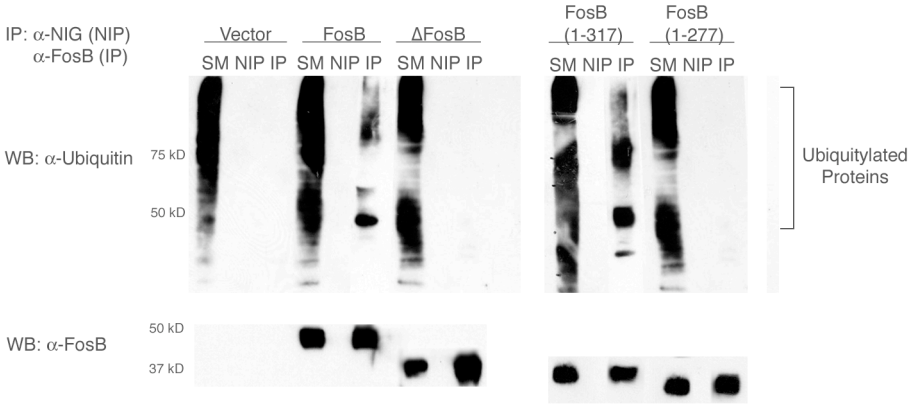


Figure 2-9. Ubiquitylation of FosB and FosB C-terminal truncation mutants Plasmids for FosB, ΔFosB, or FosB mutants, or empty vector as a control, were co-transfected with HA-tagged ubiquitin into PC12 cells. After transfection, cells were treated with the proteasome inhibitor, epoxomicin (5 μM), and immunopreciptated with non-immune goat IgG (NIp) or anti-FosB antibody (IP), then Western blotted with an anti-ubiquitin antibody or an anti-FosB antibody (SM = starting material). Results shown in the figure are representative of at least 3 independent experiments.

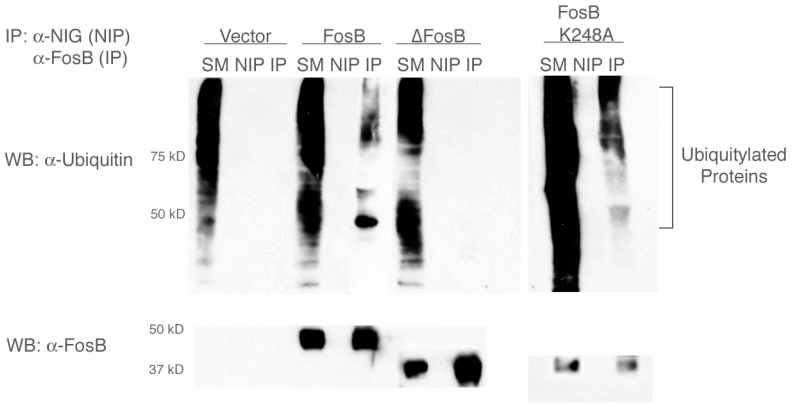
The ubiquitylation of FosB and FosB(1-317), and lack of ubiquitylation of FosB(1-277) and ΔFosB, was confirmed by utilizing both non-denaturing (data not shown) and denaturing conditions and therefore rules out the possible confounds of non-proteasomal proteolytic activity during protein extractions.

Finally, we analyzed if the FosB C-terminus serves as the direct substrate for ubiquitylation, or rather is important for proteasome machinery targeting. We favor the latter hypothesis, since the domain in the FosB C-terminus which is required for FosB ubiquitylation (amino acids 278-317) does not contain a lysine (K) residue. Thus, FosB contains 9 lysine residues, 8 of which are shared with ΔFosB (Fig. 2-10B). Only one lysine is unique to FosB, and that is lysine 248. We therefore tested whether this lysine

residue is important for FosB ubiquitylation. To this end, we mutated FosB lysine 248 to alanine (A), and analyzed whether this mutation influenced the ubiquitylation of FosB. We found comparable levels of ubiquitylation in wildtype FosB compared with FosB(K248A) (Figure 2-10A). These findings suggest that the FosB C-terminus is important for targeting FosB to the proteasome machinery, but does not itself undergo ubiquitylation.

Figure 2-10

A



B

```
MFQAFPGDYD SGSRCSSSPS AESQYLSSVD SFGSPPTAAA SQECAGLGEM PGSFVPTVTA
ITTSQDLQWL VQPTLISSMA QSQGQPLASQ PPAVDPYDMP GTSYSTPGLS AYSTGGASGS
GGPSTSTTTS GPVSARPARA RPRRPREETL TPEEEEKRRV RRERNKLAAA KCRNRRRELT
DRLQAETDQL EEEKAELESE IAELQKEKER LEFVLVAHKP GCKIPYEEGP GPGPLAEVRD
LPGSTSAKED GFGWLLPPPP PPPLPFQSSR DAPPNLTASL FTHSEVQVLG DPFPVVSPSY
TSSFVLTCPE VSAFAGAQRT SGSEQPSDPL NSPSLLAL
```

Figure 2-10 FosB lysine 248 is not required for FosB ubiquitylation. (A) Plasmids for FosB, ΔFosB, FosBK248A or empty vector as control, were analyzed for ubiquitin incorporation as described above. (B) FosB protein sequence. Highlighted region indicates C-terminal 101 amino acids truncated by formation of ΔFosB. Lysine residues are indicated in red. (Non-immune goat IgG = NIP, anti-FosB antibody = IP, SM = starting material). Results shown in the figure are representative of at least 3 independent experiments.

Casein Kinase 2 is important for endogenous ΔFosB stabilization

We were curious about the differences in stability between the endogenous ΔFosB protein, which disappeared by 50% in approximately 24-30 hrs, and the overexpressed protein, that had a calculated half-life by pulse chase of 13.3 hr. A recent study has indicated that casein kinase 2 (CK2) phosphorylation of ΔFosB at serine 27 is also important for stabilization of the protein (Ulery, *et al,* 2006). We considered the possibility that CK2 may be a limiting factor that stabilizes ΔFosB, which may be saturated by ΔFosB overexpression. To explore this possibility, we used RNAi to knockdown CK2 expression in our PC12 cell induction system. An approximately 50% reduction of CK2 in our system, the maximum that we could achieve with this approach, accelerated the rate of endogenous ΔFosB disappearance by 50% from ~28 hrs to ~20 hrs (Figure 2-11). These data more closely reconcile the discrepancy in stabilities between the endogenous and overexpressed protein half-lives in our PC12 systems.

Figure 2-11

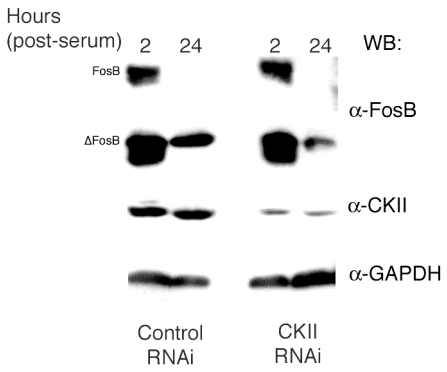


Figure 2-11. RNAi knockdown of CKII accelerates degradation of endogenous ΔFosB PC12 cells were transfected with either control or CKII targeting RNAi. 24 hrs later, cells were serum starved, then 48 hrs post-transfection, cells were serum stimulated to induce production of FosB and ΔFosB. Cells were harvested for Western blot at indicated times.

Proteasome-Independent FosB/ΔFosB degradation

To determine what other cellular degradation pathway may be involved in proteasome-independent FosB and ΔFosB degradation, we analyzed the relative stabilities of FosB and ΔFos in the presence of various lysosome and protease inhibitors. First, the lysosome inhibitor, chloroquin (25μM), had no significant effect on ΔFosB stability, as analyzed by pulse chase. We also measured protein accumulation by Western blot for FosB and ΔFosB following transient transfection or serum stimulation in the presence of chloroqine and found no significant accumulation. In addition, we analyzed FosB and ΔFosB accumulation in the presence of the trypsin-like and cystein protease inhibitor, leupeptin (50-200 μM), the calpain inhibitor, calpeptin (25-75 μM), and the lysosomal inhibitor, pepstatin A (10-50 μM). Unfortunately, we did not see any appreciable protein stabilization in the presence of any of these inhibitors following 4-24 hrs of treatment.

Discussion

ΔFosB accumulates in specific brain regions important for reward, such as the nucleus accumbens, after several types of chronic stimulation (see Introduction). Unique among all other Fos family proteins, ΔFosB persists in these regions for weeks following the cessation of the stimulus. These observations have led to the suggestion that ΔFosB may act as a sustained ”molecular switch” that first initiates and then maintains an altered neural and behavioral phenotype in response to chronic perturbations (Nestler *et al,* 2001; McClung *et al*, 2004). The biochemical mechanisms mediating ΔFosB’s unusual persistence has remained unknown.

In this study, we set out to examine the relative stability of ΔFosB in cultured cells. We first established a cell culture system that provided differential protein stabilities of FosB and ΔFosB, as observed in brain. Using quantitative RT-PCR, we found that ΔFosB protein remains in the cell long after its mRNA is degraded, confirming that ΔFosB is, indeed, a stable transcription factor that persists long after mRNA levels are returned to basal levels. In addition, we found that serum-induced endogenous FosB protein and overexpressed FosB protein are stabilized by inhibition of the proteasome, whereas endogenous or overexpressed ΔFosB protein levels are unaffected by proteasome inhibition. We then identified two degron domains in the C-terminus of FosB that promote its protein degradation: one proteasome-independent domain (aa 238-277) and one proteasome-sensitive domain (aa 278-338) (Fig. 2-11). Within the proteasome-sensitive domain, we identified two distinct regions: amino acids 278-317 and amino acids 318-338. Amino acids 278-317 are necessary to signal FosB poly-ubiquitylation and direct FosB proteasome-mediated degradation. Amino acids 318-338 further destabilize FosB, although in a ubiquitin-independent manner. This region is homologous to the C-terminal 20 amino acids of c-Fos that has been reported to mediate ubiquitin-independent proteasomal degradation of the protein (Bossis *et al*, 2003), and the same may operate for FosB based on our present findings. Finally, we determined that overexpression of ΔFosB in a cell culture system saturates an additional stabilization mechanism for ΔFosB – phosphorylation by CK2. These two factors, truncation of FosB degrons by alternative splicing and phosphorylation by CK2, are critical for the overall stabilization of ΔFosB *in vivo*.

Figure 2-12

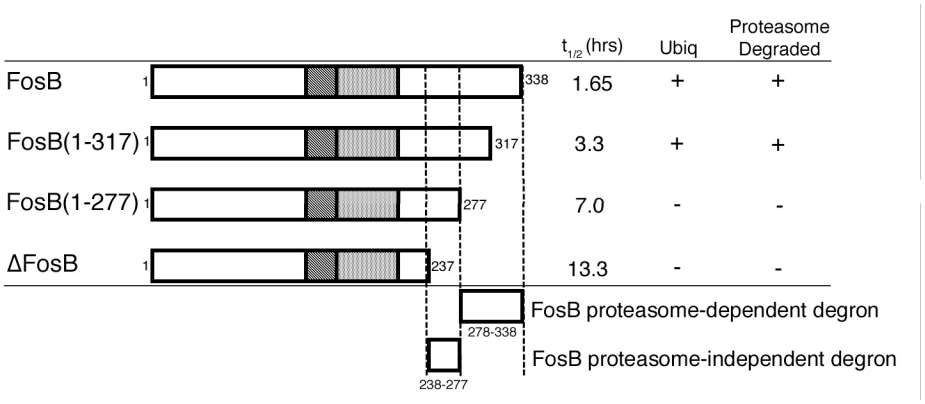


Figure 2-12. Identification of FosB degron domains.
Schematic representing full-length FosB, FosB C-terminal truncation mutants, and ΔFosB. Each protein half-life, ubiquitylation capacity, and sensitivity to proteasome degradation is indicated. FosB proteasome-dependent and –independent degron domains are shown.

The stability of many transcription factors is regulated by a PEST sequence. The high scoring PEST sequence found upstream of the FosB leucine zipper is conserved among Fos family members. We found that this sequence did not significantly contribute to the instability of FosB (Fig. 2-4A). Although the unique C-terminus of FosB regulates proteasome-dependent degradation, the precise residues that regulate this process are unclear. There are several potentially interesting amino acid regions within the proteasome-sensitive degron domain, including two WW domain interaction motifs at amino acids 294-299 and 329-334. WW domains are small protein modules that recognize proline-containing ligands. These modules are found in many signaling and structural proteins, localized in both cytoplasm and nucleus (Macias *et al,* 2001), and have been reported to mediate the interaction with the HECT (homologous to E6-AP carboxyl terminus) family of E3 ligases (Ingham *et al*, 2004). E3 protein-ubiquitin ligases select specific proteins for ubiquitin conjugation. In addition, there are polyproline rich regions that could recruit SH3-containing proteins, a PDZ domain

binding motif, and one SH2 domain binding motif. Each of these protein interaction motifs may be involved in targeting unknown binding partners to FosB that modulate its stability. In the future, it will be important to elucidate the precise molecular mechanisms that regulate FosB instability through these distinct FosB degrons. Additionally, it will be important to determine directly whether the proteasomal degradation of FosB requires poly-ubiquitylation, or whether the amino acid sequences within the FosB C-terminus target the protein to the proteasome via some other mechanism, as reported recently for c-Fos (Bossis *et al,* 2003).

Inhibition of FosB proteasomal degradation or removal of its degron domain stabilized the protein to approximately 60% the total stability of ΔFosB. This finding indicates that amino acids 238-277, present in the FosB(1-277) mutant, but absent in ΔFosB, also contributes to the destabilization of FosB, independent of its proteasome degradation (Fig. 2-12). The termination of FosB at residue 277 may result in an unstable or misfolded secondary structure component, making it less stable than ΔFosB. However, even full-length FosB in the presence of proteasome inhibitors is only as stable as FosB(1-277), indicating that this region (aa 238-277) does contribute to the overall destabilization of full-length FosB independent of the proteasome. There are other proteasome-dependent mechanisms that affect ΔFosB stability, such as the phosphorylation of Serine 27 by casein kinase 2, which protects ΔFosB from proteasomal degradation (Ulery *et al*, 2006); however, mutation of this site in full-length FosB has no affect on its stability (Ulery, P.G., personal communication). In the future, it will be important to understand the relationship between these different protein stability/instability mechanisms to the overall stability of ΔFosB in neurons.

While the difference in protein stability affects the duration of FosB and ΔFosB transcriptional activities, the duration is not the only important aspect. In addition to temporal differences, FosB and ΔFosB also appear to activate their own specific (albeit partially overlapping) genetic profiles (Chen *et al*, 1997; McClung, *et al,* 2003). In particular, FosB and ΔFosB can have different effects on the same gene promoter (Chen *et al*, 1997). As well, ΔFosB can repress AP-1-dependent transcription in some cases, but activate it in others (Dobrazanski *et al*, 1991; Nakabeppu & Nathans, 1991; Yen *et al*, 1991; McClung, *et al,* 2003). The molecular mechanisms governing the different transactivation capabilities of FosB and ΔFosB have not yet been fully explored. It is conceivable that the differing stabilities between FosB and ΔFosB may be an important factor.

Several studies have observed that extremely potent transcription factors are quickly targeted for proteasomal degradation by their transcription activation domains. In contrast, transcription factors with weaker transactivation activity are less efficiently targeted for degradation and are much more stable (Salghetti *et al*, 2000; Tyers & Thomas, 2000). In general, FosB's and ΔFosB's activity and protein stability agree with this model, as our data and others show that ΔFosB's transcription activity in reporter assays is ~60% of the transcription activity of FosB (Dobrzanski *et al,* 1991). In addition, we observed the transcriptional activity of the FosB mutants decreased as their stability increased (Figure 2-6C). However, the more stable ΔFosB was a more potent transactivator than both of the FosB truncation mutants, suggesting that the FosB C-terminus may contain some repressive element that is deleted with the generation of ΔFosB.

In summary, we report that the unique C-terminus of FosB contains distinct domains that contribute to its protein instability by both proteasome-dependent and proteasome-independent mechanisms. Our findings suggest that alternative splicing of *fosb* generates a protein form (ΔFosB) that evades the cell's degradation machinery and promotes long-lasting AP-1-dependent transcriptional responses that are critical for neuronal and behavioral plasticity. While both N- and C-terminal destabilizing elements have been reported for c-Fos (Bossis *et al.,* 2003), this is the first report to identify destabilizing elements for any other Fos family member. Future studies will be necessary to elucidate the precise molecular mechanisms that regulate FosB instability, and to determine whether additional mechanisms contribute to the long-lived ΔFosB levels observed in brain after chronic stimulation.

