

***Cited2 and PFTAIRE, two Ways in Which
Cyclin Dependent Kinases Impact on Development and
Degeneration in the Central Nervous System***

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“Very few beings really seek knowledge in this world. Mortal or immortal, **few really ask**. On the contrary, they try to wring from the unknown the answers they have already shaped in their own minds (...). **To really ask is to open the door to the whirlwind**. The answer may annihilate the question and the questioner.”

Anne Rice.

A man should look for what is, and not for what he thinks should be.
Information is not knowledge.

Albert Einstein

Abstract.

Cyclin dependent kinases (Cdks) belong to a large family of evolutionary conserved kinases that are at times divided into mitotic and post-mitotic Cdks, classifications based on their expression/function profile. Neuroblast proliferation rates, differentiation, developmental apoptosis, migration, axogenesis and maintenance are some of the processes which Cdks regulate in the Central Nervous System (CNS). Additionally, Cdks have a role in mature neurons mediating survival/degeneration. Their deregulation has been functionally linked to conditions such as Stroke, Alzheimer's disease and Parkinson's. However, the mechanisms underlying Cdk's involvement in neurons are far from being clear. Accordingly, this thesis research explored two subjects relevant to the functions of Cdks in the CNS: first, the mechanism through which Cited2, a Cdk4-dependent signal, mediates neuronal apoptosis after DNA damage and second, the potential role of PFTAIRE, a post-mitotic Cdk, in CNS development.

Cited2, a CBP (cAMP response element-binding protein-binding protein)/p300 interacting transactivator, was identified as a signal upregulated in neurons after DNA damage. We showed that Cdk4 activation is required for Cited2 upregulation and that this event is upstream of mitochondrial cytochrome c release. Additionally, we report that Cited2 activates peroxisome proliferator-activated receptor- γ (PPAR γ), an activity that proved to be critical for DNA damage-induced death. We show that these two molecules require each other, forming an active complex that ultimately triggers the mitochondrial pathway of death. Our results not only define a novel Cdk4-mediated neuronal prodeath pathway but report for the first time functional data on Cited2 biological roles in neurons.

In the second project, we explored the effects of the deficiency of PFTAIRE, a novel Cdk highly expressed in neurons, in development of the *Drosophila* ventral nerve cord (VNC). Using two different PFTAIRE *Drosophila* mutant lines, we demonstrated that

the deficiency leads to CNS defects as early as stage 11 of embryonic development. Our findings show that PFTAIRE mutation leads to premature axon outgrowth, axon misguidance and defasciculation accompanied by disorganization of neuronal and glial cell bodies that affect both commissural and longitudinal axons of the VNC. Our data confirms for the first time that PFTAIRE has an essential role in CNS developmental processes and it may be required in neurons for proper axogenesis to occur.

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List of Manuscripts

- I. **Gonzalez YR**, Zhang Y, Behzadpoor D, Cregan S, Bamforth S, Slack RS, Park DS. (2008) CITED2 signals through peroxisome proliferator-activated receptor-gamma to regulate death of cortical neurons after DNA damage. **Journal of Neuroscience**, 28(21):5559-69.

- II. **Gonzalez YR**, JafarNejad P., Hawari D, Slack RS, Sonnenfeld M., Park DS. Eip63E, the Drosophila PFTAIRE, is required for the proper development of the Central Nervous System. **(Manuscript under preparation)**.

Appended Articles:

- III. Biswas SC, Zhang Y, Iyirhiaro G, Willett RT, **Rodriguez Gonzalez Y**, Cregan SP, Slack RS, Park DS, Greene LA. (2010) Sertad1 plays an essential role in developmental and pathological neuron death. **Journal of Neuroscience**, 30(11):3973-82.

- IV. Zhang Y, Parsanejad M, Huang E, Qu D, Aleyasin H, Rousseaux MW, **Gonzalez YR**, Cregan SP, Slack RS, Park DS. (2010) Pim-1 kinase as activator of the cell cycle pathway in neuronal death induced by DNA damage. **Journal of Neurochemistry**, 112(2):497-510.

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List of Abbreviations

A	Alanine
AA	Aminoacid
AAV	Adeno-Associated Virus
Ab	Antibody
AD	Alzheimer's Disease
Apaf-1	Apoptotic protease activating factor-1
ATM	Ataxia telangiectasia-mutated
ATP	Adenosine-5'-triphosphate
BAX	BCL2-associated X protein
BDNF	Brain-derived neurotrophic factor
C	Cysteine
CAK	Cdk Activating Kinase
cAMP	cyclic AMP
cDNA	complementary DNA
Cdc-2	Cell division cycle 2
Cdc25	Cell division cycle 25
Cdc28	Cell division cycle 28
Cdk	Cyclin-dependent Kinase
CDKL	Cdk like
Chk1	Checkpoint kinase 1
Cited2	CBP/p300 interacting transactivator with Aspartic and Glutamic acid (ED) rich termini 2
CNS	Central Nervous System
D	Aspartic Acid

DIV	Days in-vitro
DN	Dominant-Negative
DNA	Desoxi-ribonuceic Acid
E	Glutamic Acid
F	Phenyl-Alanine
gcm	glial cells missing
GFP	Green Fluorescent Protein
gmc	ganglion mother cell
h	hour
HIF1- α	Hypoxia-Inducible factor 1- α
I	Iso-leucine
JNK	c-jun N-terminal Kinase
K	Lysine
kDa	KiloDalton
KO	Gene knockout
LRP	Low-density lipoprotein receptor related protein
mRNA	messenger RNA
N	Asparagine
NF- κ B	Nuclear factor kappa beta
NGF	Nerve Growth Factor
NLS	Nuclear localization sequence
P	Proline
PARP	Poly ADP-ribose polymerase
PD	Parkinson's disease
Pftk	PFTAIRE kinase

PPAR	Peroxisome proliferator-activated receptor
R	Arginine
Rb	Retinoblastoma gene
RNA	Ribonucleic Acid
RNAi	Interference RNA
S	Serine
shRNA	Short hairpin RNA
Stk9	Serine/Threonine kinase 9
T	Threonine
VNC	Ventral Nerve Cord
UAS	upstream activated sequence
Y	Tyrosine

Thesis format

Following the guidelines of the Departments of Neuroscience and Cellular and Molecular Medicine, this thesis was written as a compendium of manuscripts preceded by a general introduction and followed by a general discussion of thesis research as it relates to current knowledge in these fields.

Chapter one, the introductory section, is a summarized review of the scientific literature relevant to the research presented on chapters two and three. It includes a general section dedicated to Cdks, their structure, regulation and functions in the CNS which is separated into of the knowledge preceding the research presented in following chapters work basic to the subjects accumulated to date on the subjects relevant to the

Chapter two presents a manuscript entitled: “CITED2 signals through peroxisome proliferator-activated receptor-gamma to regulate death of cortical neurons after DNA damage”. This work was published on the Journal of Neuroscience in the year 2008 and it is included here exactly as published.

Chapter three presents a manuscript entitled: “Eip63E, the *Drosophila* PFTAIRE, is required for the proper development of the Central Nervous System”. This manuscript is still in preparation for publication.

An overview of the major findings of this thesis is presented in chapter four. A discussion on the potential direction that each of the project could take in the future is included as well.

CHAPTER 1.

General Introduction.

Cyclin Dependent Kinases.

Cyclin Dependent Kinases (Cdks) were first identified in yeast (CDC28 and cdc2) and initially described for their regulatory role in cell cycle progression. Cdks are, by definition, members of a cdc2-related family of serine/threonine kinases that require binding to cyclin proteins (positive regulatory subunits) for their activation. Since their initial identification, more than 21 family members have been discovered (Liu and Kipreos, 2000).

Several Cdk members: CDC2/Cdk1, Cdk2, Cdk3, Cdk4 and Cdk6, indeed regulate cell cycle progression directly (Grana and Reddy, 1995; John et al., 2001) and so are regarded as prototypical Cdks. They appear to mediate the transition from one distinct phase to another and their activation is refereed by the phase-specific expression and binding of a cyclin partner (Pines, 1993b, a; van den Heuvel and Harlow, 1993; Morgan, 1997). For example, Cdk4/cyclinD regulates progression through the restriction point during G1 phase by phosphorylating and inactivating the tumor suppressor factors of the retinoblastoma (Rb) gene family (p105^{Rb}, p107, p130^{Rb2}), the only characterized in-vivo substrates for this Cdk (Weinberg, 1995; Bartek et al., 1996; Farkas et al., 2002). During cell cycle, phospho-Rb (pRb) acts as a signal transducer between several Cdks and the transcription machinery, promoting the expression of sets of genes that mediate the progression of the cell through the critical growth phases (Weinberg, 1995; Bartek et al., 1996). Hyperphosphorylation of Rb by cyclinD/Cdk4/6 leads to the release and activation of the E2F family of transcription factors and the expression of genes required for cell cycle progression (Chan et al., 2001; Farkas et al., 2002; Ren et al., 2002).

Further research into Cdks' functions revealed that not all Cdks are involved in cell cycle regulation. Cdk7 to Cdk12 participate in the regulation of transcription and RNA splicing (Loyer et al., 2005; Bagella et al., 2006; Chen et al., 2006; Even et al.,

2006; Loyer et al., 2008) and Cdk7 has the additional function of being the only known eukaryotic Cdk-activating kinase (CAK) (Kaldis, 1999).

It was believed for many years that Cdks were irrelevant to neurons once these cells exited cell cycle and fully differentiated. Nevertheless, additional investigation revealed that such was not the case (Herrup and Yang, 2007). Surprisingly, other Cdks were identified that appear to have biological functions in postmitotic cells unrelated to cell cycle regulation. Cdk5, PCTK1-3 (PCTAIRE), and several others of still unknown functions (PFTK1-2 (PFTAIRE) (Besset et al., 1998), CDKL1 (KKIALRE) (Yen et al., 1995), CDKL2 (KKIAMRE) (Sassa et al., 2004) and CDKL5 (STK9) (Montini et al., 1998)) were found to be highly expressed in the Central Nervous system (CNS). Due to the discovery of these neuronal Cdks, family members have been divided by researchers into cell-cycle related and post-mitotic Cdks. This introductory chapter will then make use of this classification to discuss the roles of Cdks in the CNS.

Cdks structure and regulation. Generalities.

Cdks are small proteins (~34-60 kDa) with distinct structural features. Based on Cdk2 structure, these molecules have: 1) a large C-terminal lobe that contains the T-loop, a flexible conformational structure that covers and blocks the substrate binding site in the absence of cyclin binding (De Bondt et al., 1993). As part of the T-loop sequence, there is a conserved activating threonine phosphorylation site (T160 in Cdk2) (Desai et al., 1992; Gu et al., 1992). 2) A smaller N-terminal lobe that is usually drawn above the C-terminal lobe and contains a glycine-rich loop and a helix with a conserved PSTAIRE amino acid sequence that is part of the cyclin binding region (De Bondt et al., 1993; Jeffrey et al., 1995). Variations on this sequence have been used to denominate novel family members (see above). Inhibitory threonine and tyrosine phosphorylation sites are present in the N-terminal lobe as well (T14 and Y15 in Cdk2) (Gu et al., 1992). And 3) The active site as a deep cleft defined by the lobes that contains the ATP binding residues (aspartic acid (D)127, lysine (K)129 and asparagine (N)132, a conformational amino acid (AA) triad, plus K33 and D145). Substrate binds to this region as well (De Bondt et al., 1993) (reviewed in (Morgan, 1997)). These general structural features have been so far confirmed for all studied Cdks, including Cdk5 (Tarricone et al., 2001), the leader of the post-mitotic/neuronal Cdks.

Cdks are inactive as monomers. Their activation is subjected to complex and multilayered regulatory mechanisms.

Activating mechanisms:

- Binding to regulatory subunit: cyclin or non-cyclin partner.

As mentioned before, cyclins are members of a family of proteins which levels oscillate during cell cycle (Murray, 2004; Fung and Poon, 2005). Originally, they were the only known partners to Cdks. Later on, the identification of postmitotic Cdks

pointed to the existence of non-cyclin partners. p35 and p39, the known Cdk5 activators, are the only ones that have been so far characterized (Lew et al., 1994; Tsai et al., 1994; Delalle et al., 1997; Patrick et al., 1998).

In the case of Cdk2, cyclinA interacts with regions on both lobes and this leads to conformational changes that impact both ATP orientation and substrate binding. 1) The PSTAIRE helix is pushed inwards and drives proper orientation of the ATP phosphates for phosphotransfer to occur and 2) the T-loop moves out of the cleft entrance, allowing substrate interaction (Jeffrey et al., 1995; Morgan, 1996). It is now known that this Cdk2/cyclinA model of interaction is not completely true for all Cdk complexes (Echalier et al., 2010). For example, the extension of the Cdk9/cyclinT interface area is only a 40% of that of Cdk2/cyclinA, reduced only to the N-terminal lobe, but still constitute an activating interaction (Echalier et al., 2010).

For most studied cases, cyclin binding is enough to render a partially active Cdk complex, the exception being, so far, the Cdk4/cyclinD complexes. In these cases, cyclinD isoforms bind only to the N-terminal lobe region as it happens for Cdk9/cyclinT, but do not drive enough conformational changes to render an active Cdk complex (Day et al., 2009; Takaki et al., 2009).

Finally, other Cdk activating protein partners exist apart from cyclins. For Cdk4, Sertad1 (also known as p34^{SEI1} and TripBr1) is such an interactor (Sugimoto et al., 1999; Li et al., 2004). It has been reported that Sertad1 directly binds and activates Cdk4 even in the inactivating presence of p16 (Sugimoto et al., 1999). It appears as well that the Sertad1 effect on Cdk4 activity is concentration-dependent, since higher concentrations of Sertad1 are inactivating instead of activating over Cdk4 (Li et al., 2004).

- Phosphorylation of conserved threonine residue at the T-loop.

CyclinA binding to Cdk2 is enough to render an active Cdk2/cyclinA complex (Connell-Crowley et al., 1993). Nevertheless, phosphorylation of the T160 residue

enhances Cdk2/cyclinA level of activity ~80-300 folds (Gu et al., 1992; Morgan, 1997). In eukaryotic cells, this is an event regulated by the Cdk7/cyclinH/Mat1 complex (Kaldis, 1999). T106 phosphorylation stabilizes the Cdk/cyclin interaction, the T-loop is flattened and moves closer to cyclinA, leading to critical changes in the shape and girth of the substrate-binding site (Russo et al., 1996).

The level on which the activity of other Cdks depends on this phosphorylation event varies. For Cdk4/cyclinD complexes, phosphorylation is required but still not enough for full activity (Takaki et al., 2009; Echaliier et al., 2010). Cdk5/p25 does not require this phosphorylation at all (Tarricone et al., 2001) and Cdk8 even lacks the phosphorylatable equivalent threonine residue (Tassan et al., 1995). Additionally, Cdk9/cyclinT (Baumli et al., 2008) and PFTK1 (Lazzaro et al., 1997) show autophosphorylation activity, which may render them independent of the Cdk7 CAK activity.

- Dephosphorylation by Cdc25 phosphatases.

Removal of the inhibitory phosphorylation at T14 and Y15 sites in Cdk2 constitutes a rapid way to activate readily available Cdk/cyclin complexes (Lew and Kornbluth, 1996). This event is regulated by the Cdc25 family of phosphatases (Sebastian et al., 1993), isoforms A, B and C, that are in turn activated by Cdk/cyclin complexes and other kinases (reviewed in (Karlsson-Rosenthal and Millar, 2006)).

Inactivating mechanisms:

- Phosphorylation of the glycine-rich loop residues.