Figure 2-12

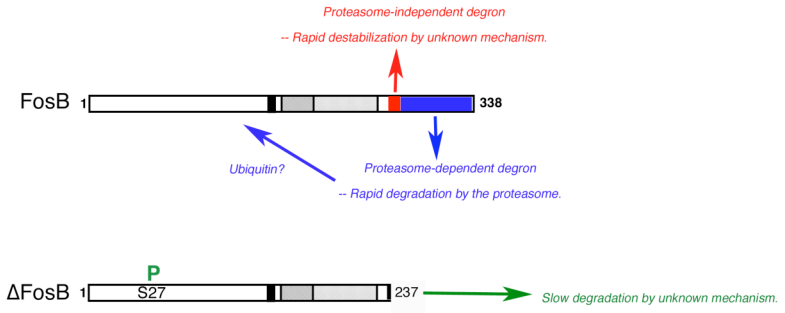


Figure 2-13. FosB and ΔFosB degradation. FosB is targeted for proteasome degradation by its C-terminal proteasome-dependent degron (blue). Ubiquitylation may or may not be required for targeting. The proteasome-independent degron destabilizes FosB by an unknown mechanism (red). ΔFosB is slowly degraded by an unknown mechanism following CK2 phosphorylation of Serine 27.

CHAPTER 3

Identification of FosB/ΔFosB Interaction Partners

Abstract

Although FosB and ΔFosB are products from the same gene, they are different proteins with some distinct properties, such as transformation activity and transcriptional regulation. Here, we use two different approaches to identify novel interacting partners for each protein that may offer some insight to the different functions of each protein. First, we designed a proteomic screen to identify *in vivo* interacting partners from rat cortical tissue. Using this approach, we identified then confirmed the interaction of heat shock cognate protein (Hsc70) with FosB, but not ΔFosB. Next, we pursued a candidate approach. Previous studies have observed that *c-fos* is desensitized following chronic drug treatments, when ΔFosB is highly induced. We used to ChIP to analyze the mechanism of *c-fos* repression. Interestingly, we found both HDAC1 and ΔFosB both occupied the *c-fos* promoter following chronic amphetamine treatment. Using co-immunoprecipitation assays both *in vitro* and *in vivo*, we determined that ΔFosB and HDAC1 are interacting partners. These data suggest an intriguing model where ΔFosB and HDAC1 may form a repressive complex on the *c-fos* promoter in response to chronic drug administration. Taken together, these approaches revealed novel interacting partners that offer new insight to the different biochemical properties of FosB and ΔFosB.

Introduction

FosB and ΔFosB possess many differing characteristics beyond their temporal properties of induction and protein stability that have not been fully explored. Specifically, FosB has been well characterized along with all other Fos family members in tumorigenesis. Overexpression of FosB is transforming in rat fibroblasts; however, overexpression of ΔFosB is not, similar to FRA-1 and FRA-2 (Wisdom, *et al,* 1993; Milde-Langosch, 2005). This effect may be explained by the absence from ΔFosB of most of the Fos family C-terminal transactivation domain that is required for transformation (Wisdom, *et al,* 1993). In addition, FosB and ΔFosB have been characterized in many different tissues other than the brain. Changes in FosB levels have been identified in breast and skin cancer lines, while ΔFosB has been implicated in bone formation and osteoblast differentiation (Bamberger, *et al,* 1999; Milde-Langosch, 2005; Kveiborg, *et al,* 2004) and in cataract formation (Kelz, *et al*, 2000). These effects may be attributed to differences in FosB and ΔFosB transcriptional activities and the different genetic profiles activated by each protein, some of which have already been described (Chen *et al*, 1997; McClung, *et al,* 2003). For example, FosB has been shown to downregulate AP-1 promoter activity in a stably transfected cell line, while ΔFosB upregulates AP-1 promoter activity (Chen *et al*, 1997).

The crystal structures of FosB and ΔFosB are still unknown. Computer analysis suggests that the FosB C-terminal domain consists of partial β-strand and α-helical

secondary structure that contain numerous protein interaction domains, such as WW domain interaction motifs, an SH2 domain, and a PDZ domain (www.cmpharm.ucsf.edu; www.prosite.com). In addition, there are numerous putative phosphorylation sites present in the C-terminus. We hypothesized that truncation of this region by alternative splicing to generate ΔFosB may have drastic changes on the secondary structure of the protein, revealing new binding motifs, while removing or obscuring others and consequently altering both proteins' functions. One known example of a FosB to ΔFosB functional change is serine 27. Phosphorylation of serine 27 on ΔFosB stabilizes the protein by protecting it from proteasome degradation (Ulery, *et al*, 2006); however, mutation of the same residue on FosB has no effect on its protein stability (Ulery, P., personal communication). Interaction with different binding partners may be a critical factor in the differing transcriptional activities of FosB and ΔFosB, and may be an additional factor contributing to their very different stabilities *in vivo.* In addition, truncation of the C-terminus may be responsible for differences in the relative affinities of FosB and ΔFosB to Jun dimerization partners and to DNA. These differences would have a great impact on the transcriptional activities of each protein.

I wanted to explore the possibility that differing binding partners are important for mediating the different biochemical properties of FosB and ΔFosB. In addition, differences in interaction partners may be responsible for ΔFosB mediating both AP-1 activation and repression on different promoters. To this end, I carried out initial, exploratory studies to identify novel ΔFosB interaction partners. I employed two approaches: first, an open-ended proteomics approach, and second, a candidate approach.

3A. Proteomics Approach

Results

Protocol

I chose to directly immunoprecipitate ΔFosB and FosB from rat brain in order to detect interaction partners. Briefly, rats were treated with sham or chronic electroconvulsive seizure to induce high amounts of FosB and ΔFosB in brain. Animals were sacrificed 4 hrs or 24 hrs after the last treatment. This protocol would induce high amounts of both FosB and ΔFosB (4 hrs) or primarily ΔFosB (24 hrs) in brain. Prefrontal cortex was grossly dissected then subcellularly fractionated (Figure 3-1). Soluble P1 nuclear fractions were immunoprecipitated with non-immune control or anti-FosB antibody. Immunoprecipitates were washed, then run on an SDS-PAGE gel and silver stained (Figure 3-2). Bands differing between non-immune and immune preciptations, or between mock and chronic treatments, were excised and identified by mass spectrometry.

Figure 3-1

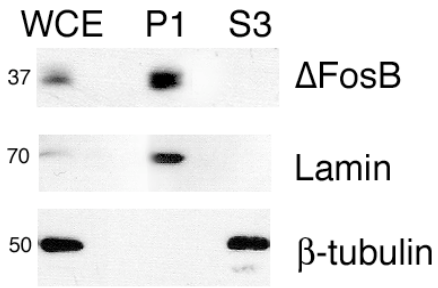


Figure 3-1. Subcellular fractionation of rat cortical tissue.
After gross dissection, cortical tissue was homogenized in isotonic sucrose by a Dounce homogenizer, then centrifuged (2000 x G, 10 min) to obtain the crude nuclear fraction (P1). The supernatant (S1) was saved. The resulting P1 pellet was homogenized in hypotonic solution, then pelleted again (20,000 x G, 10 min) to obtain the soluble P1 fraction. The S1 fraction was pelleted, (100,000 x G, 1 hr) and the supernatant saved to obtain the crude cytosolic fraction (S3). (WCE = whole cellular extract).

Pros and Cons

This protocol had advantages and disadvantages. The primary disadvantage was the extremely crude co-immunoprecipitation. We saw countless non-specific and non-replicable bands. It was also very difficult to optimize the ratio of antibody to lysate, such that I could pull down as much material as possible with as little antibody as possible, to minimize the amount of IgG bands visible at 75, 50, and 25 kD. The large IgG bands at these molecular weights may have masked interesting co-migrating proteins. Although eluting off the material with a competitive peptide seemed to work nicely by western blot, the direct pull-down and boiling always gave best results by silver stain. Another disadvantage was the expense. At $300 a band, excision and identification of numerous bands from many gels was very expensive. (However, the expense is only a fraction of the cost of the various ChIP on chip, laser capture, and behavior experiments going on in our lab every day!) Finally, direct immunoprecipitation with the Santa Cruz antibody, which recognizes an N-terminal epitope to FosB, was not ideal because we could not specifically immunoprecipitate FosB versus ΔFosB. This antibody pulled down both FosB and ΔFosB from the sample lysate.

The main advantage to this protocol was its efficiency. We were able to identify candidate binding partners in a matter of weeks, rather than the months required for a yeast two-hybrid screen. In addition, we appreciated the novelty of identifying candidates *in vivo* from brain, rather than from an artificial system. Lastly, although the protocol was crude, we were able to consistently co-immunoprecipitate JunD, the known FosB/ΔFosB AP-1 heterodimerization partner, so we were satisfied that our experimental system was reliable and capable of identifying novel interaction partners. All proteins listed in Table 3-1 were identified in at least 3 independent experiments.

Figure 3-2

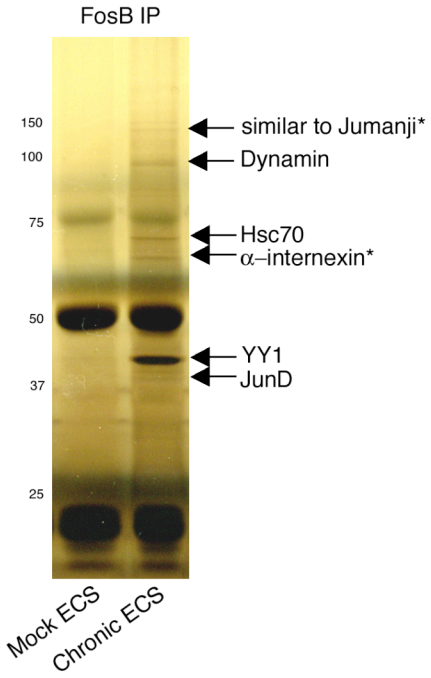


Figure 3-2. Proteomic Approach to identify ΔFosB binding partners
Sample silver stained gel from P1 fraction immunoprecipitation. Mock or chronically treated ECS cortical tissue was immunoprecipitated with an α-FosB antibody, then run on an SDS-PAGE gel and silver stained. Bands differing between the mock or ECS treated FosB pull downs (indicatd by arrows) were excised and identified by mass spectrometry. (Asterisks indicate proteins that were not reproduced).

Table 3-1 Candidate Binding Partners Identified by Mass Spectrometry

MW ON GEL	PROTEIN NAME	PROTEIN MW
102	Dynamin	95.5
100	Drebrin 1	77.4
75	**Hsc 70**	70.9
45	YY1	44.4
44	Actin beta	41.5
40	Tropomodulin 2	39.5
37	**JunD**	34.5
37	Protein Phosphatase 2a	35.6
24	DJ-1 protein	20.0

Table 3-1. Candidate Binding Partners Proteins identified by mass spectrometry that co-immunoprecipitated with FosB antibody. Each of these proteins was identified at least 3 times in separate experiments.

Ying Yang 1

The first candidate protein we considered as an interaction partner for FosB or ΔFosB was Ying Yang 1 (YY1). YY1 is a zinc-finger transcription factor implicated in both positive and negative gene regulation, depending on the promoter context and intracellular environment, similar to ΔFosB. Typically, YY1 is a gene activator in the presence of the adenovirus protein, E1A, and a repressor in its absence (Gordon, *et al,* 2005). It regulates the function of a wide variety of consensus sequences, such as CRE, AP-1, and SRE sites, similar to FosB and ΔFosB (Gordon, *et al,* 2005). In addition, YY1 has been shown to interact with c-Jun (Kang, *et al,* 2004). YY1, in a complex with HDAC1, has also been implicated in cell cycle regulation, specifically with induction of cyclin D1 (Cicatiello, *et al,* 2004). In addition, FosB has been implicated in this pathway (Brown, *et al,* 1996). I used a co-immunoprecipitation assay in PC12 cells and HEK-293 cells and found no interaction between endogenous or overexpressed YY1 with FosB or ΔFosB by this method, despite numerous attempts. We therefore concluded that the detection of YY1 in our pulldown assays is likely an artifact.

Heat shock cognate protein 70

Heat shock cognate protein (Hsc70) was identified from animals sacrificed 4 hrs post treatment, indicating that Hsc70 may interact with FosB, ΔFosB or both proteins. The 70-kDa heat shock proteins (Hsp70 and Hsc70) are involved in a wide range of folding processes, including folding of nascent polypeptide chains into their native state, prevention of protein aggregation, and solubilizing and refolding of aggregated proteins (reviewed by Mayer & Bukau, 2004). Hsc70 is constitutively expressed in the cytoplasm, but translocates to the nucleus under stress (Manzerra & Brown, 1996). Our

reasons for pursuing Hsc70 as a ΔFosB interaction partner were two-fold: First, Hsc70 functions in a complex with heat shock protein 90 (Hsp90) (Wegele, *et al*, 2004). Hsp90 is a molecular chaperone required not only for folding, but also for the stabilization of numerous signaling proteins. Continuous association with Hsp90 protects the "client protein" from proteasomal degradation (reviewed by Neckers and Ivy, 2003). Pharmacological inhibition of Hsp90, by drugs such as geldanamycin, has been shown to accelerate the proteasomal degradation of Hsp90 clients, such as the protein kinases, IRE1α and Akt, and the transcription factor, HIF-1α (Marcu, *et al,* 2002; Neckers & Ivy, 2003). We considered the possibility that ΔFosB association with Hsc70, in a complex with Hsp90, may be another stabilization mechanism for the protein.

Second, there is evidence that Hsc70 may be involved in AP-1 transcriptional regulation. One study found that Hsc70 modulates AP-1 DNA binding activity. Specifically, gel shift assays demonstrated that Hsc70 attenuated AP-1 binding by interaction with c-Fos-c-Jun heterodimers (Carter, 1997). These data are particularly interesting considering that certain stimuli, such as ECS, activate both AP-1 activation and Hsc70 translocation to the nucleus (Morgan & Curran, 1991; Gass, *et al*, 1995.) To verify a protein interaction, I obtained an HA-tagged Hsc70 plasmid from Dr Danny Manor, Cornell University, and co-transfected it with either FosB or ΔFosB into PC12 cells. Hsc70 co-immunoprecipated with FosB, but not ΔFosB (Figure 3-3). These findings support the ability of our pulldown assays to identify *bona fide* binding partners for FosB/ΔFosB. Consistent with our finding that Hsc70 interacts with FosB, and not ΔFosB, was our observation that treatment of PC12 cells with the Hsp90 inhibitors geldanamycin (5μM) and radicicol (3μM), as well as the Hsp70 inhibitor, KNK437 (*N*-

Formyl-3,4-methylenedioxy-benzylidine-g-butyrolactam) (100μM) did not affect the apparent half-life of endogenous ΔFosB induced by serum stimulation (Figure 3-4). These data provide the first direct demonstration that heat shock protein-related mechanisms do not contribute to ΔFosB's persistence.

Figure 3-3

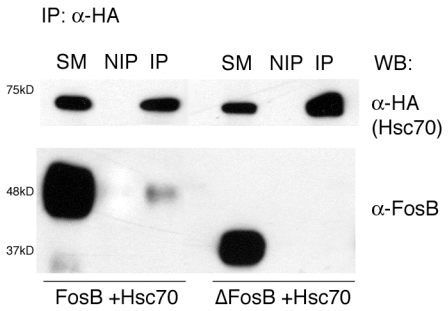


Figure 3-3. FosB, but not ΔFosB, co-immunoprecipitates with Hsc70
PC12 cells were co-transfected with HA-Hsc70, and FosB or ΔFosB plasmids. Cells were harvested after 24hrs and lysates were immunopreciptated with a non-immune (NIP) or anti-HA (IP) antibody. Immunoprecipitates were run on an SDS-PAGE gel and western blotted with an anti-HA or anti-FosB antibody. (SM = starting material)

To examine if Hsc70 is involved in FosB transcriptional regulation, I co-transfected Hsc70 or an Hsc70 dominant-negative mutant (Hsc70K71M) with FosB or ΔFosB in PC12 cells with a 4 x AP-1 luciferase reporter construct (Chen, *et al,* 1997). Substitution of Hsc70 lysine 71 for methione inhibits its ATPase activity, which is required for substrate binding (Johnson & McKay, 1999; Mayer & Bukau, 2005). Co-expression of Hsc70 or the Hsc70 mutant did not have an effect on transactivation of the luciferase reporter by FosB or ΔFosB (Figure 3-5).

Figure 3-4

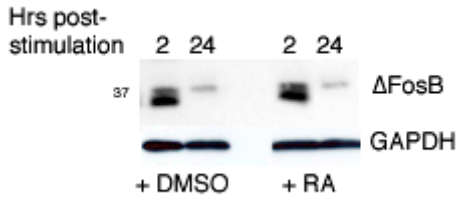


Figure 3-4. Heat shock proteins do not modulate ΔFosB protein stability. PC12 cells were serum starvfed, then serum stimulated in the presence of DMSO or 3μM radicicol (RA). Similar results were obtained with geldanamycin or KNK437.

Figure 3-5

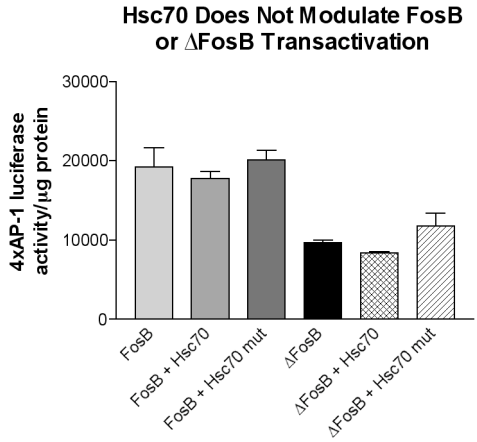


Figure 3-5 Hsc70 Does Not Modulate FosB Transcriptional Regulation
PC12 cells were co-transfected with Hsc70, a Hsc70 dominant-negative mutant or a control plasmid with FosB, or ΔFosB, and a 4 x AP-1 luciferase reporter plasmid. Luciferase activity was measured and calculated as enzyme activity per microgram of total protein.

During the course of these experiments, I analyzed Hsc70 protein levels in the NAc of the ΔFosB transgenic mice, described in Chapter 1 (Kelz, *et al,* 1999). NAc tissue was harvested from mice off doxycycline for 8 weeks, when ΔFosB levels are at their peak. I found a significant decrease in Hsc70 protein levels compared to littermate controls on doxycycline (Figure 3-6). In addition, I found a slight decrease in the levels of Hsp40, a member of the Hsc70 multi-protein complex that chaperones protein folding. In contrast, I found no change in Hsp90 levels in these mice. Since FosB levels are

unchanged in these mice, it is possible that the absence of ΔFosB modulates these effects; however, the mechanism of these changes is unclear. ΔFosB may interact with Hsc70 indirectly or the interaction may be transient, such that it is undetectable by the co-immunoprecipitation assay employed. Another possibility is that downstream effectors may be responsible, such as one of ΔFosB's target genes. Nonetheless, further pursuing possible interactions between Hsc70 and FosB or ΔFosB would be interesting for future study. My demonstration that levels of the Hsc70 chaperone protein complex are decreased in the ΔFosB expressing transgenic mice is an important characterization of these mice, which may have other as yet unknown implications in numerous biochemical pathways.

Figure 3-6

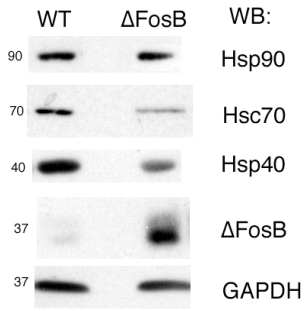


Figure 3-6. Heat shock protein levels in ΔFosB transgenic mice The NAc of transgenic mice following 8 weeks on or off doxycyclne in their drinking water were harvested and western blotted for heat shock proteins. ΔFosB protein levels are highest in transgenic animals following 8 weeks off doxycycline. (ΔFosB = animals off dox; WT = littermate controls on dox).

Discussion

In this study, we devised an ambitiously rapid *in vivo* proteomic approach to identify FosB/ΔFosB interacting partners. We directly immunoprecipitated FosB and ΔFosB from brain, then identified co-immunoprecipitating proteins by mass

spectrometry. Although a more traditional yeast two-hybrid technique may have yielded more consistent results, we preferred our method for its rapid results and *in vivo* interactions. We detected and verified at least two *bona fide* interaction partners – JunD and Hsc70 – and several other interesting candidates that warrant more critical examination.

JunD as FosB/ΔFosB Control

JunD has previously been identified as the preferred AP-1 heterodimeration partner of ΔFosB based solely on gel shift assays (Chen *et al*, 1995; Hiroi *et al,* 1998). To date, JunD (and to a lesser extent other Jun proteins) is the only known binding partner for ΔFosB; therefore, it served as a control protein for this assay. Although western blotting confirmed that the immunoprecipitation protocol efficiently pulled-down ΔFosB from the lysates, we were unable to identify ΔFosB in any of the corresponding silver stained bands by mass spec. This was troubling; however, there is some indication from a collaborator that the ΔFosB protease cleavage pattern does not yield peptides of an optimal size for detection by mass spectrometry. This property of the protein has caused difficulty in crystallization and further characterization of ΔFosB (Rudenko, G, University of Michigan, personal communication). I increased the amount of input protein or antibody in an attempt to detect ΔFosB, however, this had disastrous effects on the quality of the silver stained gel. I also altered the percentage of gel and tried different staining methods, such as SYPRO Ruby. Ultimately, we were unable to ever detect ΔFosB peptides in any of our experiments; therefore, JunD served as our control protein.

Protein Phosphatase 2a

Our screen identified several interesting candidate interacting proteins. One in particular that deserves more investigation is protein phosphatase 2a (PP2A). PP2A and protein phosphatase 1 (PP1) are the major serine/threonine phosphatases in all eukayotic cells. PP2A consists of a 36-kD catalytic subunit (α or β isoform) bound to a 65-kD regulatory subunit. These two proteins associate with a third variable domain to form the PP2A holoenzyme (Lechward, *et al,* 2001). We identified the catalytic subunit β in our screen. Previous studies have indicated that both FosB and ΔFosB are phosphorylated, and these phosphorylations are critical for the functions of each protein (Skinner, *et al,* 1997; Ulery, *et al,* 2006). Specifically, FosB transcriptional activation was regulated by phosphorylation of its C-terminus (Skinner, *et al,* 1997), while casein kinase 2 phosphorylation of ΔFosB protected ΔFosB from proteasome-meditated degradation (Ulery, *et al,* 2006). In this latter study, micromolar concentrations of okadaic acid (OA) were used as phosphatase inhibitors to identify ΔFosB as a phosphoprotein in brain. Micromolar concentrations of OA are sufficient to inhibit both PP1 and PP2A in slice culture. It would be interesting to repeat the study using a nanomolar concentration of OA or fostriecin, which specifically inhibit PP2A (Zolnierowicz, 2000). Detection of phospho-ΔFosB or FosB in the presence or absence of these treatments would be an interesting indicator that PP2A is the specific phosphatase targeted to the FosB isoforms. The generation of a phospho-specific antibody to serine 27 of ΔFosB will be extremely beneficial for these studies in the future.

The GTPase dynamin repeatedly identified by this screen is most likely a contaminating interaction. Dynamin is necessary for the biogenesis of synaptic vesicles, and plays an important role in clathrin mediated receptor endocytosis. In addition, dynamin contains a SH3 interacting domain that may interact non-specifically with the FosB C-terminus (Scaife & Margolis, 1997). Dynamin is highly enriched in brain and its interaction with FosB/ΔFosB is likely an artifact from incomplete protein fractionation. Actin and the actin binding proteins, drebrin 1 and tropomodulin 2, are also likely contaminating proteins due to their great abundance in the cell.

Lastly, I considered DJ-1 as an interacting protein for FosB or ΔFosB. Although the function of DJ-1 protein has remained somewhat obscure, loss of function mutations in the *DJ-1* gene cause early onset development of Parkinson's disease. Recent studies have indicated that DJ-1 is involved in the regulation of oxidative stress, apoptosis, protein aggregation and the transcriptional regulation of tyrosine hydroxylase (Zhong, *et al,* 2006). Considering that ΔFosB is induced in the striatum of Parkinsonian patients (Tekumalla, *et al,* 2001), we were interested in examining if ΔFosB interacts with DJ-1. Using the co-immunoprecipitation assay described previously, I was unable to detect any interaction in cell culture. Nonetheless, DJ-1 would be an interesting target to pursue in the future by other methods.

Hsc70 as FosB Chaperone

Hsc70 is a well-characterized chaperone protein that promotes the proper folding of nascent polypeptides in an ATP-dependent manner (reviewed by Mayer & Bukau, 2004). In addition, Hsc70 has recently been recognized as an important factor involved

in the activation of macrophages and induction of cytolytic T and B cells (Lagaudriere-Gesbert, *et al*, 2002). I identified Hsc70 as a FosB, but not ΔFosB, interacting partner by using co-immunoprecipitation assays in cell culture. Interestingly, I also observed the HscK71M mutant co-immunoprecipated with ΔFosB, but not FosB. These results were confusing because this mutant has an inactive ATPase domain that is required for binding substrate, indicating that this interaction was non-specific. However, the decrease in Hsc70 and Hsp40 protein levels observed in the ΔFosB transgenic mice suggests that ΔFosB may interact, perhaps indirectly, with Hsc70.

A previous study demonstrated that nuclear Hsc70 complex interacts with several transcription factors, such as CREB, the estrogen receptor (ER), the glucocorticoid receptor (GR), and CbfA1 (Niyaz, *et al,* 2003). These data give rise to the possibility that the Hsc70 multi-protein complex may be involved in the regulation of numerous transcription factors; however further study is required to verify these interactions. The discovery of FosB binding with Hsc70 is interesting, considering there is very little literature describing Hsc70 functions with transcriptional factors. The initial discovery may have been fortuitous or seemingly an artifact, due to the stickiness of heat shock proteins by nature; however, subsequent experiments demonstrated that Hsc70 does indeed interact specifically with FosB rather than ΔFosB by co-immunoprecipitation assays in cell culture. Further studies are required to examine the biological significance of this interaction. Specifically, it would be interesting to analyze whether Hsc70 chaperones FosB folding and whether the Hsc70 complex is involved in FosB translocation into the nucleus following stress.

In conclusion, the direct co-immunoprecipition assay from cortical tissue was a successful method to detect protein interaction partners for FosB and ΔFosB. We confirmed the principle of the technique by identifying JunD and identified a novel FosB interaction partner, Hsc70. In addition, the identification of PP2a may lead to further studies that will confirm this phosphatase as the enzyme that dephosphorylates FosB and ΔFosB. In the future, a more traditional protein interaction study, such as a yeast two-hybrid assay, should be performed in order to detect more FosB/ΔFosB interaction partners. These differences may be crucial to ΔFosB neurobiology, to other systems where ΔFosB has been implicated (bone, lens), and perhaps to oncology, where the function of ΔFosB is yet to be explored.

3B Candidate Approach

Results

c-Fos is Desensitized in Response to Chronic Cocaine

In 1992, our laboratory explored changes in IEG expression following acute and chronic cocaine treatments to identify biochemical changes induced by drugs of abuse. The study found that an acute injection of cocaine expectedly increased *c-fos* mRNA and protein levels in the nucleus accumbens. However, following chronic cocaine injections, *c-fos* mRNA and protein returned to control levels, suggesting that cocaine desensitized its ability to induce *c-fos*. Similar findings were found for other IEG mRNAs, *c-jun, fosB, junB,* and *zif268*. In addition, the group examined AP-1 binding after acute and chronic cocaine treatments. Expectedly, AP-1 binding increased following acute cocaine administration and reverted back to control levels within 8-12 hrs. These data correlated with increased IEG mRNA and protein levels observed. Interestingly, chronic cocaine treatments also resulted in increased AP-1 binding activity. However, the AP-1 binding remained elevated at 18 hrs after the last injection, persisting after IEG mRNA and protein levels had returned to control values (Hope, *et al,* 1992). These findings were later extended to the discovery of ΔFosB as the persistent AP-1 constituent (Hope, *et al,* 1994a,b). The mechanism for c-Fos desensitization has remained unknown.

To extend these findings, I worked in collaboration with another graduate student in the Nestler laboratory, Will Renthal. We injected rats with either saline or 4 mg/kg amphetamine once a day for 7 days. Chronic amphetamine animals were allowed to withdraw from the drug for up to 10 days before they were given a saline or amphetamine

challenge. Rat ventral striatum was dissected and c-Fos mRNA was measured by quantitative PCR (q-PCR) (Figure 3-7). As demonstrated previously, we verified that c-Fos mRNA induction after amphetamine administration is desensitized after repeated treatments. This desensitization persists for 7 days following the chronic treatment. Interestingly, there is also a gradual, but significant, repression of baseline c-Fos levels over 5 days of withdrawal, the mechanism of which may be relevant to the observed desensitization

Figure 3-7

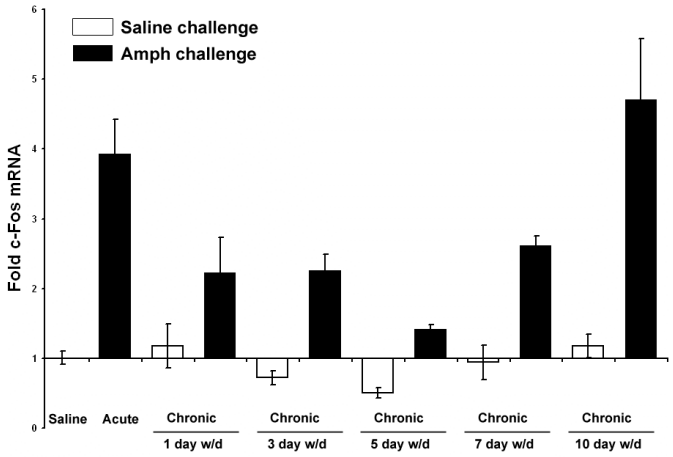


Figure 3-7. c-Fos mRNA is Repressed After Chronic Amphetamine Withdrawal
Rats were injected with either saline or 4mg/kg amphetamine once a day for 7 days. Acute amphetamine rats received 6 days of saline injections to habituate them to the stress of injection. Chronic rats were allowed to withdrawal from the drug for 1, 3, 5, 7, or 10 days before given either a saline or amphetamine challenge, after which they were sacrificed 1hr later. Rat ventral striatum was dissected and c-Fos mRNA levels were quantified by qPCR. Data are expressed as fold difference from saline control..

RNA Polymerase II

Next, Renthal and I wanted to analyze if the c-Fos promoter was desensitized as a result of inefficient recruitment of RNA polymerase II (Pol II). Since c-Fos constitutively has Pol II bound to its promoter (Fass, *et al,* 2003; Fivaz, *et al,* 2000), a significant reduction in Pol II after 1-5 days of withdrawal would be consistent with a transcriptional mechanism of c-Fos desensitization/repression. We performed a chromatin immunoprecipitation (ChIP) assay to assess its occupancy on the c-Fos

repeated amphetamine treatment. Rats were treated with chronic saline or amphetamine, then sacrificed 1 day or 5 days following the last injection. Striatal punches were dissected and fixed in 1% formaldehyde to crosslink proteins bound to DNA. The crosslinked chromatin was sheared to ~500 base pair fragments by sonication. We then performed ChIP with an antibody against Pol II and quantified the amount of DNA associated with Pol II by q-PCR. We found Pol II is significantly reduced on the c-Fos promoter after 1 day and 5 days of withdrawal from chronic amphetamine treatment, perhaps contributing to the reduced induction of the gene (Figure 3-8).