As previously mentioned, phosphorylation on threonine/tyrosine residues of the N-terminal lobe inhibits Cdk activity. This inhibition happens even in the presence of T160-equivalent phosphorylation (Morgan, 1997). For Cdk1/2, two sites have been described: T14 and Y15, which are the targets of Wee1 and Myt1 kinases (Obaya and

Sedivy, 2002). In the case of Cdk4/6, Y17 is the equivalent residue, but no kinase has been identified yet that mediates this phosphorylation.

As with other regulatory aspects discussed earlier, the consequence of the T14/Y15 phosphorylation is not as clear for other Cdks. For example, Y15 phosphorylation for Cdk5/p35 appears to be activating instead of inactivating (Zukerberg et al., 2000), the contrary to what happens when the partner is p25 (Mapelli and Musacchio, 2003), the product of p35 excision.

- Endogenous Cdks inhibitors (CKIs).

Two gene families of Cdk inhibitors have been described:

a. The INK4 family (p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}). These proteins bind to Cdk4 and Cdk6 blocking their association with all cyclinD isoforms and so inhibiting their kinase activity (Sherr and Roberts, 1999).

b. The Cip/Kip family (p21^{Cip1/Waf1/Sdi1}, p27^{Kip1}, and p57^{Kip2}). Members of this family block the kinase activity associated to cyclins A and E by binding to both Cdk and cyclin (Sherr and Roberts, 1999). Originally and based on in-vitro experiments, it was thought that these proteins had an inhibitory effect on the activity of all Cdk/cyclin complexes (Xiong et al., 1993), but further studies showed that the contrary is true for cyclin D-associated Cdk activity. It has been reported that the Cip/Kip proteins can facilitate assembly of Cdk4/cyclinD and so enable its kinase activity, as well as targeting the complex to the nucleus (LaBaer et al., 1997).

Cell cycle related Cdks in the Central Nervous System.

The initial body of research on cell cycle related Cdks was mostly focus on their roles in dividing cells. The last decade though has revealed that this group of Cdks has essential functions during development and degeneration of the CNS. Following is a discussion on those roles.

In CNS development

The proper timing of cell cycle progression and exit is critical in CNS development and so, the essential role of Cdks in proliferation and cell cycle exit of neuronal precursors should be apparent. Research has shown that that is indeed the case (Cunningham and Roussel, 2001; Calegari and Huttner, 2003; Li and DiCiccio-Bloom, 2004; Dehay and Kennedy, 2007). For instance, pharmacological inhibition of Cdks' activity leads to premature differentiation of neuroprogenitor cells (Calegari and Huttner, 2003). Additionally, in-vivo overexpression of Cdk4/cyclinD1 in neural progenitors promotes their expansion and delays neurogenesis (Lange et al., 2009). Furthermore, the E2F/Rb pathway, the convergent molecular switch for cell cycle progression signals and Cdks target, has also been implicated in regulating survival and differentiation of distinct cell types of the CNS (Swiss and Casaccia, 2010). Data show that in vivo inactivation of pRb in neuroprogenitor cells leads to increased proliferation and apoptosis, reduced glial cell population, abnormal morphology and aberrant cell patterning in the mouse brain (McLear et al., 2006).

The above mentioned evidence has led to the thesis that regulation of cyclinD-associated Cdks in dividing progenitors is key to the fate of daughter cells (Fichelson et al., 2005). Accordingly, a long G1 phase is linked to differentiation while shorter G1 correlates with proliferation (Calegari et al., 2005; Dehay and Kennedy, 2007). The mechanisms by which Cdks participate in this process are yet unknown.

In CNS degeneration

For a long period of time, it was a well accepted believe that cell cycle Cdks were generally not expressed in mature neurons, due to the non-dividing nature of these highly differentiated cells. Fairly recently correlative evidence started accumulating that negated that dogma leading to intense research on the matter.

Results revealed that cyclinD1 and its associated Cdks, Cdk4/6, are upregulated and aberrantly activated in models of neuronal excitotoxicity and ischemia (Li et al., 1997; Timsit et al., 1999; Park et al., 2000a). Upregulation of cyclinD1, Cdk4, cdc2 and cyclinB1 in several areas of post-mortem brains appeared to correlate with Alzheimer's disease (AD) (Nagy et al., 1997; Vincent et al., 1997; Busser et al., 1998; Tsujioka et al., 1999). Additionally, the use of other in-vitro models showed that Cdk4/6 activation appeared to be required for neuronal death due to proteosomal inhibition (Rideout et al., 2003), superoxide dismutase deficiency (Nguyen et al., 2003) and DNA damaging agents (discussed ahead, (Morris et al., 2001). These models are relevant to conditions such as Parkinson's disease (Alam et al., 1997; McNaught and Olanow, 2006), Huntington's disease, amyotrophic lateral sclerosis (Vucic and Kiernan, 2009), AD (Lovell and Markesbery, 2001) and Stroke (Rashidian et al., 2007), among others (Greene et al., 2007). These findings consolidated the notion of the involvement of Cdk4 activation in neurodegenerative conditions.

Mechanistic studies then showed that the upregulated cyclinD1/Cdk4 complex appeared to lead to Rb phosphorylation, since this last event was detected after ischemic injury in vivo (Osuga et al., 2000). Furthermore, pharmacological inhibition of Cdks blocked Rb phosphorylation and led to significant protection (Osuga et al., 2000). Equally, Cdk4 inhibition, either by a pharmacological agent or a dominant negative form of the kinase, protected primary cortical neurons from death induced by β -Amyloid treatment. This protection appeared to be mediated by inhibition of caspase activation (Giovanni et al., 1999; Giovanni et al., 2000). Later on, functional data was reported

showing that inhibition of Cdk4 (dominant negative approach) and cyclinD1 (germline deficiency) protected neurons from delayed/apoptotic death induced by ischemic/hypoxic stress (Rashidian et al., 2005; Rashidian et al., 2007). For most of these paradigms, the Rb/E2F pathway appeared to mediate the Cdk4-induced apoptotic events (Giovanni et al., 2000; O'Hare et al., 2000; Park et al., 2000b; Rashidian et al., 2005; Rashidian et al., 2007)

Developmental neuronal apoptosis due to the lack of neurotrophic factors support seems to be dependent as well on cell cycle Cdk activity (Greene et al., 2007). It has been reported that Cdk4 inhibition, either by virally-delivered dominant negative form (Park et al., 1997b) or shRNA (Biswas et al., 2005), promotes survival of nerve growth factor (NGF)-deprived sympathetic neurons. This is not true for cdk2/3, since dominant negative forms of these Cdks failed to protect neurons from death evoked by NGF deprivation (Park et al., 1997b). The mechanisms mediating this Cdk4-dependent death pathway appear to be similar to those activated during apoptosis of mature neurons.

The findings discussed in this section had led to the notion that, far from being permanently post-mitotic, neurons must constantly hold their cell cycle in check. Failure to do so would lead to the re-initiation of the cell cycle in adult neurons and subsequent death (reviewed in (Herrup and Yang, 2007)). Therefore, cell-cycle Cdks may be dynamically important for the 'well-being' of post-mitotic neurons.

Questions still remain regarding the mechanisms regulating the activation of Cdks after these apoptotic insults, as well as the possible downstream pathways extending from this event. Accordingly, the research discussed in chapter two of this thesis addresses this matter. We have explored the role of Cited2, a transcriptional co-activator that appears to be induced by Cdk4, as part of the pro-apoptotic events that occur in neurons after DNA damage. For the relevance they have to our work, separate sections of this introduction have been dedicated to the discussion of the neuronal DNA damage apoptotic model and Cited2.

DNA damage in neurons.

DNA damage in neurons has been identified as one important component of neurodegenerative conditions including Ischemia (Chen et al., 1997), Parkinson's (Alam et al., 1997) and Alzheimer's Disease (Lovell and Markesbery, 2001), among others.

Studies suggest that DNA damage, rather than only a consequence, is a key initiator of apoptosis in neurons. For instance, mice deficient in a variety of DNA damage repair pathways (DNA end-joining proteins: DNA ligase IV and XRCC4 and 2 and Ku proteins and DNA polymerase Beta) display aberrant neuronal loss and impaired neurodevelopment (Barnes et al., 1998; Deans et al., 2000; Sugo et al., 2000). Additionally, DNA damage sensing enzymes such as poly ADP-ribose polymerase (PARP) and p53, appear to be required for death evoked by ischemic injury (Crumrine et al., 1994; Endres et al., 1997). Finally, in vitro experiments has shown that DNA damaging agents, such as camptothecin and etoposide, induce apoptosis in murine cortical neurons through the activation of distinct signals (discussed ahead) that are not activated by other apoptotic agents such as staurosporine (Morris et al., 2001; Kruman et al., 2004).

- **The camptothecin model.**

Camptothecin is a cytotoxic quinoline alkaloid derived from the Chinese tree *Camptotheca acuminata* Decne. It is a powerful inhibitor of topoisomerase I, an enzyme that regulates DNA superhelicity during cellular processes such as DNA replication and transcription (Wang, 2002). During these processes, topoisomerase I relaxes the supercoiled DNA by transiently cleaving one strand and rotating it over the intact one while suffering topological changes (Champoux, 2001). This is done in such a manner that religation of strands is ultimately facilitated. During camptothecin toxicity, its molecules intercalate between the topoisomerase I/DNA complex and so prevent strand

religation (Staker et al., 2002). As a result, double-stranded cuts of DNA accumulate eventually leading to cell death.

In neurons, camptothecin induces DNA breakage as rapidly as 10 minutes after exposure, as shown by single-cell-gel-electrophoresis comet-assay analyses (Zhang et al., 2006). This toxicity appears to be dependent on transcription, since transcription but not replication blockers protect neurons from camptothecin-induced death (Morris and Geller, 1996). Although surprising at first, the effects of camptothecin on transcription regulation has been confirmed by numerous reports (reviewed in (Capranico et al., 2007)).

The use of camptothecin treatment of primary cortical neurons has proven to be a powerful tool as an in-vitro model of neuronal apoptosis. Many of the signals involved in neuronal DNA damage-induced death have been identified exploiting this paradigm. The pathways such identified are discussed ahead.

- DNA damage-induced apoptotic pathways.

The signaling pathways which regulate DNA damage-induced neuronal death are intricate and balanced by both pro- and anti-apoptotic signals. These early activated signals converge in the classical apoptotic pathway mediated by BAX translocation to mitochondria membrane and successive caspases activation (Fig 1.1).

The involvement of the intrinsic mitochondrial pathway in DNA damage-evoked neuronal apoptosis is widely accepted today. There is evidence that camptothecin-induced apoptosis is mediated by cytochrome c release and caspase-3 activation (Stefanis et al., 1999; Keramaris et al., 2000). Furthermore, BAX deficient neurons and caspase-3 deficient neurons show significantly delayed camptothecin-induced death (Keramaris et al., 2000; Morris et al., 2001).

Three main early pro-apoptotic pathways have been shown to regulate the mitochondrial pathway of death after DNA damage. (i) p53 activation, known for its role in a DNA damage check point in proliferating cells, appears to be induced by at least

three independent signals: nuclear factor kappa beta (NF- κ B) (Aleyasin et al., 2004), ataxia telangiectasia-mutated (ATM) (Keramaris et al., 2003) and Calpains (Sedarous et al., 2003). (ii) The Cdc42-JNK-c-jun pathway (Ghahremani et al., 2002), known as a stress response in other death models, also has an impact in DNA damage-induced apoptosis. (iii) Almost immediately after insult, activation of G1/S cyclin-dependent kinases (D1/Cdk4, 6) can be detected which leads to E2F transactivation through Rb hyperphosphorylation (Park et al., 1998; Park et al., 2000b).

Research shows that both Cdks and p53 need to be activated for death to occur (Morris et al., 2001). It is apparent as well that they are activated in an independent manner, since inhibition of one has no impact on the activation of the other (Morris et al., 2001). They both however act upstream of the mitochondrial pathway of death, since inhibition of both signals results in the inhibition of BAX translocation, cytochrome c release and caspase activation (Morris et al., 2001). Alternatively, there appears to exist crosstalk between the Cdks and the c-jun signals, since c-jun phosphorylation level seems to be dependent on Cdk activation as well as on JNK activation (Ghahremani et al., 2002; Besirli and Johnson, 2003).

Recent developments had brought some light on the signals that appear to regulate de activation of cell cycle Cdks after DNA damage. It appears that mechanisms that exist in proliferating cells for the same purpose get activated in neurons after genotoxic insult. Such is the case of the Chk1/Cdc25A pathway, where checkpoint kinase 1 (Chk1) basal activity is inhibited after DNA damage with an associated increase in cell division cycle (Cdc) 25A phosphatase activity leading to Cdk4/6 activation and Rb phosphorylation (Zhang et al., 2006). An additional signal appears to reinforce Cdc25A activation in this paradigm. After DNA damage, Pim-1 transcript, protein and kinase activity are upregulated by the NF- κ B pathway, leading to Cdc25A activation (Zhang et al., 2010). Finally, Sertad1/TripBr1, a Cdk4 activator in proliferating cells (Sugimoto et al., 1999; Li et al., 2004), is significantly upregulated

after DNA damage in neurons and appears to be required for Rb phosphorylation to occur (Biswas et al., 2010).

Figure 1.1. Pro-apoptotic pathways activated in neurons after DNA damage.

The requirement for JNK, p53 and Cdk4/6 signals for apoptosis to occur is well documented (see text for scientific evidence). Nevertheless, the upstream signals that regulate them and those that mediate their effects over the mitochondrial classical pathway of death are only now being elucidated,

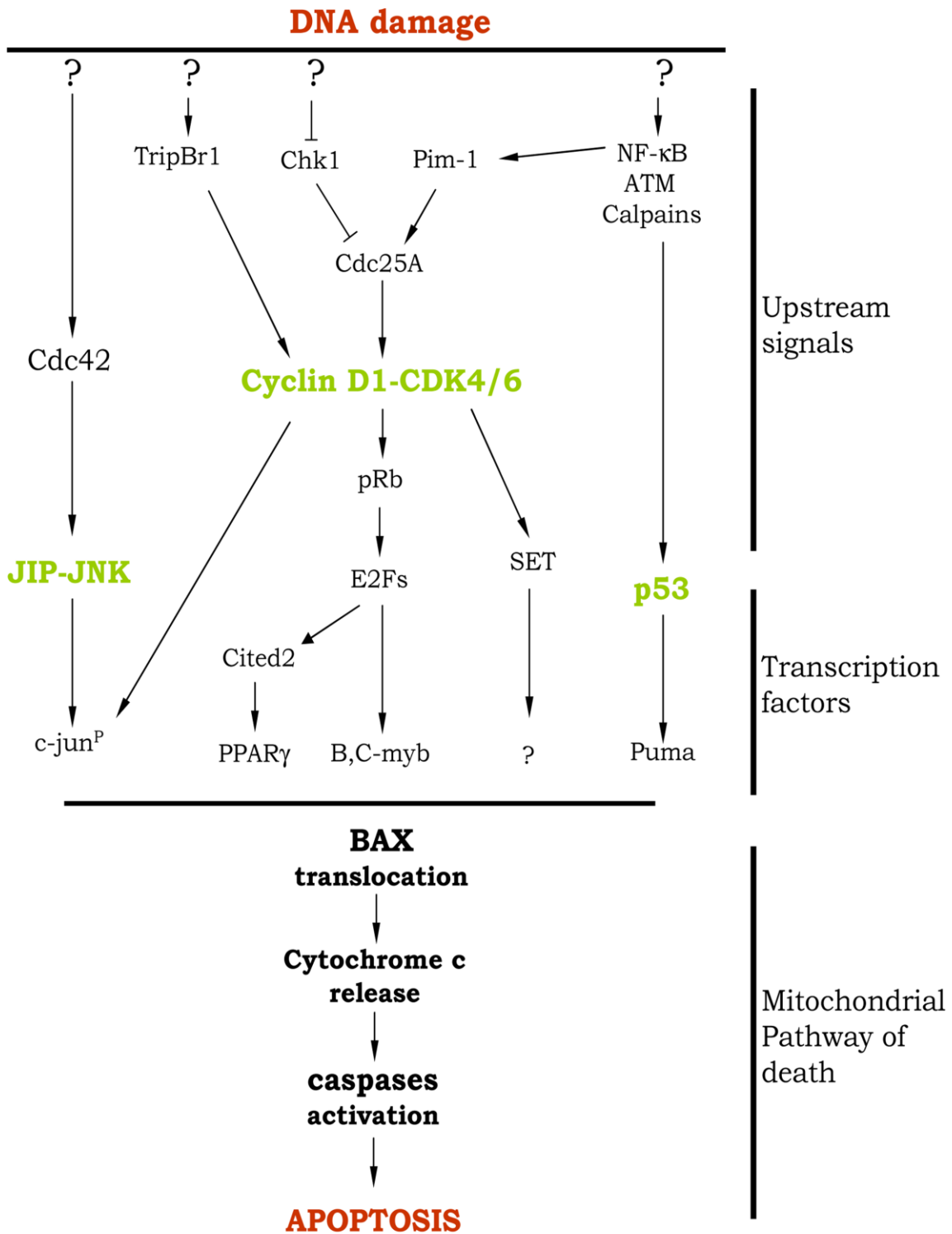


Figure 1.1. Pro-apoptotic pathways activated in neurons after DNA damage.

Cited2: CBP/p300 interacting transactivator with Aspartic and Glutamic acid (ED) rich termini 2

Cited2 was first identified as a cytokine-induced transcriptional coactivator (Shioda et al., 1997). Research showed that it is ubiquitously expressed and localized to the nucleus where it is almost entirely bound to p300 and/or CBP (cAMP response element-binding protein-binding protein), key regulators of RNA polymerase II-mediated transcription (Bhattacharya et al., 1999; Leung et al., 1999). The characterization of Cited2-deficient mice revealed that this coactivator is critical to development since its deficiency leads to embryonic lethality from stage E13.5 (Bamforth et al., 2001; Yin et al., 2002). Knockout (KO) embryos exhibit cardiac malformations, adrenal agenesis, neural crest defects and exencephaly (Bamforth et al., 2001), with the cardiac defects being the primary cause of death. Investigation on the possible mechanisms involved in Cited2 roles during development has revealed some of its targets.

Cited2 is a target and a negative regulator of the Hypoxia-Inducible factor 1- α (HIF1- α) (Bhattacharya et al., 1999). Bhattacharya and collaborators showed that HIF1- α induces Cited2 transcription and that the latter, in turn, competes with it for the interaction with the Taz1 domain of CBP/p300. This competition leads eventually to the downregulation of HIF-1 α in a negative feedback mechanism (Bhattacharya and Ratcliffe, 2003; Freedman et al., 2003). This pathway appears to be the main one behind the Cited2 role on heart development. HIF-1 α levels were found to be upregulated in hearts of Cited2 deficient mice embryos and heterozygosity for HIF-1 α almost completely rescued the heart defects (Xu et al., 2007).

Relevant to CNS development, Cited2 has been identified as a transcriptional coactivator of all isoforms of tfap2 (AP-2) (Bamforth et al., 2001), transcription factors essential in neural crest, neural tube and cardiac development. Although it could be considered a secondary effect of the cardiac malformations, the exencephaly phenotype present in Cited2 KO mice could be justified at least in part by this interaction. It has

been reported that exencephaly in *Cited2* KO mice can be rescued using folic acid supplementation (Barbera et al., 2002), a treatment normally used to prevent neural tube defects. Moreover, AP-2 deregulation also leads to exencephaly (Kohlbecker et al., 2002) and this transcription factor appears to participate in tissue folate homeostasis by regulating the expression of the human reduced folate carrier (hRFC) gene (Whetstone et al., 2002). These observations support the notion of *Cited2* having a more direct role in CNS development.

Additionally, *Cited2* can act as a ligand independent activator of peroxisome proliferator-activated receptors (PPARs) α and γ (Tien et al., 2004). This interaction could explain part of the systemic impact of *Cited2* deficiency in mice, since PPAR transcription factors have been implicated in numerous processes such as cell growth, lipid homeostasis, angiogenesis, apoptosis and differentiation of the adipose tissue, brain, placenta and skin (Michalik et al., 2002; Lehrke and Lazar, 2005; Chaturvedi and Beal, 2008; Abbott, 2009).

Extensive research has linked *Cited2* activity to tumor development and transformation (Chou et al., 2006; Bai and Merchant, 2007; Daino et al., 2009; van Agthoven et al., 2009). Nevertheless, no functional studies have ever been done to elucidate if and how *Cited2* indeed has a role in cell cycle regulation. One existing hypothesis states that *Cited2* promotes proliferation potentially by the activation of the expression of the polycomb-group genes *Bmi-1* and *Mel18* (Kranc et al., 2003), negative regulators of the *ink4a-ARF* locus. Further research would be needed to test this theory.

Previous to our own work (chapter 2), no functional studies had ever been done on the possible roles of *Cited2* in neurons regarding development or neurodegeneration. Correlative data suggested that *Cited2* was upregulated in ischemic brains, but in those regions that appear to be resistant to degeneration (Sun et al., 2006). This piece of evidence led the authors to hypothesize that *Cited2* could be a potentially protective signal. Contrary to that idea, our work suggests that *Cited2* is a proapoptotic signal, regulated by *Cdk4* activation after genotoxic insult (Gonzalez et al., 2008).

Post-mitotic Cdks in the Central Nervous System.

As mentioned before, postmitotic Cdks are regarded as atypical due to their presence in neurons. Cdk5 was almost simultaneously reported on under different names as a neurofilament kinase (Hellmich et al., 1992), a Cdk human family member (Meyerson et al., 1992), a bovine brain kinase (Lew et al., 1992) and a Tau kinase (Ishiguro et al., 1992). Later on, the Cdk5 name was adopted following its recognition as the active subunit of the complex that had been previously identified (Kobayashi et al., 1993). These findings triggered intense research on the possible roles of what appeared to be a very special Cdk (reviewed in (Dhariwala and Rajadhyaksha, 2008)). Although Cdk5 itself is ubiquitously expressed, it was initially thought to be only active in neurons due to the high expression of its activating partners (p35/p25 and p39) in the CNS compared to other tissues (Tang and Wang, 1996). Nevertheless, research has revealed that Cdk5 appears to have extraneuronal functions, such as regulation of myoblast differentiation and secretory exocytosis in pancreatic cells, among others (reviewed in (Rosales and Lee, 2006)). However, this section will concentrate on those Cdk5-related findings relevant to CNS development and degeneration.

PCTAIRE and PFTAIRE kinases share the same evolutionary ancestor with Cdk5 (Liu and Kipreos, 2000). This would suggest that they are also relevant in neurons. Accordingly, research is starting to reveal some of the potential roles of these two neuronal Cdks. Virtually nothing is known about the rest of the members of this subgroup.

In CNS degeneration

Presently, Cdk5 is the only postmitotic Cdk that has been linked to neurodegeneration. Since its identification, the Cdk5/p25 complex was known to phosphorylate Tau and promote the formation of paired helical filaments (Ishiguro et al., 1992). Such Tau transformation is an event that mediates the formation of

neurofibrillary tangles, a pathological hallmark of numerous neurodegenerative disorders, such as Alzheimer's and Parkinson's disease. Later on, the study of brains at early stages of AD revealed increased Cdk5 activity when compared to controls as well as Cdk5 co-localization with neurofibrillary tangles (Pei et al., 1998).

Additional research showed that neurotoxic events such as oxidative stress, β -amyloid treatment, and excitotoxicity triggers calpains activation. These proteases, in turn, mediate cleavage of membrane-bound p35 into p25 enabling the formation of the Cdk5/p25 cytoplasmic complex with access to a different set of substrates (Dhavan and Tsai, 2001). Investigation using in-vivo models has implicated the previously described events in Parkinson's (Crocker et al., 2003; Smith et al., 2006; Qu et al., 2007) and stroke-related neurodegeneration (Rashidian et al., 2005; Rashidian et al., 2009). Data demonstrate that Cdk5 inhibition is neuroprotective in the MPTP Parkinsonian model. Additionally, it appears that Cdk5-induced neuronal death is mediated, at least in part, by phosphorylation (and inactivation) of MEF2 (Smith et al., 2006) and Prx2 (Qu et al., 2007). In this way, Cdk5/p25 shuts down survival and antioxidant pathways in dopaminergic neurons. Equally, these two same targets, Prx2 and MEF2, were implicated in Cdk5 prodeath mechanisms during stroke (Rashidian et al., 2009)

Interestingly, recent developments have led to the hypothesis that Cdk5 mediates the maintenance of cell cycle arrest in differentiated neurons and that loss of this role would lead to cell cycle re-entry and apoptosis. It has been shown that cell cycle control is deficient in Cdk5 KO embryos, since cell cycle appears to still be active in neurons found in the cortical plate. Additionally, *in-vitro* transfection of these cells with a wild-type Cdk5 construct restored cell cycle control (Cicero and Herrup, 2005). It was later argued that this Cdk5 role is dependent on its nuclear localization and that export of Cdk5 to the cytoplasm correlates with cell cycle re-entry. Data revealed that pharmacological inhibition of Cdk5 translocation abolishes cell cycle activation (as detected by BrdU staining) in wild type neurons after β -amyloid insult (Cicero and Herrup, 2005). Furthermore, Cdk5 nuclear vs. cytoplasmic overexpression in Cdk5

deficient neurons blocks cell cycle activation after insult (Zhang et al., 2008; Zhang and Herrup, 2008). Further investigation showed that Cdk5 pharmacological inhibition blocks Cdk4 activation and Rb phosphorylation in neurons after β -amyloid treatment (Lopes et al., 2009). This group of evidence reinforces the notion that Cdk5-mediated cell cycle re-entry could be important to neurodegenerative processes relevant to AD. It would be interesting to explore if such is the case for other neurodegenerative conditions. A previous report show that inhibition of nuclear Cdk5 activity using a dominant-negative approach does not protect neurons against DNA damage induced apoptosis (O'Hare et al., 2005), a model of death that is known to be mediated by activation of cell cycle Cdks.

In CNS development

Cdk5 expression and basal activity in the mature CNS pointed to its potential roles in neuronal physiology and maintenance. Consequently, Cdk5 have been shown to regulate many essential neuronal processes, including intracellular trafficking and transport (Smith and Tsai, 2002), cytoskeletal dynamics (Xie et al., 2006) and synaptic plasticity (Hawasli and Bibb, 2007).

Relevant to CNS development, the generation of a Cdk5 deficient mouse demonstrated that the CNS requirement for this kinase starts before birth, since the deficiency leads to perinatal lethality (E18-P5) (Ohshima et al., 1996). The characterization of the brain of mutant mice revealed they have abnormal corticogenesis, with inverted cortical laminar structure (Ohshima et al., 1996; Gilmore et al., 1998). Additionally, thalamocortical axons showed to be clearly misguided (Gilmore et al., 1998). These findings suggested that Cdk5 had a role in neuronal migration and differentiation as well as axogenesis. Further research confirmed these hypotheses.

The generation of a Cortex-specific Cdk5 conditional KO mouse showed that

they replicated the same inverted layering of the cerebral cortex and the abnormal axonal trajectory and dendritic development, just as the germline KO (Ohshima et al., 2007). Furthermore, *in-utero* electroporation of green fluorescent protein (GFP) revealed that radially migrating neurons present in wild-type embryos were absent in the Cdk5 KO embryos. When specific Cdk5 inhibition (dominant-negative approach) was driven in wild-type migrating neurons, the multipolar-to-bipolar transition of these cells was impaired in an obvious cell-autonomous manner (Ohshima et al., 2007). These data confirmed that Cdk5 is required for cortical development due to its role in regulating migration and differentiation.

Moreover, Cdk5 appears to mediate the neuronal reception and intracellular signaling triggered by extracellular cues to regulate neurite outgrowth and synaptogenesis (reviewed in (Cheung and Ip, 2007)). Research revealed that Cdk5 involvement in these events is multilayered. For example, along the brain-derived neurotrophic factor (BDNF)-Trk receptors-neurite outgrowth signaling pathway, (i) Cdk5 phosphorylates (activates) and is phosphorylated by (activated) the TrkB receptor. Blocking of this event by overexpression of a TrkB molecule lacking its Cdk5-phosphorylation site abolishes BDNF signaling. Additionally, TrkB pharmacological inhibition eradicates Cdk5 activation by BDNF treatment and Cdk5 inhibition blocks BDNF-induced dendritic growth (Cheung et al., 2007) (ii) Cdk5 activation is required for BDNF-induced Cdc42 (GTPase family member) activation, and overexpression of a constitutively active Cdc42 rescues the lack of dendritic growth in Cdk5 KO neurons following BDNF stimulation (Cheung et al., 2007). This last event would put Cdk5 as the link between BDNF signaling and, potentially, cytoskeleton rearrangements to enable neurite outgrowth, since Cdc42 has a well known role in the regulation of actin dynamics (Kuhn et al., 2000). Furthermore, Cdk5 appears to regulate cytoskeletal dynamics as well, since it is capable of inactivating PAK1 by hyperphosphorylating it, in a Rac (GTPase member) dependent manner (Nikolic et al., 1998; Smith, 2003). There is evidence as well linking Cdk5 activity to the regulation of microtubules and

neurofilament dynamics (Dhavan and Tsai, 2001; Smith, 2003). It is clear that these roles of Cdk5 would as well have an impact in migration, neurite outgrowth and synaptogenesis.

Regarding the PCTAIRE kinases, their involvement in CNS development has not as yet been explored. This is probably due to the potential compensation mechanisms that would trigger the lack of only one of the three mammalian isoforms. Intriguingly, evidence shows that, in myocytes, PCTAIRE-1 interacts with p35 and is phosphorylated by Cdk5 with a resulting increase in its kinase activity. Furthermore, PCTAIRE-1 is accumulated at the neuromuscular junction, just as Cdk5 is, and Cdk5 KO mice show decreased expression of PCTAIRE-1 both in brain and muscle (Cheng et al., 2002). Additionally, both PCTAIRE-1 and Cdk5 have been associated with exocytosis mechanisms (Lilja et al., 2004; Palmer et al., 2005; Liu et al., 2006). Although studies need to be done using CNS relevant models, these findings suggest that PCTAIRE and Cdk5 may be part of the same pathways.

It is the main objective of chapter three of this thesis to explore the potential roles of PFTAIRE during CNS development. Consequently, the evidence related to previous studies on this Cdk, as well as the description of the animal model used for our studies, have been included as separate sections of this chapter.

PFTAIRE

PFTAIRE was first identified in a screen for neuronal cdc2-like cDNA's (Lazzaro et al., 1997). Its initial denomination was taken from its variation of the conserved PSTAIRE amino acid sequence of classical Cdks (Lazzaro et al., 1997; Besset et al., 1998). Later on, it was called PFTAIRE kinase 1 (Pftk1 in mice, PFTK1 in humans, currently denominated Cdk14 (Entrez-gene, 2010)). This Cdk proved to be conserved from worms to humans (Liu and Kipreos, 2000). In invertebrates, there is only one PFTAIRE complex gene, encoding for multiple mRNA splicing forms and protein

isoforms (Stowers et al., 2000). Alternatively, vertebrates have two different PFTAIRE genes, each encoding for multiple protein isoforms (Yang and Chen, 2001; Entrez-gene, 2010). Interestingly, the second mammalian gene *Pftk2/PFTK2* (currently denominated *Cdk15* (Entrez-gene, 2010)) was only identified as a result of the genome sequencing project and no research on it has ever been published.