Figure 3-8

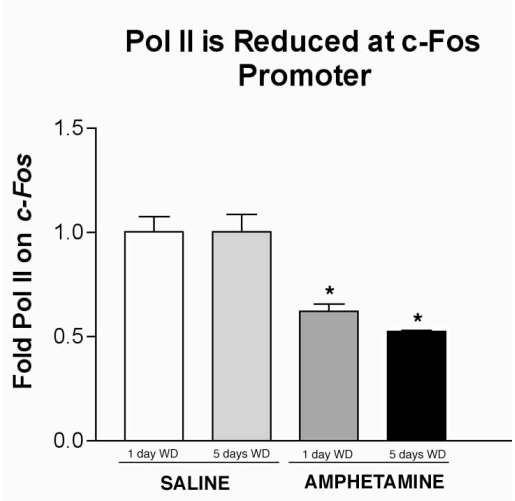


Figure 3-8. c-Fos Desensitization prevents RNA Polymerase II from Binding
Rats were treated once a day with 4 mg/kg amphetamine for 7 days. Following 1 or 5 days of withdrawal, rats were challenged with either saline or amphetamine, and then sacrificed. Striata were dissected and ChIP was performed with a Pol II antibody. Data are expressed as fold change from saline conrol. (WD = withdrawal)

Histone Modifications

Chromatin remodeling through histone modification is a critical mechanism underlying transcriptional regulation. Specifically, acetylation reduces the net positive charges of the core histones, reducing their binding affinity for DNA. Subsequently, histones are unfolded from the nucleosome, providing access for transcription factors to

the DNA (Wade, *et al,* 1997; Thiel, *et al,* 2004). Recent reports have indicated the histone acetylation-deacetylation is regulated in response to acute and chronic cocaine, seizures, and psychotropic drugs (Kumar, *et al,* 2005; Huang, *et al,* 2002; Tsankova, *et al,* 2004; Li, *et al,* 2004). We considered the possibility that c-Fos repression may also be regulated by this mechanism. As reported recently by Kumar *et al* (2005), acute cocaine induces a profound induction of H4 acetylation at the c-Fos promoter, whereas this induction is desensitized after chronic cocaine. Acetylation loosens the interaction of histones with DNA; therefore, more acetylation permits the transcriptional machinery access to gene promoters (Jenuwein and Allis, 2001). Reduction in acetylation following chronic cocaine correlates with reduced c-Fos mRNA transcription (Figure 3-9).

Figure 3-9

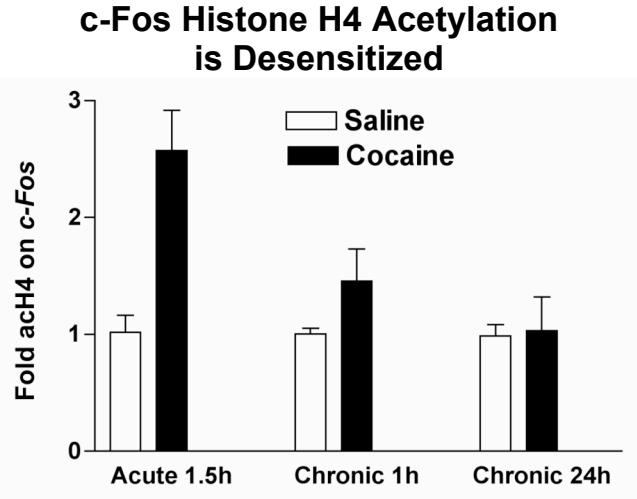


Figure 3-9. Histone Acetylation at c-Fos Promoter is Desensitized by Chronic Amphetamine
ChIP was performed on striata of acute and chronic-saline, acute- and chronic-amphetamine treated rats using an AcH4 antibody to assess its occupancy on the c-Fos promoter after repeated amphetamine treatments. Acute rats were sacrificed 1.5 hrs after the last injection, and chronic rats were sacrificed 1 hr or 24 hrs after the last injection. Data are expressed as fold difference from saline control. (From Kumar, *et al,* 2005).

Kumar *et al* (2005) also provided direct evidence that these changes in histone acetylation could contribute to regulation of the *c-fos* gene. Histones are acetylated and deacetylated by enzymes called histone aceyltransferases (HATs) and histone deacetylases (HDACs), respectively. HATs transfer an acetyl group to the ε-amino group of a lysine on a histone (Wade, *et al*, 1997). HDACs remove these acetyl groups, thereby condensing the chromatin and repressing gene transcription (Jenuwein & Allis, 2001). Kumar *et al* (2005) demonstrated that systemic administration of the widely used HDAC inhibitor, sodium butryrate (NaB), significantly restored the c-Fos repression seen after 5 days of withdrawal (Figure 3-10).

Figure 3-10

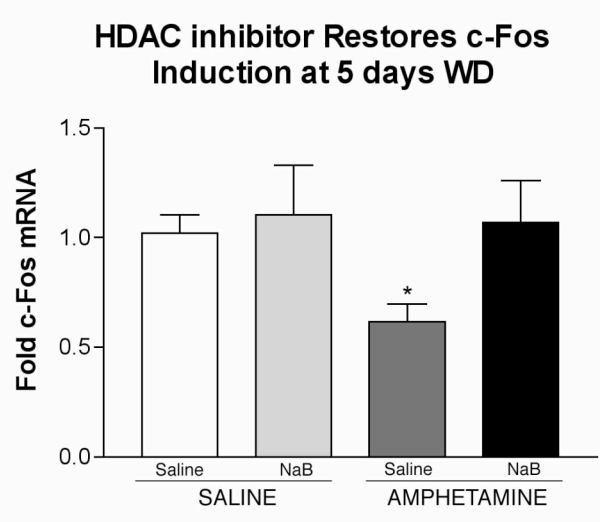


Figure 3-10, HDAC Inhibitor Restores c-Fos Induction Following Chronic Amphetamine Rats were administered saline or amphetamine 7 days. Following 5 days of withdrawal, rats were challenged with saline or 300mg/kg sodium butyrate and sacrificed 0.5-1hr later. RNA was extracted from striatum as described previously and c-Fos mRNA was quantified by q-PCR. Data are expressed as fold difference from saline control. (WD = withdrawal, NaB = sodium butyrate). (From Kumar, *et al,* 2005).

Renthal and I next investigated candidate HDACs that may be specifically involved in c-Fos repression. A previous study indicated that HDAC1 was recruited to AP-1 sites by an adaptor molecule to repress transactivation (Lee, *et al,* 2000). HDAC1

is a member of class I HDACs (HDACs 1, 2, 3, and 8) that localize to the nucleus and are similar to the RPD3 protein in yeast (Thiagalingam, *et al,* 2003). We decided to use a ChIP assay to determine if HDAC1 is recruited to the c-Fos promoter during amphetamine withdrawal. In addition, histone 3 lysine 9 dimethylation (diMeK9) has been reported to be sufficient for transcriptional repression and recruitment of other transcriptional repressors, such as HP1 and SUV39H1 (Steward, *et al,* 2005). We found that both HDAC1 and diMeK9 are recruited to the c-Fos promoter after 5 days of withdrawal from chronic amphetamine, but not after 1 day, perhaps explaining why there are reduced c-Fos levels in the unstimulated state after 5 days of withdrawal (Figure 3-11). These data still leave open the question of why c-Fos is desensitized at 1 day of withdrawal even though these markers are not yet present.

Figure 3-11

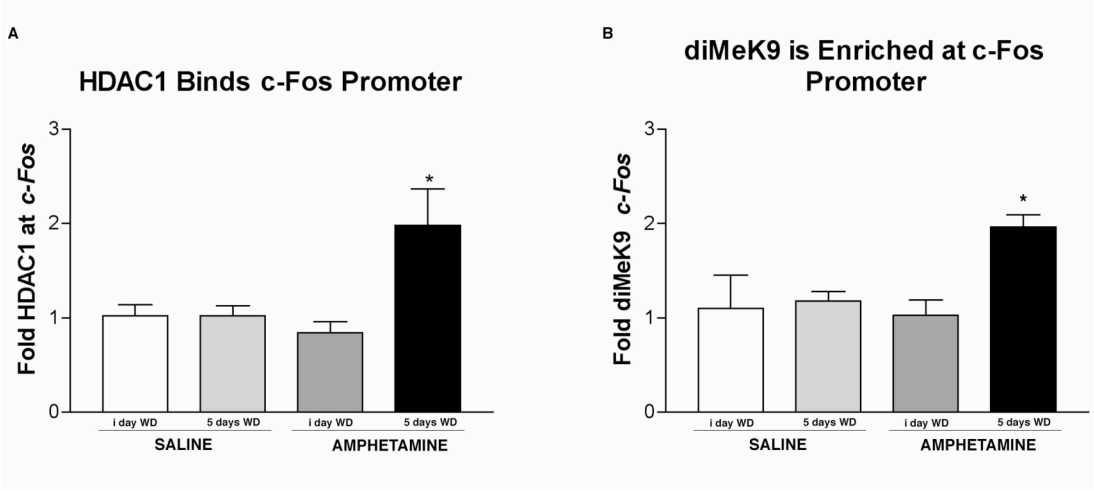


Figure 3-11. HDAC1 and diMeK9 are recruited to the c-Fos Promoter 5 days Following Withdrawal from Chronic Amphetamine Administration

Following chronic saline or amphetamine administration and one or 5 days of withdrawal, rat striata were dissected and ChIP was performed with (A)HDAC1 or (B)diMeK9 antibodies. Data are expressed as fold difference from saline control. (WD = withdrawal)

c-Fos Repression and ΔFosB

Although HDAC1 appeared important for c-Fos repression by 5 days of withdrawal, the initial c-Fos repression factor, critical for c-Fos repression after 1 day of withdrawal, was still elusive. To identify this factor, we used a candidate approach and analyzed the consensus sites present in the primary sequence of the c-Fos promoter. We were very interested to find several AP-1 sites. Previous studies have demonstrated that persistent AP-1 binding is induced while c-Fos desensitizes in response to chronic cocaine treatment (Hope, *et al,* 1992). ΔFosB has been shown to be responsible for this AP-1 binding activity (Hope, *et al,* 1994a). In addition, ΔFosB has been well characterized as a transcriptional repressor of AP-1 activity (McClung, *et al,* 2003). We, therefore, considered the possibility that ΔFosB may be repressing c-Fos through prolonged interaction at its AP-1 sites. We used a ChIP assay (the immunodepletion method, Kumar, *et al,* 2005) to determine if ΔFosB is present at the c-Fos promoter following chronic amphetamine treatment. We found an increase in ΔFosB on the c-Fos promoter after both 1 day and 5 days of withdrawal (Figure 3-12). These data place ΔFosB on the promoter at the same time we see desensitization and repression of c-Fos mRNA.

ΔFosB/ HDAC1 Complex

Previous literature has indicated that HDACs can be recruited to specific sites by proteins in order to modulate transcriptional activity (Lee, *et al,* 2000). c-Fos mRNA induction decreased following 1 day withdrawal -- when we identified ΔFosB bound to

the promoter -- and 5 days withdrawal, when we identified both ΔFosB and HDAC1 bound to the promoter. We hypothesized that following chronic amphetamine, ΔFosB accumulates in the cell and binds the c-Fos promoter to repress transcription. Over time, as more ΔFosB accumulates, it recruits HDAC1 to the promoter to further repress c-Fos by deacetylating histones. To test this hypothesis, we used two systems to demonstrate that ΔFosB and HDAC1 are indeed interacting partners.

Figure 3-12

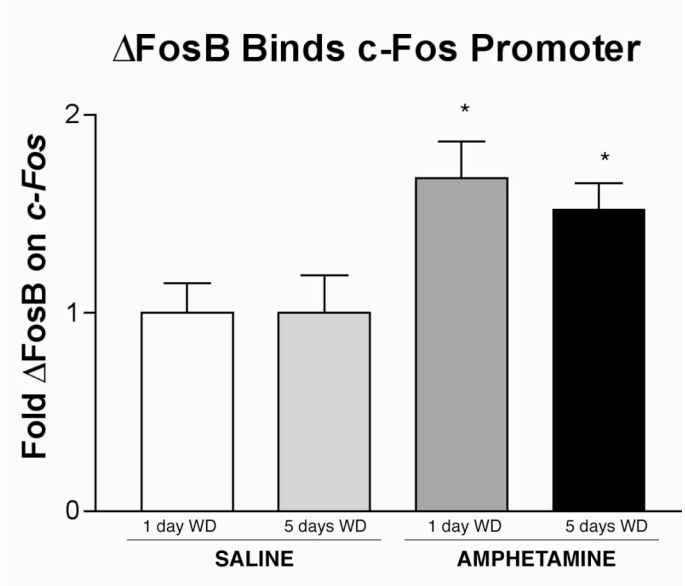


Figure 3-12. ΔFosB Occupies the c-Fos Promoter After 1 and 5 Days of Amphetamine Withdrawal
ChIP with a ΔFosB antibody was performed on the striata of rats sacrificed after 1 or 5 days of withdrawal following 7 days of saline or amphetamine treatment. Data are expressed as fold difference from saline control. (WD = withdrawal)

First, we used a cell culture system to identify *in vitro* that HDAC1 co-immunoprecipitates with ΔFosB (Figure 3-13A). PC12 cells were co-transfected with a V5-tagged HDAC1 plasmid and with either a FosB or ΔFosB plasmid, then harvested and immunoprecipitated with an anti-FosB antibody. HDAC1 co-immunoprecipitated with

ΔFosB but not appreciably with FosB (Figure 3-13A). We completed the reciprocal experiment *in vivo.* We treated animals with chronic ECS to induce high amounts of ΔFosB. Animals were sacrificed, then cortical tissue was grossly dissected, subcellularly fractionated, and nuclear fractions were immunoprecipitated with an anti-HDAC1 antibody. ΔFosB co-immunoprecipitated with HCAC1 in chronic ECS treated tissue, but not in mock treated tissue (Figure 3-13B).

Figure 3-13

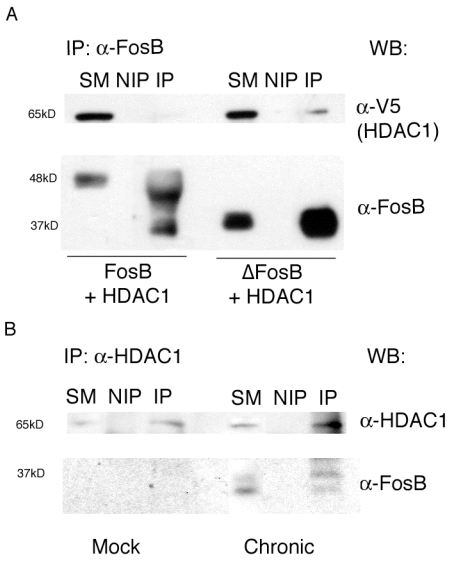


Figure 3-13. ΔFosB and HDAC1 are interaction partners
(A) PC12 cells were co-transfected with V5-tagged HDAC1 and FosB or ΔFosB plasmids. 24hrs post-transfection, cells were harvested and lysates were immunopreciptated with a non-immune (NIP) or anti-FosB (IP) antibody. Immunoprecipitates were run on an SDS-PAGE gel and western blotted with an anti-V5 or anti-FosB antibody. (SM = starting material) (B) Rats were administered chronic ECS or sham treatment then sacrificed. Cortical tissue was grossly dissected and subcellularly fractionated. Soluble nuclear fractions were immunoprecipitated with a non-immune (NIP) or anti-HDAC1 (IP) antibody, run on an SDS-PAGE gel and western blotted with an anti-HDAC1 or anti-FosB antibody.

Discussion

I

n the present study, Will Renthal and I extended previous observations that c-Fos is highly induced following acute drug treatments and is desensitized in response to chronic

drug treatments (Hope, *et al,* 1992). Withdrawal from chronic amphetamine administration gradually represses c-Fos mRNA induction, with maximal repression after 5 days. To examine the mechanism of transcriptional repression, we first analyzed occupation of RNA polymerase II at the c-Fos promoter. Since Pol II binds the c-Fos promoter under basal conditions (Fivaz, *et al,* 2000), any persistent loss of Pol II might suggest an active transcriptional mechanism preventing the pre-initiation complex from forming on *c-fos*. Indeed, we did observe decreased Pol II binding at the c-Fos promoter following both 24 hrs and 5 days of withdrawal from chronic amphetamine treatment. These findings are consistent with the results of a recent study by Kumar, *et al* (2005), who analyzed histone modifications at the c-Fos promoter. Histone proteins and their associated modifications contribute to mechanisms that alter chromatin structure, leading to changes in transcriptional "on-off" states (Jenuwein & Allis, 2001). Using ChIP, these authors determined that acetylation of histone H4 was also desensitized after chronic amphetamine administration, possibly implicating histone acetylation in this process. Treatment with the histone deacetylase (HDAC) inhibitor, sodium butyrate, restored c-Fos mRNA induction to control levels, indicating that an HDAC is likely involved in c-Fos repression. To follow up on these observations, we found that, following 5 days, but not 1 day, of amphetamine withdrawal, HDAC1 is recruited to the c-Fos promoter. HDAC1 has previously been identified in a repressive complex recruited to AP-1 sites (Lee, *et al,* 2000). We then examined the consensus sites in the c-Fos promoter sequence to identify candidates that may be involved in c-Fos repression after 1 day of withdrawal. We discovered several AP-1 binding sites and decided to analyze ΔFosB occupation of the c-Fos promoter. ΔFosB is a well-characterized repressor of AP-1 activation in some

physiological conditions (Dobrazanski *et al*, 1991; Nakabeppu & Nathans, 1991; McClung, *et al,* 2003). In addition, ΔFosB protein is abundant in striatum following chronic drug treatments due to its enhanced protein stability, and is capable of persistent AP-1 binding (Hope, *et al,* 1994a,b; Chen, *et al,* 1997). We identified ΔFosB bound to the c-Fos promoter following 1 and 5 days of withdrawal from chronic amphetamine. Finally, using both *in vitro* and *in vivo* systems, we were able to co-immunoprecipitate HDAC1 with ΔFosB to demonstrate that the two proteins are interacting partners. These data establish the groundwork for an intriguing model where HDAC1 and ΔFosB interact as a repressive complex to desensitize c-Fos induction in response to chronic drug administration (Figure 3-14).