Pftk1 transcripts appear to be highly expressed, but not exclusively, in the CNS during and after development (Lazzaro and Julien, 1997; Besset et al., 1998). Interestingly, protein expression studies suggest a tight posttranscriptional regulation, since mRNA expression does not always correlate with detectable protein levels. For example, in lung murine tissue very high mRNA levels can be detected while no protein seems to be expressed (Lazzaro et al., 1997). Additionally, although PFTK1 appears to have two nuclear localization sequences (NLS), the protein seems to be mainly cytosolic (Lazzaro and Julien, 1997; Yang and Chen, 2001).

Research using *Drosophila melanogaster* revealed that PFTAIRE is essential for developmental processes (Stowers et al., 2000). Flies deficient for *Eip63E*, the *Drosophila* PFTAIRE homolog, die during embryonic/larval stages. This lethality could be rescued by re-introducing the expression of the conserved kinase domain common to all isoforms, but not when the PFTAIRE sequence which potentially mediates cyclin binding was mutated. This led authors to suggest that the role of this kinase in development might be mediated by its potential involvement in cell cycle regulation (Stowers et al., 2000). Nevertheless, none of the classical known cyclins appeared to interact/activate *Eip63E* (Rasclé et al., 2003).

In addition to *Eip63E* apparent requirement for development, several other observations imply that PFTAIRE could be as relevant for CNS development as *Cdk5*. (i) PFTAIRE and *Cdk5* are two of the three major subbranches from a same evolutionary ancestor (Liu and Kipreos, 2000). (ii) PFTAIRE and *Cdk5* share the highest amino acid sequence identity between Cdks (50-52%) (Besset et al., 1998). (iii) PFTAIRE expression profile during murine embryonic development is far closer to that of *Cdk5* than to *Cdc2*

expression profile (Lazzaro et al., 1997; Besset et al., 1998). (iv) Both Cdk5 and PFTAIRE seem to have a role in axogenesis (see chapter three for PFTAIRE related evidence). Nevertheless, they may function in opposing pathways, since inactivation of Cdk5 in cortical neurons results in inhibition of axonal growth (Nikolic et al., 1996); the opposite to the one obtained in our lab after expression of kinase-dead PFTAIRE in cortical neurons (unpublished data). (v) Although Cdk5 deficiency has an effect on axon guidance, it does not totally prevent axons from projecting (Gilmore et al., 1998; Connell-Crowley et al., 2000). This could suggest that other signals including PFTAIRE may be involved in the regulation of the axon guidance mechanisms.

Looking for clues on PFTAIRE potential biological functions, several approaches have been used to identify its interacting proteins (Yang et al., 2002; Barrios-Rodiles et al., 2005; Gao et al., 2006; Ewing et al., 2007; Jiang et al., 2009). However, the functional relevance of very few of these interactions has been explored. PFTK1 specifically interacts with cyclin D3 (CCND3) in mammalian neuroblastoma cells (Shu et al., 2007). This interaction appears to enable PFTK1 to phosphorylate Rb, and this activity is inhibited by the formation of a ternary complex with p21. Additionally, PFTK1 siRNA caused cell cycle arrest at G1, whereas ectopic expression of PFTK1 promoted cell proliferation. These findings suggest that this Cdk has a role in cell cycle regulation in transforming cells but does not confirm if that would be the case under physiologic conditions, specially since PFTK1 does not appear to interact with any of the rest of the cyclinD isoforms.

Three of these yeast-two hybrid approaches to find PFTAIRE interactors led to the identification of a novel cyclin: CCNY or cyclinY (Stanyon et al., 2004; Gao et al., 2006; Jiang et al., 2009). PFTK1 association to cyclinY, appears to mediate the kinase sequestration to the plasma membrane and its further activation (Jiang et al., 2009). Although the cytoplasmic PFTK1/CyclinY complex was also active, the targets specificity seems to depend on cellular localization, since cyclinY itself appears to always be a target for phosphorylation, but Rb is phosphorylated only by the

cytoplasmic complex (Jiang et al., 2009). Even though PFTK1 interaction to cyclinY has been confirmed in brain tissue, all the findings associated to the kinase activity were done in kidney cell lines (Jiang et al., 2009) and so the relevance of the interaction for the CNS-related PFTK1 functions remains to be explored. Nevertheless, the PFTK1/cyclinY complex has been shown to phosphorylate, *in-vivo*, the Low-density lipoprotein receptor related proteins 6 (LRP6), a transmembrane receptor that mediates Wnt/catenin signalling (Davidson et al., 2009). LRP6 phosphorylation appears to be dependent not only on the presence of cyclinY but on cell cycle activity, since downregulation of both cyclinY and Cdc25 inhibited LRP6 phosphorylation (Davidson et al., 2009). Additionally, research in *Drosophila* showed that cyclinY is essential for proper development, with a lethality effect for the deficiency very similar to that of Eip63E (Liu and Finley, 2010). Furthermore, the Wnt pathway appears to be required for proliferation/differentiation of neuroprogenitor cells (Ille et al., 2007; Grigoryan et al., 2008). Put together, this group of evidences points to the possibility of a cell cycle-related PFTAIRE role that could be relevant in neuroprogenitor cells and dependent on cyclinY interaction and Wnt signalling.

Finally, our own data included in chapter three of this thesis confirms that Eip63E is required in differentiated neurons for *Drosophila* CNS development, especially axogenesis, since Eip63E downregulation in neurons leads to precocious axon outgrowth, misguidance and defasciculation.

The *Drosophila melanogaster* model

Drosophila melanogaster has become a powerful research model due mainly to its rapid generation time (~10 days at 25°C), inexpensive culture requirements and large progeny number from a single cross. This organism provides for a vast battery of genetic tools: rapidly expanding collection of mutants, transposon-based methods for gene manipulation, systems that allow controlled ectopic gene expression and balancer chromosomes (see ahead). Flies, like worms, facilitate the carrying out of large-scale

functional genetic screens, inexpensively and rapidly, to find mutations that affect relevant processes. Additionally, the small *Drosophila melanogaster's* brain (~ 250000 neurons) is organized in a way similar to mammals, with areas specialized in learning, memory, olfaction and vision (Tanaka et al., 2004; Sanchez-Soriano et al., 2007). These facts have led to the use of *Drosophila melanogaster* for research of mechanisms relevant to neurodegenerative conditions such as Alzheimer's' and Parkinson's diseases (reviewed in (Cauchi and van den Heuvel, 2006))

Additionally and more relevant to us, the ventral nerve cord (VNC) provides for an excellent *in situ* model to study CNS-related processes. Although much remains to be clarified on the mechanisms involved, the main developmental events of the *Drosophila* VNC have been well described. Moreover, a plethora of specific markers to light up different structures are readily available. This enables for relatively easy characterization of phenotypic effects associated to the deficiency/manipulation of any given gene on VNC development. Since results in chapter three of this thesis are based on the use of this model, a description of neurogenesis and axogenesis at the VNC has been included, as well as a review on the genetic tools used for the research.

- Embryonic development of the Ventral Nerve Cord

Neurogenesis.

The first events in *Drosophila* CNS development occur during blastoderm (embryonic stage 5). At this stage, the dorso-ventral specification mechanisms (Flores-Saaib and Courey, 2000) give rise to the presumptive neurogenic (ectoderm), midline (mesectoderm) and mesoderm regions (Weigmann et al., 2003). Later on during gastrulation (stages 6-7), the mesoderm anlage (characterized by expression of the twist gene (Furlong et al., 2001)) invaginates and cells migrate and proliferate leading to mesoderm formation (Leptin et al., 1992).

This last event brings together the midline cells (characterized by expression of

the single-minded gene (Nambu et al., 1990)). Until that moment, midline anlage cells are organized as two parallel rows dividing the mesoderm and the presumptive neurogenic regions. Once they meet at the ventral furrow, precursor cells from it will give rise to a variety of neurons (Jacobs and Goodman, 1989b) and midline glial cells (Jacobs and Goodman, 1989a) that will have a key role in axon development at the VNC.

Once the ventral midline is formed (stage 8), the cells of the neurogenic anlage (characterized until that moment by stripes of expression of the rhomboid gene (Ip et al., 1992)) undergoes enlargement and reorganization into proneural clusters of 5-7 cells with the potential to form neuronal precursors. Cellular interactions (Delta-Notch pathway (Campos-Ortega and Knust, 1990)) will lead to only one of these cells expressing neurogenic genes (achaete-scute and others (Campos-Ortega, 1995; Hassan and Vaessin, 1996)). This unique cell will then migrate internally (delamination) to position itself between the mesoderm and the ectoderm and will constitute a neuroblast.

From this first delamination event and for approximately 3 hours (stages 8-11) neuroblasts will segregate from the ectoderm in 5 pulses (segregation waves) to complete a set of 30-32 neuroblasts per hemisegment (Campos-Ortega, 1993). After each consecutive segregation wave, neuroblasts will form a monolayer between ectoderm and mesoderm in such a way that they can be distinguished from each other based on their size and location within the array (SI to SV neuroblasts) (Doe, 1992). Nevertheless, proliferation and differentiation of neuroblasts starts scarcely 10-20 minutes after the beginning of segregation, and so SIII-SV neuroblasts insert themselves into a much more complex cellular arrangement than that one encountered by SI-SII neuroblasts. Although not completely understood, this process appears to be dependent as well on the 'checker board-like' embryonic outline imposed by the anterior-posterior and dorso-ventral patterning genes expressed since early embryonic stages (Skeath and Thor, 2003). For instance, deficiency for engrailed-invented, two of

the segment polarity genes, leads to identity change of row-five neuroblasts (N5-3) to row-four ones (N4-2) (Bhat and Schedl, 1997).

It is through embryonic stage 11 that neuroblasts originated from the mesectoderm or midline start to segregate (Klambt et al., 1991). These comprise the unpaired median neuroblast (MNB), the midline precursor cells (MPs), three midline glial precursors (MGP, MGM, MGA) and the ventral unpaired median precursors (VUM) (Klambt et al., 1991). These cells will give rise to a very distinct progeny (discussed ahead).

Based on their progeny, neuroectoderm-derived neuroblasts can be divided into two types: those that will only give rise to glial cells, called glioblasts, and those with the potential to give rise to both neurons and glial cells, called neuroglioblasts. Shortly after delamination, most neuroglioblasts divide asymmetrically up to 12 times, giving birth each time to a ganglion mother cell (gmc) and a new neuroblast that again undergoes asymmetrical division. A mechanism involving the *miranda* and *prospero* genes is responsible for the asymmetry of this division (Ikeshima-Kataoka et al., 1997; Shen et al., 1997)). A tightly regulated gene cascade of transcription factors (Hunchback → Kruppel → Pdm → Castor → Grainyhead, (Brody and Odenwald, 2002; Skeath and Thor, 2003)) confers temporary identity to both daughter cells (new neuroglioblast and gmc) and determine their ultimate fate (Grosskortenhaus et al., 2005; Tran and Doe, 2008). In the case of midline-derived precursors, the gene cascade mechanism described above does not apply, since they appear to be fate-constricted from birth and some of them only divide once before differentiating (Jacobs and Goodman, 1989a; Klambt et al., 1991).

At the time of their birth, gmcs are committed to a distinct differentiation pathway and when they terminally divide they may produce either two neurons, one neuron and one glial, or two glial cells (Jones, 2001). Not much is understood about the mechanisms that regulate the pattern of glial versus neuronal progeny determination. Nevertheless, it is well accepted that the decision to make a glial cell instead of a

neuron depends on the activation of the glial cells missing gene (*gcm*) (Hosoya et al., 1995). *Gcm* is a transcriptional activator that is transiently expressed in all glial cells, except midline-derived ones. This nuclear factor acts as a binary switch, turning on glial fate and off the default neuronal fate, since *gcm* deficiency leads presumptive glial cells to develop into neurons and *gcm* overexpression results in all presumptive neurons becoming glial cells (Kim et al., 1998). The regulation of the expression of *gcm* varies in lineages derived from different populations of neuroglioblast. Evidence suggests that *gcm* upregulation in some of the presumptive glial cells is dependent on the expression of the *prospero* gene (Akiyama-Oda et al., 2000), although the mechanism linking both events is still unknown. The alternative mechanism regulating *gcm* expression in lineages from the rest of the neuroglioblasts involves inhibition of Notch signaling by the asymmetric factor *Numb*. In that scenario, Notch positively regulates *gcm* expression, while *Numb* promotes neuronal fate (Udolph et al., 2001). Little is known about the genes downstream *gcm* activation. Most of those identified so far are transcription factors that are differentially expressed in different glial cells populations (Jones, 2001; Egger et al., 2002). The most ubiquitously expressed of them is the reverse-polarity gene (*repo*), present in all glial cells, except for midline-derived ones (Xiong et al., 1994; Halter et al., 1995).

Even less is known of the mechanisms involved in full differentiation of neurons and no master gene for regulation of general neuronal fate has ever been identified. Evidence suggests that all neurons in a hemisegment have a distinct identity, with a unique combination of lineage, location, axon pathfinding and target tissue (Landgraf et al., 1997). Genes that appear to be involved in the specification of subsets of neurons; i.e. *islet* and *apterous* (Thor and Thomas, 2002), seem to play a role both in axon pathfinding as well as neurotransmitter and neuropeptide expression (Thor and Thomas, 1997; Benveniste et al., 1998).

The result of the above described complex pattern of neurogenesis is the presence of approximately 30 glial cells (midline and lateral glial (Halter et al., 1995))

and 350 neurons (motor neurons and interneurons (Schmid et al., 1999)) per hemisegment (at each side of the midline). These cells are organized in a stereotypical pattern that is repeated in each segment or neuromere. Axon tracts (discussed ahead) run along the synaptic area (neuropile) while neuronal cell bodies are excluded to the surrounding area, mainly ventrally (cell cortex) (Campos-Ortega and Hartenstein, 1997; Sanchez-Soriano et al., 2007).

Axogenesis.

The final structure of the main axonal pathways of the *Drosophila* VNC is a ladder-like scaffold with the midline running at the centre. Each segment is crossed by two commissures (anterior and posterior) and all segments are connected by longitudinal tracts (longitudinal connectives). The intersegmental and segmental nerves are the axon bundles leaving the VNC laterally towards target tissues.

As it happens in vertebrates, axogenesis in the VNC starts with pioneer axon tracts projected by single neurons that will subsequently be used as guidance cues by follower axons. Midline derived cells play a key role during this process with functions similar to those of the neural tube or floorplate in vertebrate embryos. A discreet number of neuroectoderm derived neurons complete the list of pioneer cells involved in the first stages of axogenesis. Accordingly, Median glial (MGA, MGM and MGP), MP1 neurons and VUM neurons from midline precursors; as well as longitudinal glial, RP neurons, MP2 neurons and anterior and posterior corner cells (aCC and pCC) of neuroectoderm origin, play a key role in early axogenesis (Jacobs and Goodman, 1989b; Klambt et al., 1991; Weigmann et al., 2003).

Development of anterior and posterior commissures.