Figure 3-14

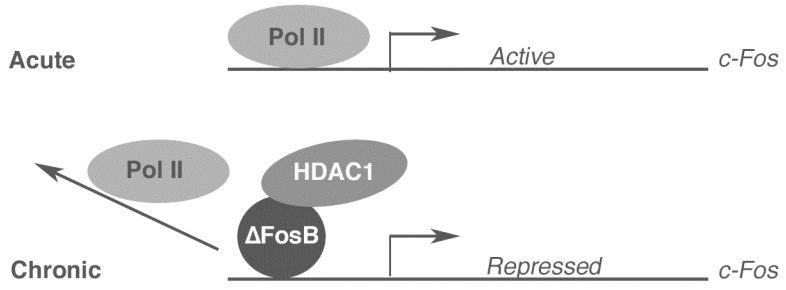


Figure 3-14 Mechanism of *c-fos* Activation and Repression. Following an acute stimulation, *c-fos* is activated by Pol II. In contrast, following chronic stimulations, *c-fos* is repressed when ΔFosB recruits HDAC1 to the *c-fos* promoter.

Future Directions

To establish ΔFosB and HDAC1 as the *bona fide* transcriptional repressive complex functioning at the c-Fos promoter, future experiments are necessary. First, interaction between ΔFosB and HDAC1 will be demonstrated biochemically following 5 days of amphetamine withdrawal *in vivo* using co-immunoprecipitation experiments.

Second, to establish ΔFosB as the initial repressive factor recruited to the c-Fos promoter, ΔFosB-AAV will be infected into rat nucleus accumbens. Animals will be challenged with acute amphetamine, and then sacrificed. Laser capture will be used to isolate specific infected cells by co-overexpression of GFP, then c-Fos mRNA will be amplified and quantified by qPCR. We expect that overexpression of ΔFosB will repress c-Fos induction. Finally, to establish HDAC1 as the ΔFosB co-repressor, we will use ΔFosB transgenic mice. This transgenic mouse line overexpresses ΔFosB in striatum in the absence of doxycycline (dox). ChIP will be performed using an HDAC1 antibody to assess its occupancy on the c-Fos promoter. We expect more HDAC1 present at the c-Fos promoter in animals off dox than on dox.

Our present study establishes the capacity of ΔFosB to interact with HDAC1. This interaction suggests one mechanism by which ΔFosB may repress gene transcription. Following repeated drug treatment, ΔFosB is induced and gradually accumulates in the cell. 24 hrs following chronic drug treatment, c-Fos desensitization correlates with a decrease in RNA polymerase II and the appearance of ΔFosB at the c-Fos promoter. We hypothesize that ΔFosB represses c-Fos transcription 24 hrs post-chronic drug administration by preventing Pol II recruitment to the promoter. Following 5 days of withdrawal, more ΔFosB accumulates in the cell and is capable of recruiting HDAC1 to the c-Fos promoter. In addition, other repressive mechanisms, such as dimethylation of histone H3 lysine 9, are also recruited to the complex to prevent c-Fos transcription.

ΔFosB Transcription and Chromatin Remodeling

The mechanism by which ΔFosB mediates both activating and repressive effects on gene transcription has long puzzled the field. The mechanism by which another transcription factor, YY1, mediates both effects has been demonstrated to somewhat straight forward: YYI induces gene activation only when associated with E1A, a protein that activates the AAV P5 promoter. In its absence, YY1 represses gene transcription (Gordon, *et al,* 2005). Here, we describe a similarly simple mechanism that may be involved with the dual effects of ΔFosB: ΔFosB may selectively activate or repress specific promoters by recruitment of HATs or HDACs, although we have little insight into what determines these two distinct actions, as will be discussed below.

Recently, Kumar, *et al*, (2005) observed chronic cocaine administration induces hyper-acetylation of histone H3 at the promoter of the ΔFosB target gene, *Cdk5*. In addition, association of ΔFosB at the *Cdk5* promoter is correlated with increased binding of the Brg-1-containing SWI-SNF chromatin remodeling proteins (Kumar, *et al,* 2005). Brg1 is the core ATPase subunit in the SWI-SNF multi-protein complex that is involved in chromatin remodeling of numerous mammalian genes (Neely & Workman, 2002; Kadam & Emerson, 2003). The CREB binding protein and p300 (CBP-p300) are key regulators of Pol II-mediated transcription through their HAT activity. CBP-p300 is recruited to promoters by transcription factors to interact with chromatin remodeling machinery, such as Brg1, and Pol II, to stabilize the transcriptional complex (Neish, *et al,* 1997; Kalkhoven, *et al,* 2004). Kumar *et al* demonstrated that both ΔFosB and Brg1 are present at the C*dk5* promoter when histones at the promoter are hyper-acetylated. CBP-p300 is known to interact with Brg1 in a transcriptional complex with the Pol II

holoenzyme (Neish, *et al,* 1997). It is important to examine if ΔFosB directly interacts with CBP-p300. ΔFosB recruitment of CBP to target gene promoters, such as *Cdk5,* may be one mechanism by which ΔFosB promotes acetylation of histones and transcriptional activation.

Here, we show that ΔFosB interacts with HDAC1, and by this mechanism may recruit HDAC1 to the c-Fos promoter to deacetylate histones and repress transcription. The specific mechanism governing which genes ΔFosB activates and which genes ΔFosB represses is still completely unknown. Other factors are clearly involved. The ΔFosB AP-1 dimerization partner *in vivo* has still not been fully explored. Studies have focused on Jun family members, namely JunD, possibly due to its abundance in the cell; however, over 50 AP-1 interacting proteins have been identified (Chen *et al*, 1995, 1997; Chinenov & Kerppola, 2001). In addition, some preliminary studies have indicated that ΔFosB may heterodimerize with FosB or perhaps even homodimerize (McClung C.A., Rudenko, G., personal communications). One hypothesis is that the ΔFosB AP-1 dimerization partner may change as ΔFosB accumulates in the cell over time. This may explain how ΔFosB represses the *c-fos* promoter by two different mechanisms following 24 hrs of withdrawal and 5 days of withdrawal. In addition, the ΔFosB complex at the *Cdk5* promoter intuitively must be different than that at the *c-fos* promoter, because it does not recruit HDAC1, but likely CBP-p300. In addition, cell signaling cascades and ΔFosB post-translational modifications may also be important for mediating these differential effects.

The major finding of this study is that ΔFosB may be responsible for the desensitization of c-Fos induction in response to chronic drug administration by two distinct mechanisms: first, ΔFosB could reduce Pol II binding to the *c-fos* promoter

following 24 hrs of withdrawal. Second, ΔFosB represses the *c-fos* promoter by recruitment and interaction with HDAC1. These data suggest a model by which ΔFosB may mediate activation and repression of gene transcription. Through recruitment of histone-modifying enzymes (HATs and HDACs), ΔFosB activates genes, such as *Cdk5*, or represses genes, such as *c-fos*. Future studies are required to assess the conditions necessary for interaction with HDAC1 and transcriptional repression, and for analysis of ΔFosB interaction with CBP-p300.

CHAPTER 4

CONCLUSIONS

In this thesis, I characterized two biochemical properties of the ΔFosB protein that are critical for its function in neuronal plasticity: enhanced protein stability and interaction with HDAC1. Together, these two properties help establish ΔFosB as the longest-lived adaptation known in the adult brain, both as a physical, persistent presence, and possibly as a mediator of chromatin remodeling, which could lead to more permanent changes in gene expression.

FosB Destruction

In Chapter 2, I identified two distinct degron domains on the FosB C-terminus that contribute to its rapid degradation by both proteasome-dependent and proteasome-independent mechanisms. The proteasome-dependent degron domain (amino acids 277-338) contains two regions that destabilize FosB through separate mechanisms. Amino acids 277-317 are necessary to signal poly-ubiquitylation of FosB and proteasomal degradation of the protein in asynchronous cells. Amino acids 318-338 also signal FosB degradation by the proteasome, but through an ubiquitin-independent mechanism. This model is consistent with the mechanism of c-Fos degradation. In asynchronous cells, c-Fos destruction is under the control of a C-terminal destabilizer that does not need an active ubiquitin cycle (Bossis, *et al,* 2003).

Interestingly, the c-Fos destabilizers that operate in cells undergoing the G_0/S_1 phase transition are largely different than those in asynchronous cells. Synchronous cells are under the control of both N and C-terminal destabilizing elements. The N-terminal

destabilizer is ubiquitin sensitive and may be limited by phosphorylation of downstream residues. The C-terminal destabilizer is ubiquitin independent and is the only destabilizer active in asynchronous cells (Bossis, *et al,* 2003). Further studies are necessary to examine if similar mechanisms are involved with FosB degradation. Here, we have established that FosB is degraded by the proteasome in both synchronous and asynchronous cells and that ΔFosB is not. The C-terminal 61 amino acids of FosB direct its proteasome-mediated degradation in asynchronous cells, however the targeting of FosB to the proteasome during the G_0/S_1 phase transition is not yet fully explored. Although we have demonstrated that FosB can be ubiquitylated, it is not certain if this event is necessary to direct FosB proteasomal destruction or if FosB can be degraded independent of ubiquitin signaling, similar to c-Fos and other proteins, such as p53. In addition, it would be interesting to determine if FosB contains an N-terminal destabilizing element analogous to c-Fos. There are some observations that suggest that it may.

Thus, a recent study by Ulery *et al* (2006) found that ΔFosB was protected from rapid proteasome-mediated degradation by phosphorylation at serine 27 by casein kinase 2 in asynchronous cells. Mutation of this site decreased the stability of ΔFosB from approximately 10 hrs to 4 hrs. In addition, my work demonstrated that CK2 knockdown in cells during the G_0/S_1 phase transition also decreased the stability of endogenous ΔFosB. These studies indicate that serine 27 is critical for destabilization of ΔFosB in both asynchronous and synchronous cells, similar to the C-terminal destabilizer in c-Fos. Mutation of this site in full-length FosB did not have any observable effect on FosB stability in asynchronous cells; (Ulery P.G., personal communication), however, a more

intensive analysis of FosB stability, ubiquitylation, and phosphorylation during the G_0/S_1 phase transition following mutation of this site is required. Serine 27 and the region surrounding it may serve as the FosB N-terminal destabilizer, analogous to the N-terminal destabilizer in c-Fos. This region may play an important role in proteasome-mediated degradation of FosB specifically in the G_0/S_1 phase transition. In contrast, in ΔFosB, this N-terminal region appears to be the major destabilizing element that functions in both asynchronous and synchronous cells.

Enhanced Protein Stability

The identification of the FosB degron domains and the observation that ΔFosB is stabilized by CK2 phosphorylation offer great insight to ΔFosB's long-term expression in cells. Together, these studies present a general model of the enhanced stabilization of ΔFosB. Repeated perturbations to the brain induce the *fosB* gene. Apparently constitutive alternative splicing of the primary transcript generates ΔFosB mRNA. This mRNA specifically lacks the FosB C-terminal degron domains, indicating that protein translated from this template will only contain the N-terminal FosB destabilizing element. By this mechanism, ΔFosB can only be directed to proteasomal degradation by one destabilizer, instead of two; therefore, ΔFosB will be more stable than FosB or c-Fos. Once ΔFosB is translated, the N-terminal destabilizer is either silenced by serine 27 phosphorylation and ΔFosB is stabilized, or ΔFosB is degraded by the proteasome. Future studies are necessary to assess the validity of these interpretations and, specifically, the existence of the FosB N-terminal destablilizer.

This model is in general agreement with the first hypothesis proposed by Nestler and colleagues in 1994 (Hope, *et al,* 1994a; see also Nestler, *et al,* 2001). This

hypothesis stated that the native state of ΔFosB is 33kD, when it is acutely induced at very low levels. Post-translational modifications alter the protein to its more stable forms at 35 and 37kD, which is detected following chronic perturbations. The nature of these modifications has remained obscure. Although recent data indicate that serine 27 phosphorylation contributes to ΔFosB stabilization, my analysis of the S27AΔFosB mutant indicates that there is no change in ΔFosB molecular weight following mutation of this site, at least in my gel system (Figure 4-1). In addition, my general observations from working with endogenous ΔFosB in cell culture has indicated that treatment with phosphatase inhibitors or the CK2 inhibitor, DRB, does not increase or decrease the ratio of the 35kD to 37kD bands. Although these observations are hardly conclusive, my overall indication is that phosphorylation at serine 27 is not solely responsible for the 4 kD difference in apparent molecular weight among the various ΔFosB isoforms. An additive effect of phosphorylation at other residues and/or glycosylation, acetylation, or some combination of other post-translational modifications are likely responsible for this shift in molecular weight from 33kD to 37kD.

Figure 4-1

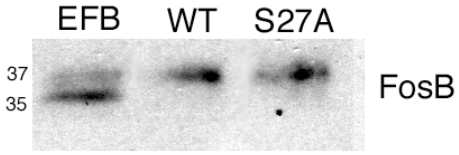


Figure 4-1. Mutation of serine 27 does not alter ΔFosB molecular weight PC12 cells were transfected with wild-type FosB (WT) or the serine 27 to alanine (S27A) ΔFosB mutant, then western blotted with a FosB antibody 24hrs later. PC12 cells were also serum stimulated to generate endogenous ΔFosB (EFB) for comparison.

Another question raised by this model is the identity and fate of the 35kD ΔFosB isoform. The endogenous 37kD ΔFosB isoform is resistant to proteasomal degradation and extremely stable, with 50% remaining detectable in cells for approximately 24-30 hrs. Interestingly, the endogenous 35kD ΔFosB isoform also appears insensitive to proteasomal degradation; however, it only persists in the cell for approximately 10-12 hrs after serum stimulation (Figure 4-2). These data suggest that this protein may be phosphorylated at serine 27, yet still lacks some other stabilizing modification that would increase its apparent molecular weight by ~2 kD. These observations bolster the hypothesis that the increasing apparent molecular weight is associated with something that is critical for ΔFosB's enhanced stability *in vivo*; however, the mechanism that mediates this change remains elusive.

Figure 4-2

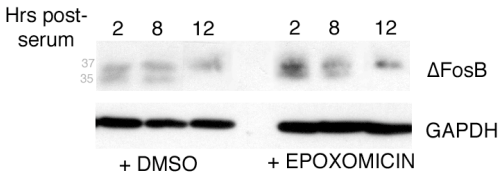


Figure 4-2. The 35kD ΔFosB Isoform is Proteasome Insensitive, yet less Stable than the 37kD Isoform
Endogenous production of ΔFosB protein was induced in PC12 cells by serum starvation for approximately 20 hr, followed by serum stimulation for 2 hr. Cells were then incubated in serum-free DMEM (+/- 2.5 μM epoxomicin or vehicle) for indicated time points and run on a 10% acrylamide gel for Western blotting. Membranes were probed for FosB/ΔFosB, then stripped and re-blotted for GAPDH as a protein loading control.

One finding worth noting here is that I observed prolonged exposure of cells to proteasome inhibitors re-induced FosB and ΔFosB mRNAs after approximately 12-16 hrs. I confirmed this observation by qPCR. This phenomenon confounded my stability analyses and proteasome sensitivity analyses of the endogenous proteins by western blot for the long time points. Pulse chase is clearly the best method to avoid this issue and

analyze protein stability, however, the artifacts associated with an overexpression system have also been described in my thesis. The half-life I calculated for overexpressed ΔFosB is likely somewhat underestimated due to the saturation of CK2 by overexpression of the protein. Overexpression of both proteins in the assay may be one option to calculate another, more accurate ΔFosB half-life; however, innumerable, unaccounted for phosphoproteins could also exacerbate the system. The best experiment to calculate the half-life of ΔFosB is a pulse chase of the endogenous protein and, unfortunately, I was never able to detect a linear signal with endogenous protein using this assay.