The pioneering of the posterior commissures is the first event. Once midline glial and neurons are specified (~7:40h into development, early stage 12), early differentiated lateral neurons extend axons that elongate towards the anterior VUM neurons at the

midline. Once there, growth cones change direction to extend anteriorly around VUM and across the space previously occupied by MP1 neurons (which immediately before moved laterally and ventrally away from the midline). That location constitutes the fasciculation point of the contralaterally growing axon bundles. Once they contact each other and fasciculate, they cross the midline (towards peripheral nervous system) and thus form the posterior commissures (Klamt and Goodman, 1991; Klamt et al., 1991). At this point (~8:20h into development, mid-stage 12), the posterior commissures has a 'V' shape pointing anteriorly.

After the migration of MP1 neurons, MGA and MGM glial move closer to the developing posterior commissures and VUM neurons move slightly anteriorly and ventrally while extending their own axons anteriorly along the midline. At the same time, the anterior commissures pioneer axons start growing from lateral neurons towards the midline. Once there, they contact and fasciculate with the VUM axons which bifurcate and extend laterally, while anterior commissures pioneers cross the midline. In this way, the anterior commissures are formed straight across the midline. Therefore, at mid-stage 12, the anterior and posterior commissures meet at the midline, right between MGA and VUM cells (Klamt and Goodman, 1991; Klamt et al., 1991).

Once both commissures are formed, MGM glial migrate posteriorly over the MGA, while RP1 neurons migrate medially. Therefore, at the end of stage 12, MGM and RP1 cell bodies come to ultimately lie between the two commissures, leading to their progressive separation. At stage 14 (~10:40h into development), the separation of the commissures is completed and the posterior commissures are totally straighten across the midline (Klamt and Goodman, 1991; Klamt et al., 1991).

The mechanisms regulating midline crossing in *Drosophila* are highly conserved (from worms to humans) and have been extensively studied (Hummel et al., 1999; Araujo and Tear, 2003; Parker and Auld, 2004; Sanchez-Soriano et al., 2007). Since this section mostly concentrates on the description of the sequence of events leading to VNC formation, we will only mention the generalities of these mechanisms. Two major

regulatory pathways are involved: the Netrin/Frazzled and the Slit/Robo ones. In general, the first pathway attracts axons to the midline while the second one inhibits them from doing so. Midline glial at the VNC express both Netrins (A and B) and Slit simultaneously. The first signal is recognized by Frazzled receptors at the membranes of commissural growth cones and attracts them to the midline. Alternatively, Slit molecules are recognized by Robo receptors leading to the inhibition of longitudinal cells and axons from elongating medially.

It is apparent that the proper development of both anterior and posterior commissures relies on these afore mentioned regulatory pathways. Added to this, research has shown that there are distinct mechanisms operating as early as neuroblasts segregation to differentially regulate the development of posterior versus anterior commissures (Joly et al., 2007; Colomb et al., 2008). For instance; deficiency of *gooseberry-neuron*, a segment polarity gene, leads to the absence of posterior but not anterior commissures. Furthermore, ectopic *gooseberry-neuron* expression in neuroblasts of KO embryos rescues the posterior commissures defect, while ectopic expression in mature neurons does not (Colomb et al., 2008).

Development of longitudinal connectives.

A different set of neurons pioneers the axons that will constitute the longitudinal connectives of the VNC: MP1 from the midline, and the MP2 (ventral and dorsal) and pCC from neuroectoderm precursors. At stage 12, all these neurons are already differentiated and located around the commissures of each segment (Thomas et al., 1984).

During stage 13 (~9:40h into development (Jacobs and Goodman, 1989b)), the pCC neuron at each hemisegment extends its axon anteriorly to contact the vMP2 axon that until that moment had been extending laterally. Both growth cones continue to extend anteriorly to reach the lateral glial cell #5 (LG5) (Jacobs and Goodman, 1989a, b). Meanwhile, at 9:50-10h into development, the MP1 neuron, and shortly after the

dMP2, extend axons laterally, away from the midline, until they contact the pCC axon. At this point, these growth cones turn posteriorly along the pCC axon until reaching the pCC cell body and then the LG5 glial. There, they meet and fasciculate with the pCC/vMP2 axons coming from the adjacent segment (Jacobs and Goodman, 1989b; Hidalgo and Brand, 1997; Hidalgo and Booth, 2000). In this way, the pioneer longitudinal tracts are formed.

From stage 14 on, a series of defasciculation/re-fasciculation events occur. First, the two axon pathways (pCC/vMP2 (inner) and MP1/dMP2 (outer) separate, maintaining the contact only at the segment boundaries (Hidalgo and Brand, 1997). Between stages 14-15, these two fascicles come closer to each other but completely separate, adopting parallel pathways at each side of the midline. Finally (stage 16), re-fasciculation happens and some axons unite the alternative partner: dMP2 with pCC, while vMP2 runs closely but independently along the same tract and the MP1 runs dorsally along the middle tract (Hidalgo and Brand, 1997). This configuration remains till the end of embryogenesis. Nevertheless, at the end of stage 16 a third axon tract appears of still unknown origin (Hidalgo and Brand, 1997; Sanchez-Soriano et al., 2007).

All the events described above require glial-glial and glial-neuron interactions to occur (Hidalgo et al., 1995; Hidalgo and Booth, 2000). Longitudinal glial differentiated from the neuroectoderm, play a key role both in formation and maintenance of the axon connectives. At first, they appear to provide guidance cues, since they are already located along presumptive axon tracts before any axon is pioneered (Jacobs and Goodman, 1989a). Furthermore, early ablation of these cells leads to longitudinal axon defects (Hidalgo and Booth, 2000). Once longitudinal axons start to extend, these glial cells undergo shape changes that enables them to ensheath and support the neuropile (Jacobs and Goodman, 1989a). Later on, it seems that they are still necessary for guidance of secondary axons and maintenance of the axon scaffold, since late ablation of these cells leads to disruption and degeneration of longitudinal connectives (Hidalgo

et al., 1995). Additionally, evidence show that the sequential fasciculation and defasciculation events are also dependent on glial (Hidalgo and Booth, 2000). For instance, specific ablation of glial normally located at the point of fasciculation of the pCC/vMP2 and MP1/dMP2 pathways leads to axons misrouting along the intersegmental nerve (Hidalgo and Booth, 2000).

- Genetic tools.

One of the most remarkable strengths of *Drosophila melanogaster* as a model is its flexibility to genetic manipulation. The genetic tools that have been developed during the last years have placed *Drosophila* at a relevant position for studies of *in-situ* gene action. Presently, researchers can take advantage of balancer chromosomes, specific anatomical antibody probes, varied systems for tissue/cell specific ectopic transgenes' expression, P-element transformation techniques, the FLP/FRT strategy for generation of mosaic animals, sophisticated genetic screen strategies, stock centres and bioinformatics tools (Fujita et al., 1982; Ryder and Russell, 2003; McGuire et al., 2004; Beckingham et al., 2005; Matthews et al., 2005). Two of these methodologies were used for the research described on chapter three of this thesis and so will be further described.

Balancer chromosomes

Balancer chromosomes are chromosomes that have been modified in at least two ways. (1) Multiple inversions and rearrangements have been induced to prevent synapsis between homologous chromosomes during meiosis. As a result, minimal or no recombination occurs, so alleles in the 'balanced' homolog would never segregate together with those in the balancer. (2) A recessive lethal gene mutation has been introduced that leads to lethality of homozygous balancer flies. The mutation involved is usually accompanied by a dominant phenotypic defect in one of the many adult's

anatomical structures, such as wings and bristles. This phenotype then acts as a marker for the presence of the balancer and allows for visual 'genotyping'. Consequently, balancer chromosomes allow for mutant or transgenic flies to be kept without constant screening. In addition, lethal mutations can be maintained as heterozygous lines without danger of wild type flies taking over the colony. (Greenspan, 2004; Beckingham et al., 2005)

Looking for balancers that would provide for embryonic markers, a new generation was created (Reichhart and Ferrandon, 1998; Casso et al., 2000; Halfon et al., 2002). For the purpose, classical balancer chromosomes have been modified to include either a fluorescent protein under the regulation of an well-characterized promoter (e.i, actin Ac5, armadillo) or an internal GAL4-UAS system (explained ahead) that drives the expression of a fluorescent protein in a tissue-specific manner (Fig 1.2A). The latter has the additional advantage of enabling the identification of the embryonic stage, since the Gal4 regulatory element is usually associated to structures that change along development. The invention of these new balancers has significantly simplified the task of linking lethal mutations to embryonic developmental processes.

The Gal4-UAS system.

The Gal4- upstream activated sequences (UAS) system is a result of the application of the P-element transformation techniques in *Drosophila* to enable targeted gene expression (Brand and Perrimon, 1993; Beckingham et al., 2005). This system is based on the galactose-induced gene expression mechanism in yeast, where the Gal4 protein binds to the UAS enhancer-like elements to induce a discreet set of genes. Once the system proved to be separable and defined (Ptashne, 1988) and Gal4 expression in *Drosophila* appeared to be phenotypically inert; the Gal4-UAS system in *Drosophila* was created (Brand and Perrimon, 1993).

In general, one fly is transformed with a tissue specific "enhancer trap" P construct that contains the Gal4 gene (instead of the original lacZ one) and called

'Driver fly'. A second fly is alternatively transformed in a second P element with the gene of interest under the control of a promoter that contains several copies of the UAS sequences. This second line would be the 'responder fly'. Once driver and responder flies are crossed, the resulting progeny would express both constructs and the expression of the gene of interest would only occur in those tissues expressing Gal4 (Fig 1.2B) (Brand and Perrimon, 1993; Duffy, 2002).

The maintenance of driver and responder as separate parental lines has several advantages. First, responder lines can be developed for genes that are toxic or lethal. This allows for strategies such as targeted cell ablation (Hidalgo et al., 1995; Hidalgo and Brand, 1997) and it facilitates the elucidation of cellular functions of lethal genes (e.i., our own data, chapter three). Additionally, rescue and functional cell autonomy experiments are permitted, since the expression of genes of interest can be spatially targeted by using different driver lines for the approach. Presently, a wide array of driver lines that include almost every major tissue type is available from *Drosophila* stock centers.

Besides this classical methodology, two additional approaches have been developed. In one, the Gal4-UAS system is modified so that Gal4 expression is activated only when a hormone analog (RU486) is present. In the other one, Gal4 activity is repressed by Gal80 at accommodating temperatures (~19°C) but not at higher ones (~30°C) (McGuire et al., 2004; Elliott and Brand, 2008). These new strategies add temporal targeting to the existing spatial one provided by the system, making it into an even more powerful tool.

The above discussed *Drosophila* model and genetic tools were used as methodology for the PFTAIRE-related research presented in chapter three of this thesis (Fig 1.2). Mutants deficient for Eip63E (fly PFTAIRE) and UAS-RNAiEip63E transgenic lines were characterized for VNC developmental stages/structures. This allowed for the evaluation of the potential relevance of PFTAIRE on CNS development.

Figure 1.2. Examples of application of *Drosophila melanogaster*'s genomic tools included in chapter three experiments.

Simplified schematic representation of crossing strategies. **(A)** Identification of Eip63E deficient embryos using a green balancer for chromosome three. Embryonic progeny is a mix of possible genotypes where only KO embryos are negative for GFP expression. The lethal nature of homozygosity for either balancer or Eip63E deficiency leads to 100% heterozygous adult populations. **(B)** Functioning of the Gal4-UAS system as applied to driving tissue-specific downregulation of Eip63E expression (RNAi for Eip63E) on a wildtype background.

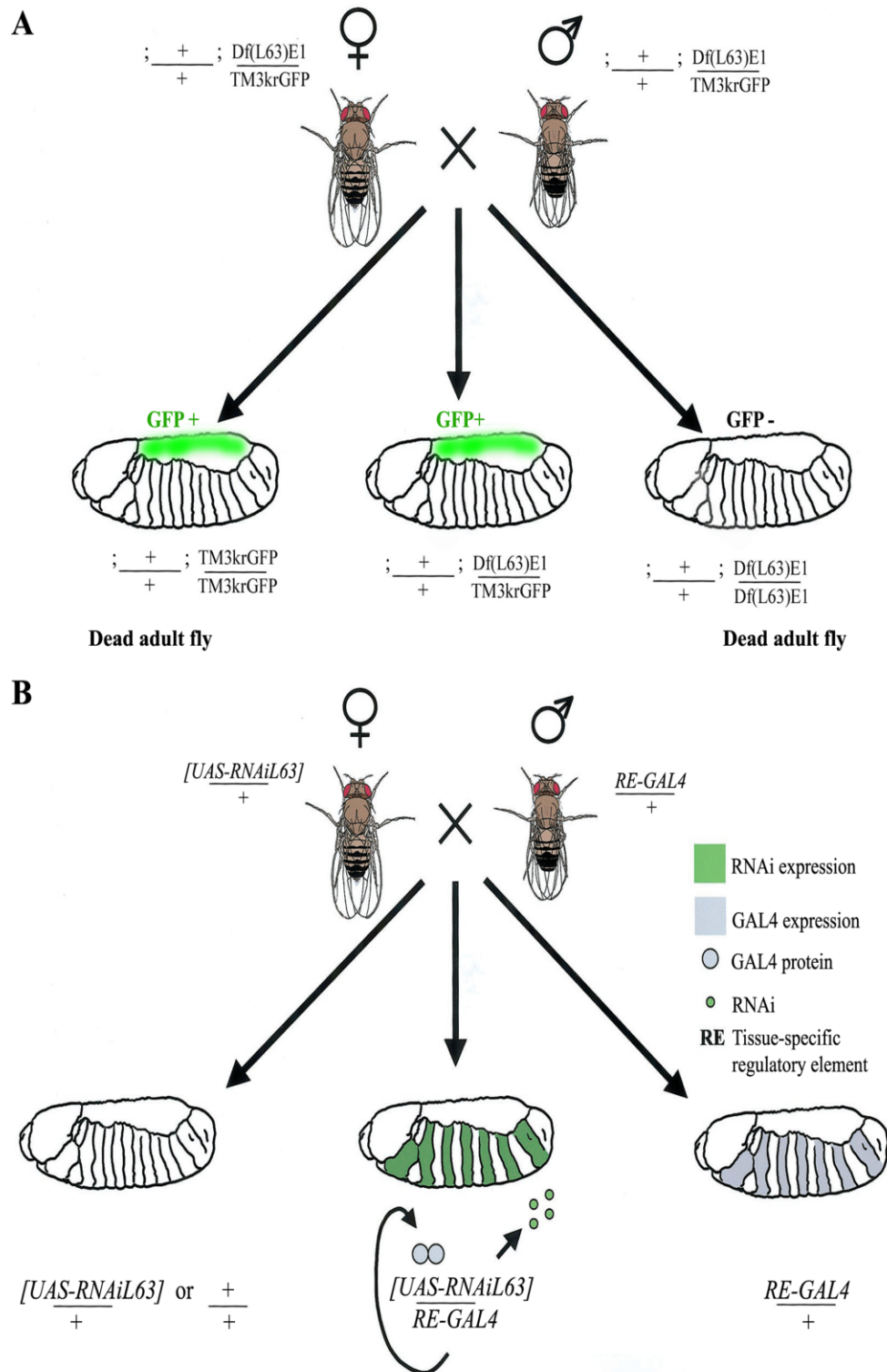


Figure 1.2. Examples of application of *Drosophila melanogaster*'s genomic tools included in chapter three experiments.

Statement of research problem, rationale and objectives.

The research content of this thesis is part of the general objective of exploring the roles of Cdks in CNS development, maintenance and apoptosis. Since two independent projects are included, the problem, rationale and objective of each are presented separately.

Cell cycle Cdk4 in neurodegeneration. The Cited2 project.

Previous to this work, it had been reported that Cdk4 activation was one of the main pro-apoptotic signals to be activated in neurons after DNA damage. Data showed that Cdk4 activation was independent of the p53 pathway and required for neuronal death to occur. Additionally, c-jun phosphorylation levels appeared to be dependent on Cdk4 activation. Although the Rb/E2F pathway was shown to mediate the Cdk4-induced death, it was possible that other signals might also be involved in the death process. Accordingly, a Gene Array screening was then performed looking for leads into potential Cdk targets with roles in the DNA damage-induced neuronal death pathways. Data revealed that Cited2, a CBP/p300 interacting transactivator with ED rich termini, was significantly up-regulated 8 hours after insult and that the upregulation appeared to be blocked by flavopiridol (Cdk inhibitor) treatment but unaffected by p53 deficiency.

A project was started driven by the hypothesis that Cited2 was a pro-death signal in cortical neurons exposed to DNA damage, induced by Cdk4 activation. Several objectives were established: (1) To confirm Cited2 up-regulation after DNA damage and to test the relevance of the signal to DNA damage-induced neuronal death; (2) To determine where in the known DNA damage-death pathway network Cited2 was functioning, i.e. in relation to Cdk and caspase activity; (3) To specifically test the effects of the downregulation of endogenous Cdk4 activity on the Cited2 DNA damage-induced upregulation and (4) To identify the possible(s) Cited2 target(s) mediating its neuronal pro-apoptotic effects. The results associated to this project are included in chapter two of this thesis.

Post-mitotic Cdks in CNS development. The PFTAIRE project.

The identification of post-mitotic Cdks challenged the original belief that Cdks were molecules exclusively related to cell cycle regulatory processes in dividing cells. Accordingly, the characterization of the neuronal functions of these molecules is clearly necessary for our full understanding of the development and functioning of the brain. Cdk5, the first identified neuronal Cdk, is involved in a variety of cellular processes key to CNS development and maintenance. PFTAIRE is the one post-mitotic Cdk closest to Cdk5 in sequence, with a common evolutionary molecular ancestor. Additionally, PFTAIRE deficiency in *Drosophila melanogaster* had proved to be lethal at embryonic-larval stages. These observations suggested that PFTAIRE may potentially be as relevant to CNS development as Cdk5. Nevertheless, nothing was known about its regulation and biological functions.

A project was designed triggered by the hypothesis that PFTAIRE was required for CNS developmental processes. Based on the availability of two *Drosophila* lines deficient for Eip63E, the fly PFTAIRE, a first objective was established: to characterize the effects of PFTAIRE deficiency in CNS development using the *Drosophila* VNC model. The main aim of this objective was to understand when (during development) and where (which CNS cells) Eip63E functions during CNS development. The approach was to reveal and score any abnormal CNS phenotype of KO embryos at different stages of embryonic development using immunostaining techniques for markers specific to neuroblasts, neurons, glial and axons. A second, but closely related objective was to determine if Eip63E function was autonomous to neurons and/or glial. For this purpose, controlled ectopic expression of RNAi(Eip63E) in either neurons or glial would be driven using the Gal4:UAS system. The results obtained for this project are presented in chapter three of this thesis.

CITED2 signals through PPARgamma to regulate death of cortical neurons after DNA damage.

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Statement of author contribution

This manuscript defines the role of Cited2 as a signal induced by DNA damage in neurons. It is reported here for the first time that Cdk4 regulates Cited2 induction as part of a pro-apoptotic pathway. Subsequently, Cited2 interacts with and activates PPAR γ leading to cytochrome c release and the activation of the mitochondrial pathway of death.

The experiments presented were predominantly performed by Y. Rodriguez Gonzalez, with the assistance of D. Behzadpoor for the caspase activity assays and immuno-precipitation techniques. Experiments involving calcium-phosphate mediated Cited2 transfection, lipofectamine-mediated Cited2/PPRE-luciferase transfection and estimation of cytochrome c release were done by MSc Y. Zhang. Dr S. Cregan performed the assessment of Cited2 levels by qRT-PCR on p53 deficient background. Cited2 deficient mice were generated by Dr. S. Bamforth, as previously reported. Original Gene Array data was provided by Dr. R.S Slack. All figures and text for the manuscript were prepared by Y. Rodriguez Gonzalez with the direction and assistance of Dr. David S. Park.

CITED2 Signals through Peroxisome Proliferator-Activated Receptor- γ to Regulate Death of Cortical Neurons after DNA Damage

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Abstract

DNA damage is an important initiator of neuronal apoptosis and activates signalling events not yet fully defined. Using the camptothecin-induced DNA damage model in neurons, we previously showed that cyclin D1-associated cell cycle cyclin-dependent kinases (Cdks) (Cdk4/6) and p53 activation are two major events leading to activation of the mitochondrial apoptotic pathway. With gene array analyses, we detected upregulation of Cited2, a CBP (cAMP response element-binding protein-binding protein)/p300 interacting transactivator, in response to DNA damage. This upregulation was confirmed by reverse transcription-PCR and Western blot. CITED2 was functionally important because CITED2 overexpression promotes death, whereas CITED2 deficiency protects. Cited2 upregulation is upstream of the mitochondrial death pathway (BAX, Apaf1, or cytochrome c release) and appears to be independent of p53. However, inhibition of the Cdk4 blocked Cited2 induction. The Cited2 prodeath mechanism does not involve Bmi-1 or p53. Instead, Cited2 activates peroxisome proliferator-activated receptor- γ (PPAR γ), an activity that we demonstrate is critical for DNA damage-induced death. These results define a novel neuronal prodeath pathway in which Cdk4-mediated regulation of Cited2 activates PPAR γ and consequently caspase.

Introduction

DNA damage in neurons is acknowledged as an important component of neuronal disease and injury. The pathways that regulate neuronal DNA damage-induced death converge on the classical intrinsic apoptotic pathway. For example, we showed that death induced by the DNA-damaging agent camptothecin, a topoisomerase I inhibitor, is mediated by BAX translocation to mitochondria, cytochrome c (cyt c) release, and caspase-3 activation (Stefanis et al., 1999; Keramaris et al., 2000).

Several major proapoptotic signals have been shown to precede this conserved mitochondrial pathway of death after DNA damage. For example, p53 regulates death, at least in part, by modulating prodeath Bcl2 members such as Puma (Keramaris et al., 2003; Sedarous et al., 2003; Aleyasin et al., 2004; Cregan et al., 2004).

Another signal, regulated independent of p53, is the activation of abnormal cell cycle signals. Accumulating evidence indicates that inappropriate activation of cell cycle cyclin-dependent kinases (Cdks) lead to neuronal death. For example, we previously showed that cyclin D1/Cdk4 is activated and required for death after β -amyloid toxicity, delayed ischemic insult, and DNA damage (Park et al., 1998; Giovanni et al., 2000; Ghahremani et al., 2002; Rashidian et al., 2005). The manner by which this occurs is not completely clear. In models of DNA damage, we showed that cyclin D1/Cdk4 activation is regulated by a Chk1/cdc25a pathway (Zhang et al., 2006). Cdk4 then phosphorylates the tumor suppressor retinoblastoma (Rb) protein leading to E2F transactivation (Park et al., 1998; Park et al., 2000b). Although these findings provide a skeletal structure to death regulation induced by cell cycle signalling and DNA damage, additional players are likely involved.

To this end, we undertook a gene array study to examine for differentially expressed genes from embryonic cortical neurons exposed to camptothecin (campto) (our unpublished data). One of the genes significantly upregulated was Cited2, a CBP (cAMP response element-binding protein-binding protein)/p300 interacting

transactivator. Cited2 was first identified as a transcriptional coactivator expressed in the nucleus (Bhattacharya et al., 1999). Cited2 has a critical role in development and its deficiency is embryonic lethal. Knock-out (KO) embryos exhibit cardiac malformations, adrenal agenesis, neural crest defects, and exencephaly (Bamforth et al., 2001). Cited2 is also a target/negative regulator of hypoxia-inducible factor (HIF) (Bhattacharya et al., 1999; Freedman et al., 2003). Additionally, Cited2 promotes proliferation at least in part via activation of expression of the polycomb-group genes Bmi-1 (Kranc et al., 2003), negative regulator of the ink4a-ARF locus. Finally, Cited2 can act as a ligand independent activator of peroxisome proliferator-activated receptors (PPARs) α and γ (Tien et al., 2004). PPARs are nuclear receptors with transcriptional activity that have been implicated in a wide range of biological processes including cell growth, lipid homeostasis, angiogenesis, and neuronal apoptosis.

The main goal of this research project, then, was to elucidate the role and mechanism of action of Cited2 in neuronal apoptosis induced by DNA damage. We show that Cited2 is part of the cell cycle pathway and acts to promote death through activation of PPAR γ . This defines a novel pathway by which abnormal cell cycle activation regulates neuronal death.

Materials and Methods

Primary cultures and drug treatments. Cortical neurons were dissected from embryonic day 14 (E14) to E15 mouse embryos, plated at $1.25\text{--}1.8 \times 10^6$ cells/ml, and cultured in supplemented Neurobasal media (Invitrogen) at 37°C and $5\% \text{CO}_2$. After 2–4 d *in vitro* (DIV), cells were treated with 1 or $10 \mu\text{M}$ camptothecin and/or CDK inhibitor [$1 \mu\text{M}$ flavopiridol (Stefanis et al., 1999)], caspase inhibitor [$50 \mu\text{M}$ Boc- Asp(OMe)-fluoromethyl ketone (BAF) (Keramaris et al., 2000)], mixed lineage kinase (MLK) inhibitor [200 nM CEP11004 (Ghahremani et al., 2002)], PPAR γ inhibitor [$10\text{--}30 \mu\text{M}$ 2-chloro-5-nitrobenzanilide (GW9662) (Leesnitzer et al., 2002)], or PPAR γ agonist [$20\text{--}50 \mu\text{M}$ Ciglitazone (Willson et al., 1996)].

Transgenic mice. All transgenic mice used were in a C57BL/6J background. Cortical neurons from each embryo derived from heterozygous crosses were dissected and plated individually. Embryos were genotyped by PCR using specific primers for wild-type (WT) and KO alleles. Embryos from Cited2 crosses were genotyped using the following primers and program: 247 bp WT product: upper, 5'-aaaggcgctaaggatagacac-3'; lower, 5'-atactgaggtccctggcac-3'; 372 bp KO product: upper, 5'-ctaccggtagaattgacctg- 3'; lower, 5'-tgctgtaagaccttcttggc-3'; PCR program: 94°C for 10 min; 15 cycles of 95°C for 30 s, $67.5\text{--}0.5^\circ\text{C}/\text{cycle}$, 72°C for 45 s; 15 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s. This was followed by 72°C for 10 min. Embryos from p53, BAX, and Apaf-1 transgenic mice crosses were genotyped as previously described (Cregan et al., 1999; Fortin et al., 2001; Aleyasin et al., 2004).

Semiquantitative reverse transcription-PCR. Total RNA was extracted/ purified using TriPure isolation reagent (Trizol) per the manufacturer's protocols (Invitrogen). Fifty nanograms of total RNA were used for the cDNA synthesis and gene amplification reactions using SuperScript One-Step with Platinum Taq from Invitrogen. cDNA synthesis was performed at 48°C for 45 min, followed by a 2 min initial denaturation step at 94°C . This was followed by 30 cycles (Noxa, HIF1- α , cyt c), 26 cycles (PUMA), or

25 cycles (Cited2, PPAR γ , Bmi-1, S12) at 94°C for 30 s, melting temperature (T_m) (°C) for 30 s, and 72°C for 1 min. Targeting primers were as follows: for Cited2, 5'-tagggcagcggaggaaaagaa-3' and 5'-ccctcgccgtagtgtatgtgctc-3' (T_m, 60°C); for Bmi-1, 5'-agagcagattggatcgga-3' and 5'-gactgggcaaacaggaagag-3' (T_m, 58°C); for PPAR γ , 5'-aggcgagggcgatcttgacag-3' and 5'-aggcttccgcaggtttttgag-3' (T_m, 57°C); for PUMA, 5'-cctcagccctccctgtcaccag-3' and 5'-cggcgcctcgactgcgcggtg-3' (T_m, 55°C); for HIF1- α , 5'-tgctcatcagttgccacttc-3' and 5'-cttcacgttctgacttga-3' (T_m, 59°C); for cytochrome c, 5'-cctcagccctccctgtcaccag-3' and 5'-gtctgccctttctccttct-3' (T_m, 59°C). Products were resolved on a 2% agarose (Invitrogen)-ethidium bromide gel, and correct band for Cited2, Bmi-1, PPAR γ , HIF1- α , and cyt c were processed by densitometry. Transcript levels were normalized against S12 signals and results were reported as times fold increase in reference to control values (untreated control for each individual experiment) or as relative mRNA level (normalized value). Data are presented as mean \pm SEM of at least three independent experiments.

Quantitative reverse transcription-PCR. Twenty nanograms of RNA were used in one-step SYBR green reverse transcription (RT)-PCR as per manufacturer's instructions (QuantiTect; QIAGEN). RT-PCR was performed on a Chromo4 system (MJ Research/Bio-Rad) and changes in Cited2 mRNA were determined by the $\Delta(\Delta C_t)$ method using S12 transcript for normalization. PCRs exhibited high amplification efficiency (>90%) and the specificity of PCR products was confirmed by sequencing. Data are reported as fold increase in mRNA levels in campto-treated samples relative to corresponding untreated control cells and expressed as mean \pm SEM (n = 4 for each genotype).

SDS-PAGE and Western analysis. Cells were harvested after wash with cold PBS by scraping in 62.5mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM dithiothrietol, and 0.1% w/v bromophenol blue, heated at 95–100°C for 5 min, and then resolved on 7.5, 10, or 20% SDS-PAGE. Transference was done to polyvinylidene difluoride membranes

(PolyScreen; PerkinElmer Life and Analytical Sciences) that were then incubated in 1% casein-Tris-buffered saline (TBS) for blocking. Primary antibodies were diluted in 5% bovine serum albumin (BSA)/TBS as follows: anti- CITED2, 1:500 (Novus Biologicals; JA22); anti-PPAR γ , 1:200 (Santa Cruz; H-100:sc-7196); anti- β -actin, 1:3000 (Sigma-Aldrich); anticytochrome c, 1:2000 (BD Biosciences Pharmingen); anti-OxPhos-complex I subunit, 1:1000 (Invitrogen); anti-PPAR α , 1:150 (Santa Cruz); and anti-HIF1- α , 1:500 (Cayman Chemical). Secondary antibodies [goat anti-mouse or anti-rabbit IgG(H_L) HRP conjugate (Bio-Rad)] were all used at 1:3000 in 3% BSA in 136 mM NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4, and 0.1% Tween 20 (PBS/T). Immunoreactivity was detected using Western blot chemiluminescence reagent system (PerkinElmer Life and Analytical Sciences). Results were processed by densitometry. Protein levels were normalized against β -actin signals, and results were reported as times fold increase in reference to control values (untreated control for each individual experiments) or as relative protein level (normalized value). Data are presented as mean \pm SEM of at least three independent experiments.

Transient transfection by calcium phosphate. Seventy-two to 96 h after plating, cortical neurons were transiently transfected using calcium phosphate coprecipitation protocol as described previously (Xia et al., 1996; Zhang et al., 2006). Cortical neurons were cotransfected with cytomegalovirus (CMV) promoter-driven green fluorescent protein (GFP)-expressing plasmid (3 μ g) and an empty vector (pcDNA3) or Cited2-expressing vector (6 μ g), generously donated by Dr. Shoumo Bhattacharya (The Wellcome Trust Centre for Human Genetics, Oxford, UK). Twenty-four hours after transfection, DNA damage was induced by adding 10 μ M campto and cells were incubated for 21 h. Cells were then fixed in 4% paraformaldehyde, 125 mM sodium dihydrogen phosphate, 125mM disodium hydrogen phosphate, and 14% picric acid for 30 min at room temperature and washed with PBS. Transfected cells were identified by green fluorescence and integrity of the nucleus was analyzed by 0.25 g/ml Hoechst

33342 staining (10 min at room temperature; Sigma- Aldrich). At least 250 GFP-positive cells per treatment were counted. Percentage survival was calculated as GFP-expressing live/GFP expressing total number of neurons. Results are presented as mean \pm SEM of three replicas per treatment.