ΔFosB Transcriptional Complexes

The extraordinary stability of ΔFosB is important for its role as a molecular switch that can initiate the sustain changes in gene expression in response to stimuli. Previous studies have established that ΔFosB both activates and represses AP-1 dependent gene transcription (Dobrazanski *et al*, 1991; Nakabeppu & Nathans, 1991; Yen *et al*, 1991; McClung, *et al,* 2003). In chapter three of this thesis, I presented data that demonstrated that ΔFosB interacts with HDAC1, and this interaction may cause repression of the ΔFosB target gene, *c-fos,* following chronic amphetamine treatment. A similar phenomenon has recently been described. An HDAC1-NFκB-p50 complex was recently reported to repress transcriptional initiation at the HIV long terminal repeat (LTR) by inducing histone deacetylation and impairing recruitment of RNA Pol II. Through this mechanism, long-term HIV latency is maintained (Williams, *et al,* 2006).

Interestingly, only NFκB p50 homodimers interact with HDAC1 and repress transcription, while NFκB RelA-p50 heterodimers do not (Williams, *et al,* 2006). The study presented in chapter three does not evaluate whether ΔFosB requires a dimerization

partner to interact with HDAC1, nor does it evaluate the ΔFosB dimerization partner that is bound to the *c-fos* promoter. In addition, there has been no analysis of the ΔFosB dimer that is bound to the *Cdk5* promoter that activates transcription and may interact with CBP. These data are severely lacking in the biochemical characterization of ΔFosB and are becoming more and more important to its function. The most logical explanation is that the activating and repressing ΔFosB complexes are composed of different proteins. For example, I would speculate that the activating complex may be a ΔFosB homodimer that binds CBP, while the repressive complex may be the ΔFosB/JunD complex that binds HDAC1. Another possibility is that the unexplored post-translational modifications that increase ΔFosB's apparent molecular weight are critical for the functioning and interactions of these dimers. In order to critically examine the nature of these complexes, ChIP protocols must be developed that employ primary, then secondary immunoprecipitations to pull down the specific dimeric complexes on known ΔFosB activating and repressive promoters.

While much more work needs to be done in order to understand the details of ΔFosB binding with HDAC1 and how this complex specifically regulates transcription, the discovery that ΔFosB and HDAC1 interact reveals an intriguing possibility for the role of ΔFosB in mediating long-term behavioral plasticity. Transient induction of ΔFosB may lead to more permanent changes in gene expression through modifications to chromatin. Histone deacetylation and methylation are now considered to be major factors contributing to permanently silenced chromatin structure that is associated with the permanent changes in gene transcription that occurs during development (Jenuwein & Allis, 2001). The behavioral consequences of drug abuse and other compulsive habits are

essentially life-long adaptations. The persistence of a ΔFosB-HDAC1 complex may contribute to long-term transcriptional changes by modifying chromatin structure. In addition, future investigations also must examine ΔFosB interaction with CBP. Similar, long-term adaptations may be mediated by an activating ΔFosB-CBP complex that hyperacetylates histones. Future studies are required to examine these speculations.

General Conclusion

This is the first study to examine the mechanisms for the relative stability of ΔFosB compared to FosB. I determined that two C-terminal domains promote FosB's rapid degradation in asynchronous cells: one proteasome-independent domain and one proteasome-sensitive domain. FosB degradation in the G_0/S_1 phase transition is not yet fully explored, however, evidence from c-Fos and ΔFosB literatures suggest that an N-terminal destabilization domain may be important to direct its degradation. Previous studies have indicated that serine 27 functions as the ΔFosB destabilizing element in both asynchronous and synchronous cells. Specifically, CK2 phosphorylation of serine 27 protects ΔFosB from proteasome-mediated degradation in asynchronous cells, and CK2 knockdown destabilizes ΔFosB during the G_0/S_1 phase transition. Taken together, these data suggest a model where silencing of the single known ΔFosB destabilizer is sufficient to mediate long-term stabilization of the protein. In contrast, several mechanisms would be required to mediate stabilization of FosB or c-Fos, due to the complexity of their degradation pathways. In addition, FosB increases in apparent molecular weight over time when induced by serum in PC12 cells, indicating FosB may also be post-translationally modified, similar to ΔFosB (Figure 4-3). These findings indicate that the

ΔFosB post-translational modifications may not be sufficient to stabilize the protein on their own. Taken together, these data thereby show that the absence of the FosB C-terminal degron domains is a critical cellular mechanism that stabilizes ΔFosB as a result of alternative splicing.

Figure 4-3

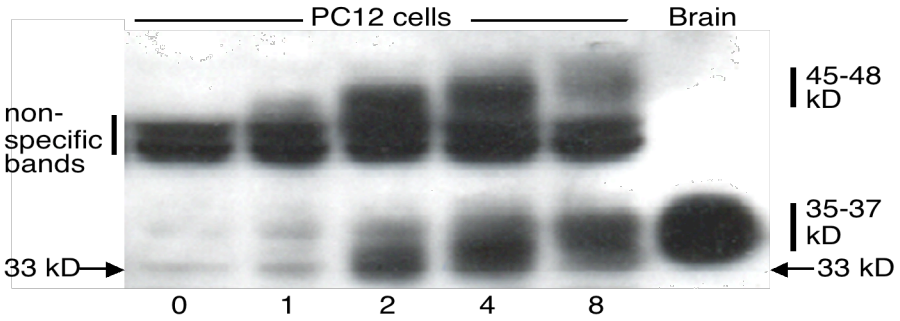


Figure 4-3. Expression of FosB Protein Isoforms Quiesent PC12 cells were stimulated for 0-8 hours with 20% serum. The molecular weight of both FosB and ΔFosB increases over time suggesting the presence of post-translational modifications. (Adapted from Alibhai I.N., 2005).

ΔFosB interaction with chromatin remodeling enzymes, such as HDAC1 or CBP, is one mechanism by which ΔFosB may both activate and repress transcription and mediate long-term changes in gene expression (Figure 4-4). Specifically, *c-fos* may be the first gene analyzed that is repressed by a ΔFosB-HDAC1 repressive complex. It would be interesting in the future to determine whether other genes repressed by ΔFosB (e.g., dynorphin) show similar recruitment of HDAC1. Future studies also must focus on ΔFosB interaction with CBP. Some evidence suggests that CBP and ΔFosB mediate similar effects in response to drugs of abuse. One study found that CBP haploinsufficient mice are less sensitive to the locomotor effects of cocaine, suggesting the presence of CBP is necessary for the rewarding effects of drugs of abuse, similar to ΔFosB (Levine, *et al,* 2005). In addition, the presence of the Brg1 chromatin remodeling protein at the

Cdk5 promoter at the same time as ΔFosB also suggests CBP and ΔFosB may interact (Kumar, *et al,* 2005). Brg1 and CBP are known to interact in the Pol II haloenzyme complex (Neish, *et al,* 1997). These interpretations are speculative, however, the possibility of ΔFosB mediating both activating and repressive effects through interactions with HATs and HDACs is an interesting possibility that fits the data presented thus far. In addition, ΔFosB interaction in these large multi-protein complexes on promoter regions may also serve to protect itself from de-phosphorylation at serine 27, thus remaining stabilized in the brain for extended periods of time.

Figure 4-4

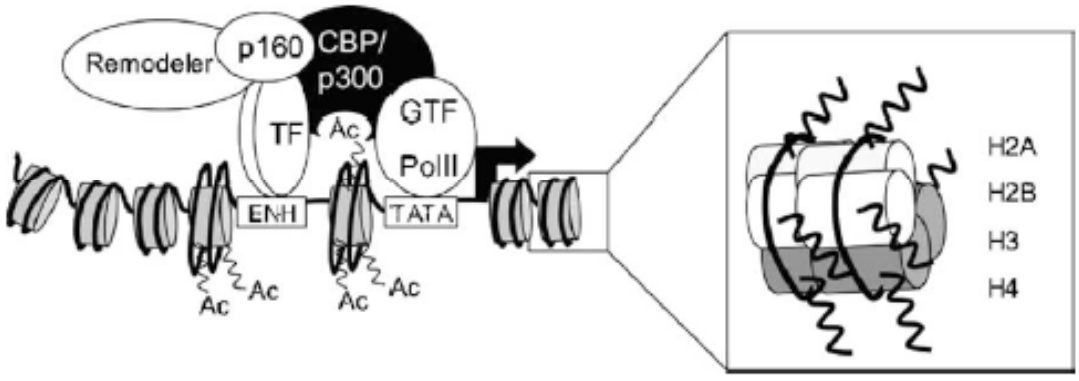


Figure 4-4. ΔFosB Regulates Transcriptional Activation or Repression. Schematic representation of the promoter region of a gene, with the DNA wrapped around nucleosomes, which consist of octamers the histone proteins H2A, H2B, H3 and H4. A ΔFosB transcription factor dimer (TF) such as ΔFosB/JunD, binds to the enhancer region (ENH), then recruits remodeling factors (remodeler), such as Brg1 and HATs, like CBP or p300 (CBP/p300), or HDACs, such as HDAC1. HATs, (CBP/p300) can make the DNA more accessible for other regulatory proteins by acetylating the histone tails (Ac), while HDACs do the opposite. In addition, CBP and p300 can form a physical bridge between transcription factors and the general transcription factors (GTF) and RNA polymerase II (Pol II). (Adapted from Kalkhoven, *et al,* 2004)

In conclusion, ΔFosB is a unique transcription factor that is derived from the *fosB* gene via alternative splicing. Although this splicing event has been shown to not be regulated in itself, the location of the specific intronic sequence and the resulting

frameshift mutation that generates ΔFosB with exactly 101 amino acids truncated cannot be deemed accidental or an unintentional event. Truncation of these amino acids is important for ΔFosB stability and interaction with protein partners. The precise formation of ΔFosB is a coordinated event for a natural purpose; however, in the age of illicit drugs and unhealthy addictions, the brain's reward system is hijacked and the accumulation of ΔFosB in unexpected, unnatural places is the result. Although numerous studies have offered some insight into the molecular consequences of this accumulation, ΔFosB as a target for addiction therapy is still a long way off.

METHODOLOGY

Animals - All animal procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by our IACUC. Male Sprague-Dawley rats (initial weight, 250 g; Charles River, Kingston, RI), and ΔFosB mutant mice off and on doxycycline (10–14 weeks old) under conditions that maximally induce ΔFosB (Kelz *et al,* 1999) were used in these studies. The animal colonies were kept on a 12 hr light/dark cycle. Mice were housed in groups (four per cage), while rats were housed in pairs; food and water were made available ad libitum.

Amphetamine treatment – Acute group: rats were given a single i.p. injection of 4 mg/kg amphetamine or saline in equal volumes and killed 1hr or 24 hrs later. Chronic group: rats were given daily i.p. injections of 4 mg/kg amphetamine or saline for 7 days and were killed 1 hr, 24 hrs, or 5 days later. Brains were removed rapidly from decapitated animals and processed immediately.

ECS treatment – Rats (250–275 g) received a single ECS via ear-clip electrodes (acute group) or daily ECS for 7 days (chronic group) between 2:00 P.M. and 4:00 P.M. each day (ECS frequency, 100 pulses/sec; pulse width, 0.5 msec; shock duration, 0.5 sec; current, 50 mA). Control animals received sham treatments: they were handled identically to ECS-treated animals but without electrical stimulation. Animals were killed by decapitation 4 hr or 24 hr after the last seizure.

Cell culture and transfections - Rat adrenal pheochromocytoma (PC12) cells (Clontech, Mountain View, CA) were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% horse serum and 5% fetal bovine serum (FBS) (Invitrogen) with antibiotics at 37°C and 5% CO2. Cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Transfection efficiency was determined by co-expression of GFP with all plasmids. The total amount of DNA within experiments was kept constant by adding empty vector to the transfection mixture when necessary. All experiments were repeated at least 3 times, and representative figures are shown. Proteasome inhibitors MG132 (Calbiochem, La Jolla, CA) and epoxomicin (Peptides International, Minoh-Shi Osaka, Japan) were dissolved in dimethyl sulfoxide (DMSO).

Expression constructs - FosB and ΔFosB cDNAs were obtained from pTetOp-constructs previously described (Chen, *et al,* 1997), then subcloned into the pcDNA 3.1 mammalian expression vector (Invitrogen). The QuickChange Site Directed Mutagenesis kit (Stratagene, LaJolla, CA) was used to generate several mutant plasmids pcDNA3.1-FosB(P152A), pcDNA3.1-FosB(1-317) and pcDNA3.1-FosB(1-277). The pcDNA3.1-JunD plasmid was a gift from Marie-Claire Peakman (Pfizer Central Research, Groton, CT) and the pcDNA-HA-ubiquitin plasmid was a gift from James Chen (UT Southwestern). The pcDNA3.1-hygro-Hsc70 and pcDNA3.1-hygro-Hsc70(K71M) were a gift from Danny Manor, (Cornell University.) The pCB6-HA-YY1 was a gift from Steve Broyles (Purdue University.)

Immunoprecipitations, Western blots, and antibodies - Approximately 24 hr following transfection, cells were incubated with 5 μM epoxomicin or DMSO for indicated times, then lysed in RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 0.1% deoxycholate). Protein concentrations were determined using the Bio-Rad (Hercules, CA) DC protein determination assay according to the manufacturer's recommendations. Lysates were split in half, incubated with either non-immune IgG (Sigma) or with a protein specific antibody (FosB (#sc-48G), HA (#sc-7392, Santa Cruz, Santa Cruz, CA); V5 (#V-8012, Sigma); HDAC1 (#ab-7028-50, AbCam, Cambridge, MA)) and rotated overnight at 4°C. Immunoprecipitates were then incubated with Protein G beads (Sigma, Saint Louis, MO) for at least 2 hr before washing. Beads were collected by brief centrifugation, then washed extensively with LSL-RIPA buffer (20 mM Na phosphate pH 7.4, 150 mM NaCl, 0.1% Triton-X), then with HSL-RIPA buffer (50 mM Tris pH 7.4, 1M NaCl, 1mM EDTA, 1% NP-40, 0.1% deoxycholate). The immunoprecipitated proteins were analyzed by Western blot with specific antibodies. (custom polyclonal FosB antibody (previously described by Perrotti et al, 2004), ubiquitin (#89899, Pierce, Rockford, IL); casein kinase 2 (#231573, Calbiochem, LaJolla, CA); V5 (#V-8012, Sigma); HA (#sc-7392), YY1 (#SC-1703, Santa Cruz); lamin (#2032, Cell Signaling); ß-tubulin (05-661, Upstate, Lake Placid, NY); GAPDH (#RDI-TRK5G4-6C5, Research Diagnostics, Concord, MA); Hsc70 (SPA-816), Hsp90 (SPA-830), Hsp70 (SPA-810, Stressgen, Victoria, BC.)) Blots were visualized using ECL Plus (Amersham Biosciences, Piscataway, NJ) reagent according to manufacturer's instructions. Bands were quantified using the NIH Scion image analysis software.

Pulse-chase experiment - Approximately 24 hr following transfection, cells were incubated at 37°C in Cys/Met- free DMEM supplemented with 10% dialyzed fetal bovine serum (Invitrogen) for at least 2 hr. Drugs or vehicle were added at this time. Following starvation, proteins were labeled by adding 0.2 mCi/ml ^{35}S (Perkin Elmer, Boston, MA) to the Cys/Met- free media for an additional 2 hr. Cells were then washed and incubated with regular media. Drugs or vehicle were re-added at this time and refreshed with new media every 6 hr. Cells were lysed in RIPA buffer at indicated times and immunoprecipitated as described. After immunoprecipitation and SDS-PAGE gel electrophoresis, the radioactive signals were quantified using Image-Quant software on the Stormscan phosphoimager (Amersham/Pharmacia, Piscataway, NJ).

Ubiquitylation assay – We followed published protocols, using both non-denaturing and denaturing conditions, to study FosB and ΔFosB ubiquitylation (Cottrell *et al*., 2006; Inoue *et al.,* 2006). PC12 cells were co-transfected with indicated plasmids and 0.875 μg of an HA-tagged ubiquitin plasmid. 24 hrs after transfection, cells were incubated with 2.5 μM epoxomicin for 24 hrs. For non-denaturing immunoprecipitation, cells were lysed in 200μL of mRIPA for 20 min on ice, scraped, and centrifuged (20,000 *g*, 10 min, 4°C). For denaturing immunoprecipitation, cells were lysed in 1% SDS and boiled for 15 min, to destroy endogenous isopeptidase activity. Lysates were mixed with 1 volume of mRIPA and centrifuged (20,000 *g*, 10 min, 4°C). Supernatants for both non-denaturing and denaturing preparations were diluted to 1 mL with mRIPA and rotated with antibodies (FosB 5μg/mL, non-immune goat IgG 5μg/mL) overnight at 4°C. Protein G was added (20μl) and samples were incubated for 2 hr at 4°C. Immunoprecipitates were

pelleted and washed 4 times as described above, then resuspended in 15µl Laemlli buffer with β-mercaptoethanol, boiled for 15 min, and analyzed by Western blot.