Transient transfection with Lipofectamine. Neuronal cultures were transfected using Lipofectamine 2000 (Invitrogen) at a ratio of 1.3 μ g of DNA to 1 μ l of Lipofectamine/well. Briefly, neurons were cotransfected with 0.3 μ g of reporter vector (CMV promoter-driven GFP-expressing plasmid) and 1.0 μ g of empty (pcDNA3), Cited2-, or DNPPAR γ -expressing vector [generously donated by Dr. Shoumo Bhattacharya and Dr. V. Krishna K. Chatterjee (Institute of Metabolic Science, Cambridge, UK), respectively]. Twenty-four hours after transfection, cells were treated with 10 μ M campto, 20 μ M GW9662, 40 μ M ciglitazone, alone or in specified combinations, and incubated for 21 h. Cells were then fixed and processed accordingly.

Viral infection. Adenoassociated virus (AAV) expressing a kinase-dead [dominant-negative (DN)] form of Cdk4 was obtained as previously described (Rashidian et al., 2005). Cortical neurons were exposed to 150 multiplicity of infection (MOI) units of AAV at the time of plating. Six to 7 DIV after infection, cells were exposed to campto as described above, and total mRNA or protein was extracted. Samples were used to detect Cited2 levels by RT-PCR and Western blot as previously stated.

Cell survival assessment. Cells were lysed in 150 μ l of cell lysis buffer (0.1X PBS, pH 7.4, 0.5% Triton X-100, 2 mM MgCl₂, 0.5% cetylmethylammonium bromide). Nuclei were evaluated under phase microscopy and healthy ones were counted (at least 150 nuclei for nontreated control wells). Percentage survival was calculated as the remaining number of live nuclei in campto-treated (12, 16, 20 h) compared with untreated control (0 h; 100%). Data are presented as mean \pm SEM of at least three independent experiments.

PPAR γ reporter experiments. TransLucent PPAR γ reporter vectors from Panomics (LR0066) were used. Embryonic cortical neurons from CD1 WT mice or transgenic Cited2 littermates were used. Three days in vitro after plating, cells were cotransfected with CMV- β -galactosidase and PPAR γ responsive element (PPRE)-luciferase-expressing plasmids as described above for Lipofectamine transfection. Twenty-four hours after, cells were then exposed to 10 μ M campto and or 1 μ M flavopiridol. At 0–4 h after treatment, cells were collected and lysed with 130 μ l of cell culture lysis reagent (Promega). For coexpression of Cited2 and PPRE-luciferase, transfection was made using 0.3 μ g of CMV- β -galactosidase and 1 μ g of each Cited2- and PPRE-luciferase-expressing plasmids for a total of 2.3 μ g of DNA/1 μ l of Lipofectamine. Samples were assayed in triplicates for luciferase and β -galactosidase activity as previously described (Fortin et al., 2001). Luciferase activity signals were standardized using β -galactosidase activity values and results were presented either as relative PPAR γ activity (luciferase/ β -galactosidase) or as fold increase in reference to control values (untreated control for each individual experiment, 0 h). Data are presented as mean \pm SEM of three independent experiments.

Caspase activity. After 2 DIV, cells were treated with 10 μ M campto and/or 20 μ M GW9662, as described above. Total protein was extracted in 1 mM KCl, 10 mM HEPES, 1.5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 10% glycerol, protease inhibitor mixture (Roche) as described previously (O'Hare et al., 2000). Each sample was analyzed in three replicas of 5 μ g each. Protein was incubated with Z-DEVD-AFC (Invitrogen) in caspase assay buffer [25 mM HEPES, 10 mM DTT, 10% sucrose, 0.1% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 15 μ M Z-DEVD-AFC] and fluorescence at 505 nm was measured at different time points (30 min to 2 h), using an excitation wavelength of 400 nm. Readings were made using a PerkinElmer Life and Analytical Sciences LS 50B Luminescence spectrometer with a slit width of 5 and 1 s integration time. Fluorescence versus time was plotted for each replica and the slope of

the curve (linear regression) was used as cleavage activity value (caspase activity). Data are presented as mean \pm SEM of four independent experiments.

Cytochrome c immunocytochemistry. After 2 DIV, cortical neurons from CD1 WT embryos or littermate embryos derived from heterozygous *Cited2* mice crosses were treated with 10 μ M campto or/and 20 μ M GW9662 for 12 h, fixed as described above, and stained with cyt c antibody (BD Biosciences Pharmingen), followed by Hoechst 33258 staining. At least 100 cells per replica were assessed by immunofluorescence and neurons positive for punctate cyt c were counted. Data are presented as percentage mitochondrial cyt c (percentage of neurons positive for punctate cyt c). The bars represent the mean \pm SEM from three independent experiments or embryos of each specified genotype.

Immunoprecipitation. Cortical neurons ($\sim 20 \times 10^6$ cells) were harvested in lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.2% NP-40) supplemented with protease inhibitors. Immunoprecipitations (IPs) were performed through incubation of anti-*Cited2* antibody (JA22) with lysates overnight followed by incubation with anti-mouse Ig IP beads (eBiosciences) at 4°C for 2 h. The beads were washed three times by lysis buffer without protease inhibitors.

Results

Cited2 signals death of cortical neurons after DNA damage

Our initial gene array screen suggested that Cited2 might be upregulated after DNA damage treatment. To test this, we first determined whether Cited2 message was upregulated after campto treatment by RT-PCR. As shown in Figure 2.1A, induction of Cited2 message was detectable as early as 2 h after initiation of camptothecin treatment and reached a maximum (~4.5-fold) induction at 4 h. We next determined whether an increase in message was also associated with increased protein levels. Consistent with an increase in message, CITED2 protein was also induced with an increase detectable as early as 2 h (p value, 0.036). Protein levels were still induced 8 h after DNA damage treatment (Fig. 2.1B).

The early induction of Cited2 suggested it might be functionally relevant for death. This is particularly important because, in this model, we have previously shown that the commitment point for death occurs 6–8 h after treatment (Morris et al., 2001). To test the functional importance of Cited2, we determined the effect of Cited2 overexpression on neuronal survival either with or without DNA damage insult. We first delivered Cited2 by calcium phosphate transfection. Importantly, in this initial experiment, there was more death in neurons expressing Cited2 when compared with GFP control either basally (42.4 vs. 27.5%) or with campto treatment (64 vs. 48.1%) (Fig. 2.2A). To confirm this, we subsequently performed three independent experiments using Lipofectamine carrier. This was done to insure that any results were not necessarily attributable to the delivery method. We obtained similar results (Cited2 vs. GFP, basally 37.2 vs. 23.6%, or with campto treatment, 74.7 vs. 55.8%) (Fig. 2.6C). This indicates that Cited2 is sufficient to induce death. To address whether Cited2 was necessary for neuronal death, we cultured cortical neurons from Cited2-deficient or heterozygous and WT littermate controls. As shown in figure 2.2B, the absence of Cited2 in neurons confers significant resistance to the genotoxic insult. Deficiency by

itself delays, but does not prevent, death. Together, these results indicate that upregulated Cited2 contributes to death of neurons after DNA damage.

Figure 2.1. Cited2 mRNA and protein levels are upregulated after DNA damage.

Total mRNA or protein was extracted from cortical neurons treated for different times with 10 μ M campto and subjected to either RT-PCR **(A)** or Western blot **(B)**. Densitometry of all signals was performed and Cited2 signal was normalized to S12 (A) and actin (B), respectively. Data are presented as fold increase relative to nontreated sample for each separate experiment. Each bar represents the mean \pm SEM from at least three independent experiments.

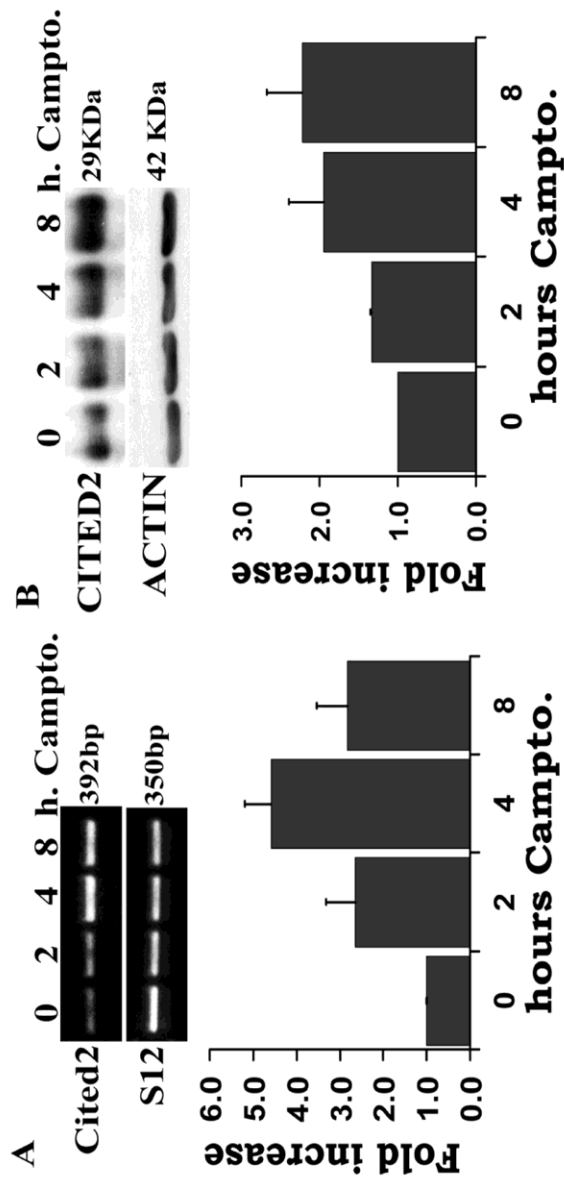


Figure 2.1. Cited2 mRNA and protein levels are upregulated after DNA damage.

Figure 2.2. Cited2 overexpression promotes neuronal death, whereas Cited2 deficiency promotes survival after DNA damage.

(A), Seventy-two to 96 h after plating, cortical neurons were transiently cotransfected with empty pcDNA3 or pcDNA3-Cited2 and pEGFP using calcium phosphate. Twenty-four hours after, 10 μ M campto was added and incubated for 21 h followed by fixation and Hoechst 33258 staining. **Left**, Photomicrographs of alive or dead cortical neurons under green fluorescence, showing GFP-transfected neurons and Hoechst-stained nuclei. At least 250 GFP-positive nuclei per treatment were assessed. Scale bar, 50 μ m. **Right**, Quantitation of death induced by Cited2 expression. Percentage survival was calculated as percentage of live over total GFP-expressing neurons. Data represent the mean \pm SEM from three replicas/treatment. ***p<0.001. **(B)**, Cortical neurons from littermate embryos derived from multiple heterozygous Cited2 mice crosses were cultured independently and treated with 10 μ M campto for 12, 16, or 24 h as indicated. Cells were lysed and the number of surviving neurons was evaluated. Percentage survival was calculated as the number of live neurons in campto-treated compared with untreated control for each embryo. Each bar represents the mean \pm SEM from n embryos as indicated. **p<0.01; ***p<0.001; N.S., no significant difference.

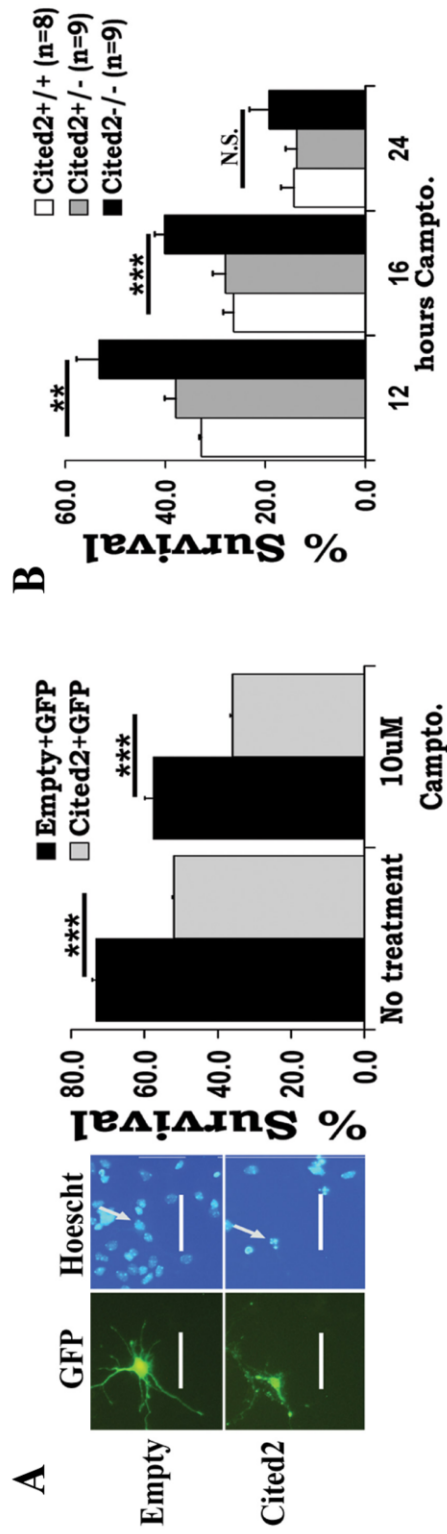


Figure 2.2. Cited2 overexpression promotes neuronal death, whereas Cited2 deficiency promotes survival after DNA damage.

DNA damage-Cited2 induction is upstream of the mitochondrial death pathway but independent of p53

We next wanted to determine the factors that regulated Cited2 induction. The early induction of Cited2 suggested that its regulatory pathways would also act early. Indeed, we previously showed caspase activation occurs later at ~6–8 h after initiation of campto treatment and coincides with death commitment (Keramaris et al., 2000; Morris et al., 2001). This suggested that the conserved mitochondrial pathway acts downstream of Cited2. Bax and Apaf-1 are both critical for neuronal death and caspase activation induced by DNA damage (Xiang et al., 1998; Fortin et al., 2001). Importantly, deficiency of either gene did not alter the induction of Cited2 consistent with the upstream nature of the Cited2 signal (Fig. 2.3A). Similarly, caspase inhibitor (BAF) treatment also did not inhibit Cited2 induction (Fig. 2.3B). To confirm whether Cited2 was upstream of the mitochondrial pathway of death, we examined the levels of mitochondrial cytochrome c release after campto treatment in Cited2-deficient neurons compared with WT littermates. Cited2 deficiency significantly inhibits mitochondrial cytochrome c release (Fig. 2.3C) after DNA damage. To ensure that the observed effects of Cited2 deficiency were not attributable to basal differences in mitochondrial content between KO Cited2 and WT neurons, we examined for levels of mitochondrial markers, cytochrome c and OxPhos mitochondrial complex I subunit. As shown in figure 2.3D, top, no significant differences were found in cytochrome c message as measured by RT-PCR between the two genotypes. In addition, Western blot analyses also showed no differences in cytochrome c and OxPhos mitochondrial complex I subunit, suggesting that Cited2-deficient neurons have mitochondria levels similar to WT cells (Fig. 2.3D, bottom). Together, these results indicate that Cited2 induction is upstream of the conserved mitochondrial death pathway.