Determination of hydrolytic activity of the proteasome -- Proteasome activity was assessed in lysates of PC12 cells using the synthetic peptide substrate, Suc-Leu-Leu-Val-Tyr (Suc-LLVY), linked to the fluorimetric reporter, aminomethylcoumarin (AMC), as described by Wojcik & DeMartino (2002). PC12 cells cultured in 6-well plates, treated with vehicle or 5 µM epoxomicin for indicated time points, were collected, washed in PBS, and lysed in Buffer H (20 mM Tris, 20 mM NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol, pH 7.6) for 30 min on ice. Cell lysates were cleared by centrifugation, and the supernatants were used for determination of protein concentration and enzymatic activity. Lysates (25 µl) were assayed by addition of 250 µl of a 50 µM solution of Suc-LLVY-AMC (in 50 mM Tris-HCl, pH 8.0, and 1 mM β-mercaptoethanol) and incubation for 30 min at 37 °C. AMC hydrolyzed from the peptides was quantified in a BioTek FL600 plate reader using 360 nm excitation and 460 nm emission wavelengths. Enzymatic activity was normalized for protein concentration and expressed as percent of activity in control lysates. Each measurement was carried out using at least three independent experiments.

Luciferase assay – Luciferase reporter assays were performed as described previously (Chen, *et al,* 1997). Transfected PC12 cells were lysed in the 1 x reporter lysis buffer (Promega, Madison, WI). Relative luciferase activity, assayed as described by the manufacturers protocol and measured in a luminometer, was calculated as enzyme

activity per microgram of total protein and expressed as fold over control.

RNA Isolation - RNA was harvested from PC12 cells using RNA STAT-60 (Tel-Test, Friendswood,TX) and precipitated with isopropanol following the manufacturers protocol. Briefly, the organic layer was extracted with chloroform and precipitated with isopropanol in the presence of Linear Acrylamide (Ambion, Austin TX). The RNA pellet was washed with 70% ethanol and re-suspended in DEPC water. The purified total RNA was DNAse treated (Ambion) and reverse transcribed to cDNA with random hexamers using a first-strand synthesis kit (Invitrogen). The amount of cDNA was quantified using q-PCR. The following primers were used to amplify specific cDNA regions of the transcripts of interest:

ΔFosB	5'-AGGCAGAGCTGGAGTCGGAGAT-3' 5'-GCCGAGGACTTGAACTTCACTCG-3'
FosB	5'-GTGAGAGATTTGCCAGGGTC-3' 5'-AGAGAGAAGCCGTCAGGTTG-3'
c-Fos	5'-GGAATTAACCTGGTGCTGGA-3' 5'-TGAACATGGACGCTGAAGAG-3'
GAPDH	5'-AGGTCGGTGTGAACGGATTTG-3' 5'-TGTAGACCATGTAGTTGAGGTCA-3'

Real-time PCR was performed in triplicate using an Applied Biosystems 7500 Real-Time PCR System (95°C- 10 min, 1cycle; 95°C-20 sec, 61°C 30 sec, 72°C- 33 sec, 35 cycles; melt curve from 60°C-95°C) with SYBR Green Master Mix (Applied Biosystems, Foster City, CA). GAPDH quantification was used as an internal control for normalization. Fold differences of mRNA levels over control values were calculated using the ΔΔCt method as described previously (Applied Biosystems manual).

Chromatin Immunoprecipitation Assay - ChIP assays were performed as described

(Kumar, *et al,* 2005; Tsankova et al, 2004). The following antibodies were used: anti-HDAC1; anti-Pol II, anti-dimethyl K9 and nonimmune rabbit and mouse IgG (all from Upstate Biotechnology). For the ΔFosB ChIP, the sequential method was employed as described by Kumar, *et al,* 2005, using two FosB antibodies (C-terminal FosB, Center for Biomedical Inventions, UTSW; SC048, Santa Cruz Biotechnology, Santa Cruz, CA). These ChIP experiments were performed in collaboration with Will Renthal.

siRNA Knockdown of casein kinase 2 - We selectively knocked down CK2 levels in PC12 cells using RNA interference. PC12 cells were transiently transfected with 20 nM (final concentration) of either non-targeting siRNA or siRNA directed toward the mRNA of the catalytic α subunit of Rat CK2, using 5 μl of the transfection agent SilentFectin (Bio-Rad) and following manufacturer's instructions. ~24 hr later, cells were serum starved (0.5% HS), then ~48 hr post-siRNA transfection, cells were serum stimulated (20% HS) for 2 hours, then incubated in serum free DMEM. The following CK2α siRNAs were used: 5'CGGAAGAUUUAUAUGACUAUU'3 and

5'P-UAGUCAUAUAAAUCUUCCGCU3'. As negative control we used si*CONTROL* Non-targeting siRNA #1 (#D-001210-01-05, Dharmacon, Lafayette, CO). The extent of CK2 knockdown was monitored by immunoblotting (described above).

Subcellular Fractionation - Rats were treated with electroconvulsive seizure (Hope, *et al*, 1994b) for 1 or 6 days. Animals were sacrificed 4 or 24 hours after the last treatment. After gross dissection, cortical tissue was homogenized by a Dounce homogenizer in 500 μl of 0.32 M sucrose, 1 mM $MgCl_2$. The homogenate was diluted with 0.32 M sucrose to 2 mL before centrifugation (2000 x g, 10 min), to obtain the crude nuclear fraction (P1).

The supernatant (S1) was saved. The pellet was re-suspended in PBS and washed twice. The resulting P1 pellet was homogenized in 0.32 M sucrose, then pelleted again (20,000 x G, 10 min) to obtain the soluble P1 fraction. The S1 fraction was pelleted, (100,000 x G, 1 hr) and the supernatant saved to obtain the crude cytosolic fraction (S3). Protein concentration was determined by the DC protein assay method (Bio-Rad) using bovine serum albumin as a standard.

BIBLIOGRAPHY

Acquaviva C., Bossis G., Ferrara P., Brockly F., Jariel-Encontre I., & Piechaczyk M., (2002) Multiple degradation pathways for Fos family proteins. *Ann. N. Y. Acad. Sci. USA,* 973, 426-434.

Acquaviva C., Brockly F., Ferrara P., Bossis G., Salvat C., Jariel-Encontre I., & Piechaczyk M. (2001) Identification of a C-terminal tripeptide motif involved in the control of rapid proteasomal degradation of c-Fos proto-oncoprotein during the G(0)-to-S phase transition. *Oncogene,* 20, 7563-7572.

Alibhai I.N., Green T

Andersson M., Westin JE., & Cenci M.A. (2003) Time course of DeltaFosB-like immunoreactivity and prodynorphin mRNA levels after discontinuation of chronic dopaminomimetic treatment. *Eur. J. Neurosci*. 17, 661-666.

Ang E., Chen J.S., Zagouras P., Magna H., Holland J., Schaeffer E., Nestler E.J. (2001) Induction of NFnB in nucleus accumbens by chronic cocaine administration. *J. Neurochem.* 79, 221– 224.

Asher G., & Shaul Y. (2005) p53 proteasomal degradation: poly-ubiquitination is not the whole story. *Cell Cycle*, 8, 1015-1018.

Atkins J., Carlezon W.A., Chlan J., Nye H.E., Nestler E.J. (1999) Region specific induction of ΔFosB by repeated administration of typical versus atypical antipsychotic drugs, *Synapse* 33,118–128.

Baichwal V.R. & Tjian R. (1990) Control of c-Jun activity by interaction of a cell-

specific inhibitor with regulatory domain delta: differences between v- and c-Jun. *Cell,* 63, 815–825.

Bamberger A.M., Methner C., Lisboa B.W., Stadtler C., Schulte H.M., Loning T., Milde-Langosch K. (1999) Expression pattern of the AP-1 family in breast cancer: association of fosB expression with a well-differentiated, receptor-positive tumor phenotype. *Int J Cancer*. 84, 533-8.

Berton O., Tsankova N.M., **Carle T.L**., Ulery P.G., Bhonsle A., Barrot M., Neve R.L., and Nestler E.J. (Submitted) Induction of ΔFosB in the periaqueductal gray by stress promotes active coping responses.

Bibb J.A., Chen J.S., Taylor J.R., Svenningsson P., Nishi A., G.L. Snyder A., Yan Z., Sagawa Z.K., Ouimet C.C., Nairn A.C., Nestler E.J., Greengard P. (2001) Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. *Nature* 410, 376– 380.

Bossis G., Ferrara P., Acquaviva C., Jariel-Encontre I., & Piechaczyk M. (2003) c-Fos proto-oncoprotein is degraded by the proteasome independently of its own ubiquitinylation in vivo. *Mol. Cell Biol.*. 20, 7425-7436.

Brown J.R., Nigh E., Lee R.J., Ye H., Thompson M.A., Saudou F., Pestell R.G., Greenberg M.E. (1998) Fos family members induce cell cycle entry by activating cyclin D1. *Mol Cell Biol*. 9, 5609-5619.

Carter, D.A. (1997) Modulation of cellular AP-1 DNA binding activity by heat shock proteins. *FEBS Lett*. 416, 81-85.

Chen J., Nye H.E., Kelz M.B., Hiroi N., Nakabeppu Y., Hope B.T., & Nestler E.J. (1995) Regulation of delta FosB and FosB-like proteins by electroconvulsive seizure and

cocaine treatments. *Mol. Pharmacol.,* 5, 880-889.

Chen J., Kelz M.B., Hope B.T., Nakabeppu Y., & Nestler E.J. (1997) Chronic Fos-related antigens: stable variants of deltaFosB induced in brain by chronic treatments. *J. Neurosci.,* 17, 4933-4941.

Chen J.S., Zhang Y.J., Kelz M.B., Steffen C.,. Ang E.S, Zeng L., Nestler E.J. (2000) Induction of cyclin-dependent kinase 5 in hippocampus by chronic electroconvulsive seizures: role of ΔFosB, *J. Neurosci*. 20, 8965– 8971.

Chinenov Y.,& Kerppola T.K. (2001) Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene*, 20, 2438-2452.

Cicatiello L, Addeo R., Sasso A., Altucci L., Petrizzi V.B., Borgo R., Cancemi M., Caporali S., Caristi S., Scafoglio C., Teti D., Bresciani F., Perillo B., Weisz A. (2004) Estrogens and progesterone promote persistent CCND1 gene activation during G1 by inducing transcriptional derepression via c-Jun/c-Fos/estrogen receptor (progesterone receptor) complex assembly to a distal regulatory element and recruitment of cyclin D1 to its own gene promoter. *Mol Cell Biol*. 24, 7260-7274.

Colby, C.R., Whisler, K., Steffen, C., Nestler, E.J., & Self, D.W. (2003) ΔFosB enhances incentive for cocaine. *J. Neurosci.,* 23, 2488-2493.

Cuevas B.D., Uhlik M.T., Garrington T.P., & Johnson G.L. (2005) MEKK1 regulates the AP-1 dimer repertoire via control of JunB transcription and Fra-2 protein stability. *Oncogene,* 5, 801-809.

Curran T., & Morgan J.I. (1987) Memories of Fos. *Bioessays.* 7, 255-258.

Dobrazanski P., Noguchi T., Kovary K., Rizzo C.A., Lazo P.S., & Bravo R. (1991) Both

products of the fosB gene, FosB and its short form, FosB/SF, are transcriptional activators in fibroblasts. *Mo.l Cell Biol.,* 11, 5470-5478.

Fang D. & Kerppola T. (2004) Ubiquitin-mediated fluorescence complementation reveals that Jun ubiquitylated by Itch/AIP4 is localized to lysosomes. *Proc. Natl., Acad. Sci. USA,* 101, 14782-14787.

Fass D.M., Butler J.E., Goodman R.H. (2003) Deacetylase activity is required for cAMP activation of a subset of CREB target genes. *J Biol Chem.* 44, 43014-43019.

Fields S. & Jang S.K. (1990) Presence of a potent transcription activating sequence in the p53 protein. *Science,* 249, 1046–1049.

Fivaz J, Bassi MC, Pinaud S, Mirkovitch J. (2000) RNA polymerase II promoter-proximal pausing upregulates c-fos gene expression. *Gene.* 255, 185-94.

Flick K., Ouni I., Wohlschlegel J.A., Capati C., McDonald W.H., Yates J.R., & Kaiser P. (2004) Proteolysis-independent regulation of the transcription factor Met4 by a single Lys 48-linked ubiquitin chain. *Nat. Cell Biol*., 6, 634-641.

Gao M., Labuda T., Xia Y., Gallagher E., Fang D., Liu Y., & Karin M. (2004) Jun turnover is controlled through JNK-dependent phosphorylation of the E3 ligase Itch. *Science,* 306, 271-275.

García-AlaiM., Gallo M., Salame M., Wetzler D.E., & de Prat-Gay G. (2006) Molecular basis for phosphorylation-dependent, PEST-mediated protein turnover. *Structure,* 14, 309-319.

Gass P, Prior P, Kiessling M. (1995) Correlation between seizure intensity and stress protein expression after limbic epilepsy in the rat brain. *Neuroscience*. 65, 27-36.

Glickman MH, & Ciechanover A. (2001) The ubiquitin-proteasome proteolytic pathway:

destruction for the sake of construction. *Physiol. Rev*., 82, 373-42.

Gordon S. Akopyan G., Garban H., Bonavida B. (2005) Transcription factor YY1: structure, function, and therapeutic implication in cancer biology. *Oncogene,* 1-18.

Hiroi N. & Graybiel A.M. (1996) Atypical and typical neuroleptic treatments induce distinct programs of transcription factor expression in the striatum. *J. Com.p Neurol.*, 374, 70-83.

Hiroi N., Brown J.R., Haile C.N., Ye H,. Greenberg M.E., & Nestler E.J. (1997) FosB mutant mice: loss of chronic cocaine induction of Fos-related proteins and heightened sensitivity to cocaine's psychomotor and rewarding effects. *Proc. Natl. Acad. Sci. USA,* 94, 10397-10402.

Hiroi N., Marek G.J., Brown J.R., Ye H., Saudou F., Vaidya V.A., Duman R.S., Greenberg M.E., & Nestler E.J. (1998) Essential role of the fosB gene in molecular, cellular, and behavioral actions of chronic electroconvulsive seizures. *J. Neurosci.*. 17, 6952-6962.

Hope B., Kosofsky B., Hyman S.E., & Nestler E.J. (1992) Regulation of IEG expression and AP-1 binding by chronic cocaine in the rat nucleus accumbens. *Proc. Natl. Acad. Sci. USA,* 89, 5764-5768.

Hope B.T., Nye H.E., Kelz M.B., Self D.W., Iadarola M.J., Nakabeppu Y., Duman R.S., & Nestler E.J. (1994a) Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatments. *Neuron,* 13, 1235-12448.

Hope B.T., Kelz R.S., Duman R. S., Nestler E.J. (1994b) Chronic electroconvulsive seizure (ECS) treatment results in expression of a long-lasting AP-1 complex in brain

with altered composition and characteristics. *J Neurosci,* 14, 4318-28.

Huang Y., Doherty J.J., Dingledine R. (2002). *J. Neurosci.* 22, 8422–8428.

Ingham R.J., Gish G., & Pawson T. (2004) The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture. *Oncogene,*. 11, 1972-1984.

Jochum W., Passegue E., Wagner E.F. (2001) AP-1 in mouse development and tumorigenesis. *Oncogene*. 20, 2401-12.

Johnson E.R. & McKay D.B. (1999) Mapping the role of the active site residues for transducing an ATP-induced conformational change in the bovine 70-kD heat shock cognate protein. *Biochemistry*. 38, 10823-10830.

Kaneko M., Abe K., Kogure K., Saito H., Matsuki N. (1993) Correlation between electroconvulsive seizure and HSC70 mRNA induction in mice brain. *Neurosci Lett* 157,195-8.

Kang J., Chang S., Yeom D., Kim S., Um S., Hong K. (2004) Weakening of the repressive YY1 site on the thrombospondin-1 promoter via c-Jun/YY1 interaction. *Exp and Molec Med*. 36, 300-310.

Kato G.J., Barrett J., Villa-Garcia M. & Dang C.V. (1990) An amino-terminal c-myc domain required for neoplastic transformation activates transcription. *Mol. Cell. Biol.,* 10, 5914–5920.

Kelz M.B., Chen J., Carlezon W.A, Whisler K., Gilden L., Beckmann A.M., Steffen C., Zhang Y.J., Marotti L., Self D.W., Tkatch T., Baranauskas G., Surmeier D.J., Neve R.L., Duman R.S., Picciotto M.R., Nestler E.J. Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. *Nature*. 401, 272-276.

Kelz M.B., Kuszak J.R., Yang Y.Q., Ma W.C., Steffen C., Al-Ghoul K., Zhang Y.-J., Chen J.S., Nestler E.J., and Spector A. (2000) ΔFosB induced cataract. *Invest. Ophthalmol. Vis. Sci.* 41, 3523-3538.

Kong X., Lin Z., Liang D., Fath D., Sang N., & Caro J. (2006) Histone Deacetylase Inhibitors Induce VHL and ubiquitin-independent proteasomal degradation of Hypoxia-Inducible Factor 1{alpha*}. Mol. Cell Biol.*, 6, 2019-2028.

Kumar A., Choi K.H., Renthal W., Tsankova N.M., Theobald D.E., Truong H.T., Russo S.J., Laplant Q., Sasaki T.S., Whistler K.N., Neve R.L., Self D.W., Nestler E.J. (2005) Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. *Neuron*. 48, 303-14.

Kuras L., Rouillon A., Lee T., Barbey R., Tyers M., &Thomas D. (2002) Dual regulation of the met4 transcription factor by ubiquitin-dependent degradation and inhibition of promoter recruitment. *Mol. Cell,* 10, 69-80.

Kveiborg M., Sabatakos G., Chiusaroli R., Wu M., Philbrick W.M., Horne W.C., Baron R. (2004) DeltaFosB induces osteosclerosis and decreases adipogenesis by two independent cell-autonomous mechanisms. *Mol Cell Biol.* 24, 2820-30.

Lagaudriere-Gesbert C., Newmyer S.L., Gregers T.F., Bakke O., Ploegh H.L. (2002) Uncoating ATPase Hsc70 is recruited by invariant chain and controls the size of endocytic compartments. *Proc Natl Acad Sci U S A.* 99, 1515-1520.

Lazo P.S., Dorfman K., Noguchi T., Mattei M.G., & Bravo R. (1992) Structure and mapping of the fosB gene. FosB downregulates the activity of the fosB promoter. *Nucleic Acids Res.,* 2, 343-350.

Lechward K., Awotunde O.S., Swiatek W., Muszynska G. (2001) Protein phosphatase

2A: variety of forms and diversity of functions. *Acta Biochimica Polonica*, 48, 921-933.

Lee S.K., Kim J.H., Lee Y.C., Cheong J., Lee J.W. (2000) Silencing mediator of retinoic acid and thyroid hormone receptors, as a novel transcriptional corepressor molecule of activating protein-1, nuclear factor-kappaB, and serum response factor. *J Biol Chem.* 17, 12470-12474.

Levine A.A., Guan Z., Barco A., Xu S., Kandel E.R., Schwartz J.H. (2005) CREB-binding protein controls response to cocaine by acetylating histones at the fosB promoter in the mouse striatum. *Proc. Natl. Acad. Sci. U.S.A.* 102, 19186–19191.

Li J., Guo, Y., Schroede F.A., Youngs R.M., Schmidt T.W., Ferris C., Konradi C., Akbarian S. (2004) Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in Drosophila polytene nuclei. *Cell* 69, 375–384.

Mandelzys A., Gruda M.A., Bravo R., & Morgan J.I. (1997) Absence of a persistently elevated 37 kDa fos-related antigen and AP-1-like DNA-binding activity in the brains of kainic acid-treated fosB null mice. *J. Neurosci.,* 17, 5407-5415.

Manzerra, P. & Brown, I.R. (1996) The Neuronal stress response: Nuclear translocation of heat shock proteins as indication of hyperthermic stress. *Experimental Cell Rsrch.* 229, 35-47.

Mayer, M.P., & Bukau, B. (2004) Hsp70: Chaperones: Cellular functions and molecular mechanisms. *C.M.L.S.,* 62, 670-684.

McClung C.A. & Nestler E.J. (2003) Regulation of gene expression and cocaine reward by CREB and ΔFosB. *Nature Neurosci.,* 6,1208-1215.

McClung C.A., Ulery P.G., Perrotti L.I., Zachariou V., Berton O., & Nestler E.J. (2004) DeltaFosB: a molecular switch for long-term adaptation in the brain. *Mol.Brain Res.,* 132, 146-154.

Milde-Langosch K. (2005) The Fos family of transcription factors and their role in tumourigenesis. *Eur J Cancer*. 41, 2449-2461.

Moratella R., Elibol B., Vallejo M., Graybiel A.M. (1996) Network-level changes in expression of inducible Fos-Jun proteins in the striatum during chronic cocaine treatment and withdrawal. *Neuron*. 17, 147-156.

Morgan J.I., & Curran T. (1995) Immediate-early genes: ten years on. *Trends Neurosci.,* 18, 66-67.

Morgan JI, Curran T. (1991) Proto-oncogene transcription factors and epilepsy. *Trends Pharmacol Sci*. 9, 343-349.

Murcu, M.G., Doyle, M., Berolotti, A., Neckers, L. (2002) Heat shock protein 90 modulates the unfolded protein response by stabilizing IRE1α. *Molec and Cell Biol.* 22. 8506-8513.

Nakabeppu Y., & Nathans D. (1991) A naturally occurring truncated form of FosB that inhibits Fos/Jun transcriptional activity. *Cell,* 64, 751-759.

Neish A.S., Anderson S.F., Schlegel B.P., Wei W., Parvin J.D. (1997) Factors associated with the RNA polymerase II holoenzyme. *Nucl Acids Rsrch.* 26, 847-853.

Nestler E.J., Barrot M., & Self D.W. (2001) ΔFosB: A sustained molecular switch for addition. *Proc. Natl. Acad. Sci. U S A,* 98, 11042-11046.

Nestler E.J., Hyman S.E., Malenka R.C. (2001) *Molecular Neuropharmacology*. 1st ed. New York: McGraw-Hill Companies, 386.

Norrholm S.D., Bibb J.A., Nestler E.J., Ouimet C.C., Taylor J.R., Greengard P. (2003)

Cocaine-induced proliferation of dendritic spines in nucleus accumbens is dependent on the activity of the neuronal kinase Cdk. *Neuroscience* 116, 19–22.

Nye, H.E. & Nestler E.J. (1996) Induction of chronic Fras (Fos related antigens) in rat brain by chronic morphine administration. *Mol Pharmacol.* 49, 636-645.

Olausson P. J., Jentsch D., Tronson N., Neve R.L., Nestler E.J., Taylor J.R. (in review) ΔFosB in the nucleus accumbens regulates food-reinforced instrumental behavior and motivation.

Peakman M.C., Colby C., Perrotti L.I., Tekumalla P., Carle T., Ulery P., Chao J., Duman C., Steffen C., Monteggia L., Allen M.R., Stock J.L., Duman R.S., McNeish J.D., Barrot M., Self D.W., Nestler E.J., Schaeffer E. (2003) Inducible, brain region specific expression of a dominant negative mutant of c-Jun in transgenic mice decreases sensitivity to cocaine. *Brain Res.* 970, 73–86.

Penrose K.J. & McBride A.A. (2000) Proteasome-mediated degradation of the papillomavirus E2-TA protein is regulated by phosphorylation and can modulate viral genome copy. *J. Virol.* 74, 6031–6038.

Perroti, L.I., Hadeishi, Y., Ulery, P.G., Barrot, M., Monteggia, L., Duman, R.S., & Nestler, E.J. (2004) Induction of ΔFosB in reward-related brain regions after chronic stress. *J. Neurosci.*. 24, 10594-10602.

Peterson C.L, & Laniel M.A. (2004) Histones and histone modifications. *Curr Biol.* 14, 546-551.

Pratt, W.B., Galigniana, M.D., Harrell, J.M., DeFranco, D.B. (2004) Role of Hsp90 and the Hsp90-binding immunophilins in the signaling proteins movement. *Cellular Signaling*. 16, 857-872.

Rechsteiner M., & Rogers S.W., (1996) PEST sequences and regulation by proteolysis. *Trends Biochem. Sci.,* 7, 267-271.

Roberts A., Picetti R., Nestler E.J., Koob G.F. (2001) *Soc. Neurosci. Abst.*

Salghetti S.E., Muratani M., Wijnen H., Futcher B., & Tansey W.P. (2000) Functional overlap of sequences that activate transcription and signal ubiquitin-mediated proteolysis. *Proc. Natl. Acad. Sci. USA*, 7, 3118-3122.

Salvat, C., Aquaviva, C., Jariel-Encontre, I., Ferrara, P., Pariat, M., Steff, A.M., Carillo, S., & Piechaczyk, M. (1999) Are there multiple proteolytic pathways contributing to c-Fos, c-Jun and p53 protein degradation in vivo? *Mol. Biol. Rep.,* 26, 45-51.

Scaife R.M. & Margolis R.L. (1997) The role of the PH domain and SH3 binding domains in dynamin function. *Cell Signal* 6, 395-401.

Skinner M., Qu S., Moore C., Wisdom R. (1997) Transcriptional activation and transformation by FosB protein require phosphorylation of the carboxyl-terminal activation domain. *Molec and Cell Biol*. 17, 2372-2380.

Spencer M.L., Theodosiou M., & Noonan D.J. (2004) NPDC-1, a novel regulator of neuronal proliferation, is degraded by the ubiquitin/proteasome system through a PEST degradation motif. *J. Biol. Chem.,* 279, 37069-37078.

Stancovski I., Gonen H., Orian A., Schwartz A.L., & Ciechanover A.*,* (1995) Degradation of the proto-oncogene product c-Fos by the ubiquitin proteolytic system in vivo and in vitro: identification and characterization of the conjugating enzymes. *Mol. Cell Biol*., 12, 7106-7116.

Steward M.D., Li J, Wong J. (2005) Relationship between histone H3 lysine 9 methylation, transcription, repression, and heterochromatin protein 1 recruitment.

Molec and Cell Biol. 25, 2525-2538.

Tekumalla P.K., Calon F., Rahman Z., Birdi. S., Rajput A.H., Hornykiewicz T., Di Paolo T., Bedard P.J., Nestler E.J. (2001) Elevated levels of ΔFosB an RGS9 in striatum in Parkinson's disease. *Biol Psychiatry*. 50, 813-816.

Thiagalingam S., Cheng K.H., Lee H.J., Mineva N., Thiagalingam A., Ponte J.F. (2003) Histone deacetylases: unique players in shaping the epigenetic histone code. *Ann N Y Acad Sci*. 983, 84-100.

Thiel G., Lietz M., Hohl M. (2004) How mammalian transcriptional repressors work. *Eur J Biochem.* 271, 2855-2862.

Thom G., Minshall N., Git A., Argasinska J., Standart N. (2003) Role of cdc2 kinase phosphorylation and conserved N-terminal proteolysis motifs in cytoplasmic polyadenylation-element-binding protein (CPEB) complex dissociation and degradation. *Biochem. J.,* 370, 91-100.

Tintignac L.A., Lagirand J., Batonnet S., Sirri V., Leibovitch M.P., & Leibovitch S.A. (2005) Degradation of MyoD mediated by the SCF (MAFbx) ubiquitin ligase. *J. Biol. Chem*., 280, 2847-2856.

Tsankova N., Kumar A., Nestler E.J. (2004) Histone modifications at gene promoter regions in rat hippocampus after acute and chronic electroconvulsive seizures. *J. Neurosci.* 24, 5603-5610.

Tsurumi C., Ishida N., Tamura T., Kakizuka A., Nishida E., Okumura E., Kishimoto T., Inagaki M., Okazaki K., & Sagata N. (1995) Degradation of c-Fos by the 26S proteasome is accelerated by c-Jun and multiple protein kinases. *Mol. Cell Biol.,* 15, 5682-5687.

Thomas D., & Tyers, M. (2000) Transcriptional regulation: Kamikaze activators. *Curr. Biol*., 10, R341-343.

Ulery, P.G., Rudenco, G., & Nestler, E.J. (2006) Regulation of ΔFosB Stability by Phosphorylation. *J. Neurosci.*.26, 5131-5142.

Wade P.A., Pruss D., Wolffe A.P. (1997) Histone acetylation: chromatin in action. *Trends Biochem Sci.* 22, 128-132.

Wegele, H., Muller, L., Buchner, J. Hsp70 and Hsp90 — a relay team for protein folding. (2004) *Rev Physiol Biochem Pharmacol*. 151, 1-44.

Werme M., Messer C., Olson L, Gilden L., Thoren P., Nestler E.J., Brene S. (2002) ΔFosB regulates wheel running. *J Neurosci.* 22, 8133-8138.

Williams S.A., Chen L., Kwon H., Ruiz-Jarabo C.M., Verdin E., Greene W.C. (2006) NF-κB p50 promotes HIV latency through HDAC recruitment of transcriptional initiation. *EMBO J*. 25, 139-149.

Winstanley C.A. Theobald D.E., Green T.A., Bachtell R.K., Perrotti L.I., DiLeone R.J., Russo S.J., Garth W.J., Self D.W., Nestler E.J. (in review) ΔFosB induction in orbitofrontal cortex regulates cocaine-induced cognitive dysfunction.

Winston S.M., Hayward M.D., Nestler E.J., Duman R.S. (1990) Chronic electroconvulsive seizures down-regulate expression of the immediate-early genes c-fos and c-jun in rat cerebral cortex. *J Neurochem.* 6, 1920-1925.

Wisdom R.,Verma I.M. (1993) Proto-oncogene FosB: the amino terminus encodes a regulatory function required for transformation. *Mol Cell Biol.* 13, 2635-43.

Wojcik C. & DeMartino G.N. (2002) Analysis of Drosophila 26 S proteasome using RNA interference. *J. Biol. Chem*., 277, 6188-6197.

Von der Lehr N., Johansson S., & Larsson L.G. (2003) Implication of the ubiquitin/proteasome system in Myc-regulated transcription. *Cell Cycle,* 5, 403-407.

Yen J., Wisdom R.M., Tratner I., & Verma I.M. (1991) An alternative spliced form of FosB is a negative regulator of transcriptional activation and transformation by Fos proteins. *Proc. Natl. Acad. Sci. USA,* 88, 5077-5081.

Zachariou, V., Bolanos, C.A., Selley, D.E., Theobald, D., Cassidy, M.P., Kelz, M.B., Shaw-Lutchmann, T., Berton, O., Sim-Selley, L.J., DiLeone, R.J., Kumar, A., & Nestler, E.J. (2006) ΔFosB: An essential role for ΔFosB in the nucleus accumbens in morphine action. *Nature Neurosci.,* 9, 205-211.

Zentrich E., Han S.Y., Pessoa-Brandao L., Butterfield L., & Heasley L.E. (2002) Collaboration of JNKs and ERKs in nerve growth factor regulation of the neurofilament light chain promoter in PC12 cells. *J. Biol. Chem.,* 277, 4110-4118.

Zhong N., Kim C.Y., Rizzu P., Geula C., Porter R., Pothos E.N., Squitieri F., Heutink P., Xu J. (2006) DJ-1 transcriptionally up-regulates the human tyrosine hydroxylase by inhibiting the sumoylation of PSF. *J Biol Chem.* In press.

Zolnierowicz S. (2000) Type 2A protein phosphatase, the complex regulator of numerous signaling pathways. *Biochem Pharmacol* 60, 1225-1233.

VITAE

Tiffany Carle Florence was born April 29, 1979 in San Antonio, Texas, daughter of Patricia Bazan Carle and Patrick Francis Carle. After graduating from Westwood High School in Austin, TX in 1997, Tiffany attended The University of Alabama in Tuscaloosa, AL to play collegiate soccer. Once her eligibility was exhausted, Tiffany completed the degree of Bachelor of Science with a major in microbiology and a minor in Spanish from The University of Alabama in May of 2001. In August of the same year, she entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas, where she joined the laboratory of Dr. Eric J. Nestler. Tiffany married Bo Lewis Florence in May of 2006. The couple currently resides in Dallas with their very large Labrador, Nina. Tiffany still plays competitive soccer every Sunday.

Permanent address: 2815 Welborn St. #106
Dallas, TX 75219