The c-Jun N-terminal kinase (JNK) pathway has also been implicated as important for DNA damage-induced death. We showed previously that the potent MLK inhibitor CEP11004 blocked the JNK/c-Jun pathway and protected neurons from death

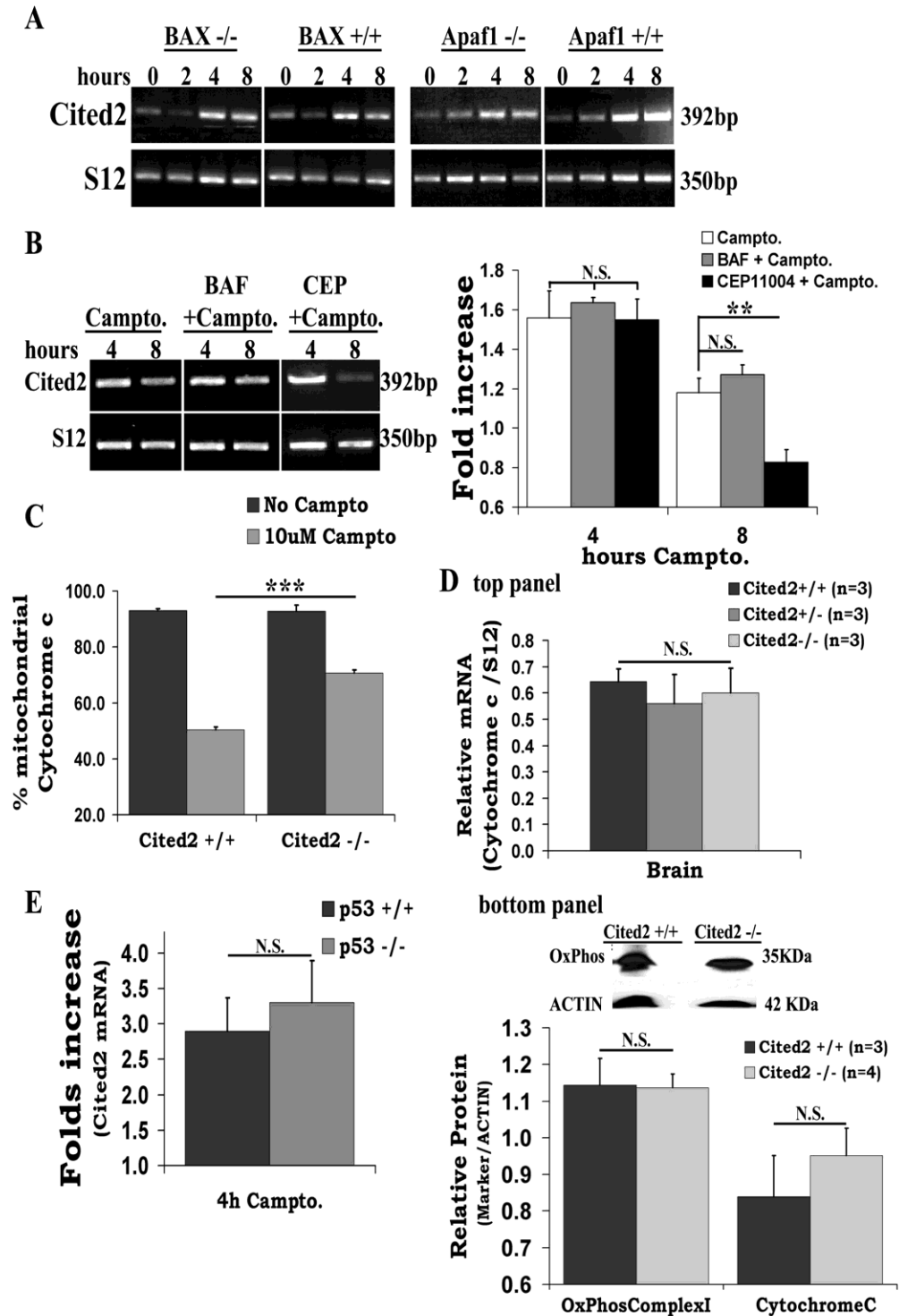
(Ghahremani et al., 2002). However, JNK inhibition also failed to significantly affect Cited2 induction at early time points (Fig. 2.3B). In contrast, at later time points (8 h), there did appear to be some effect with JNK inhibition. Therefore, although there was some modulatory effect of the JNK pathway on Cited2 late induction, it did not appear to be the major pathway regulating Cited2.

We mentioned above the relevance of the p53 pathway as a required proapoptotic signal after DNA damage in neurons. p53 activation can be detected as early as 2 h (Morris et al., 2001), making it a possible candidate as a Cited2 regulator. However, deficiency of p53 gene did not alter the induction of Cited2 suggesting that the Cited2 signal was independent of p53 (Fig. 2.3E).

Figure 2.3. Cited2 early upregulation is upstream of the mitochondrial apoptotic pathway and upstream or independent of the JNKs and p53 pathways.

(A), BAX or Apaf-1 deficiency does not alter Cited2 upregulation. Cortical neurons derived from heterozygous breeding of BAX or Apaf-1 transgenic mice were cultured and treated with 10 μ M campto for 2, 4, and 8 h as indicated. Total RNA was extracted and subjected to RT-PCR for Cited2 transcript. Results from representative embryos are presented. **(B)**, Caspases or JNK inhibition does not affect early Cited2 upregulation. Total mRNA was extracted from cortical neurons treated for the indicated times with campto and/or caspases inhibitor (50 μ M BAF) or MLK inhibitor (200 nM CEP11004). **Left**, Results of one representative experiment. **Right**, Normalized densitometry data from at least three independent experiments. Each bar represents the mean \pm SEM. **p<0.01; N.S., no significant difference. **(C)** Cited2 deficiency inhibits mitochondrial cytochrome c release. Cortical neurons from littermate embryos derived from heterozygous Cited2 mice crosses were cultured as independent experiments and campto-treated for 12h, followed by fixation and cytochrome c/Hoechst 33258 staining. Quantitation is presented as percentage of neurons positive for punctate cytochrome c. Data represent the mean \pm SEM from three embryos of each specified genotype. ***p<0.001. **(D)** Markers of mitochondria do not differ between Cited2 KO and WT neurons. Total mRNA from brains (**top**) or protein from cortical neurons (**bottom**) derived from embryos from heterozygous Cited2 mice crosses were analyzed as indicated for mitochondrial markers by RT-PCR or Western blot. mRNA results are normalized to S12 as control and Westerns are normalized to actin. Bars represent the mean \pm SEM from n embryos. N.S. No significant difference. **(E)** p53 deficiency does not alter Cited2 upregulation. WT or p53-deficient littermate cortical neurons were treated with 10 μ M campto for 4 h. Total RNA was extracted and subjected to quantitative RT-PCR for Cited2 transcript. Quantitative data from four embryos of specified genotype are presented as fold increase compared with untreated control for each embryo. The bars represent the mean \pm SEM. N.S.: No significant

difference.



DNA damage-Cited2 induction is regulated by Cdk4 activity

One of the earliest signals that changes after exposure to DNA damaging agent is cyclin D1-associated activity. We previously showed that this pathway is activated as early as 0.5–1 h after DNA damage (Park et al., 1998). To test whether the Cdk pathway might be important, we first examined the effects of treatment with the potent Cdk inhibitor flavopiridol. This general Cdk inhibitor protects neurons from campto-induced death as well as blocks downstream targets of the Cdks such as the Rb protein (Park et al., 1997a). Importantly, flavopiridol treatment blocked the induction of Cited2 mRNA (Fig. 2.4A, C) and protein (our unpublished data).

Because of potential nonspecific effects of flavopiridol, we also determined whether expression of a kinase-dead dominant negative form of Cdk4 (DNCdk4), which we also reported blocks neuronal death (Ghahremani et al., 2002), inhibits induction of Cited2. DNCdk4 was targeted to the culture via viral-mediated delivery. As shown in Figure 2.4, B and C, DNCdk4 expression also significantly blocked Cited2 induction. Remarkably, unlike flavopiridol, DNCdk4 has no effect on basal Cited2 levels (no significant difference between untreated control and infected cells; p value, 0.936). This suggests that Cdk4 specifically mediates the induction of Cited2 under genotoxic conditions. The reason why flavopiridol affects basal levels of Cited2 in addition to its induction is unknown but may be attributable to non-Cdk4-mediated effects (other Cdks, other nonspecific effects). Nevertheless, our results are consistent with the notion that Cdk4 is critical for the upregulation of Cited2 after camptothecin-induced DNA damage.

Figure 2.4. Cited2 upregulation is blocked by Cdk4 inhibition.

(A), Cortical neurons were treated with campto and/or 1 μ M flavopiridol. Total RNA was extracted at indicated times and submitted to RT-PCR. Image from one representative experiment is shown. **(B)**, Downregulation of endogenous Cdk4 activity inhibits Cited2 upregulation. Cortical neurons were infected with 150 MOI of DNCdk4 or GFP control expressing AAV as indicated in Materials and Methods. After 6–7 DIV, neurons were treated with 10 μ M campto for indicated times. Total RNA or protein was extracted and submitted to RT-PCR (**top**) or Western blot (**bottom**). Images show results from one representative experiment. **(C)**, Normalized RT-PCR densitometry data from flavopiridol and DNCdk4 experiments are presented as fold increase relative to nontreated sample. Each bar represents the mean \pm SEM from at least three independent experiments. * p <0.05; N.S., no significant difference.

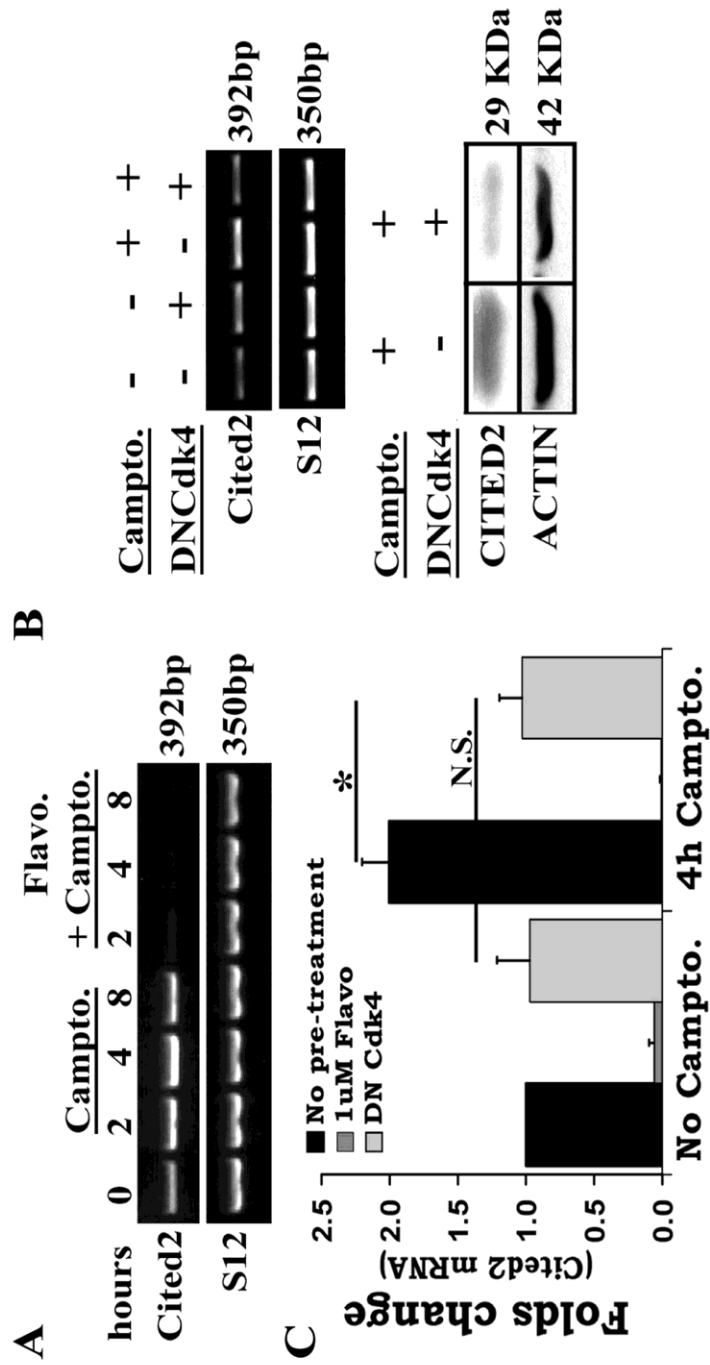


Figure 2.4. Cited2 upregulation is blocked by Cdk4 inhibition.

Cited2-mediated death does not involve Bmi-1, p53, or HIF1- α

We next determine how Cited2 may regulate death after DNA damage. Previous reports suggest that Cited2 stimulates proliferation by induction of Bmi-1 that in turn represses the INK4/ARF locus of cell cycle inhibitors (Kranz et al., 2003). We hypothesized that, in our model, Cited2 would be a signal activated by Cdk4 to ensure its own level of activation and that this would occur through Bmi-1 induction by Cited2. Indeed, our results using RT-PCR show that Bmi-1 message is slightly induced (~1.5-fold) after DNA damage (Fig. 2.5A). This also confirmed results in our original gene array screen (our unpublished data).

To test the hypothesis that Bmi-1 may be a downstream signal of Cited2 to mediate death after DNA damage, Bmi-1 expression levels were analyzed by RT-PCR in presence or absence of endogenous Cited2. However, no differences were observed between Cited2-deficient and WT neuronal cultures (Fig. 2.5A). This indicates that, although Bmi-1 message levels are changed after DNA damage, this is not relevant for a Cited2-induced pathway of death.

It has been reported that p53 is one major player in neuronal DNA damage-induced apoptosis (Morris et al., 2001; Aleyasin et al., 2004; Vaughn and Deshmukh, 2007). Additionally, we previously showed that there appears to be cross talk between Cdk4/6 and p53 apoptotic pathways (Morris et al., 2001). Here, we explored the possibility of Cited2 being part of the cross talk mechanisms. To assess the consequences of Cited2 deficiency on p53 activity, we looked at the profile of induction of two known p53 targets in our model: PUMA and Noxa (Aleyasin et al., 2004; Cregan et al., 2004), in the presence or absence of endogenous Cited2 (Fig. 2.5B). In this case, as well as with Bmi-1, Cited2 deficiency does not appear to inhibit the induction of these two genes when compared with WT littermates. This suggests that Cited2 mechanism of death does not include p53 activation.

Cited2 has also been reported to regulate and be regulated by HIF1- α (Bhattacharya and Ratcliffe, 2003; Freedman et al., 2003). Importantly, Cited2

deficiency has been shown to result in increased message of HIF1- α -responsive genes in the heart (Yin et al., 2002). Interestingly, a recent report suggests that HIF1- α expression protects hippocampal neurons (HT22) against DNA damage (Aminova et al., 2005). Accordingly, we wanted to examine whether the protection conferred by Cited2 deficiency was related to differences in HIF1- α levels. Using RT-PCR and Western blot, we looked at the basal levels of HIF1- α in the brain and cultured cortical neurons of embryos from Cited2 heterozygous crosses. As shown in figure 2.5C, top, we did not find any differences on HIF1- α transcript levels in the brain of KO versus WT embryos. Moreover, cortical neurons treated with DNA damaging agent show decreased HIF1- α transcripts (our unpublished data). Interestingly, HIF1- α protein levels did not appear to differ either between Cited2 KO and WT littermates (Fig. 2.5C, bottom). These data suggest that Cited2 deficiency is not protective simply because it leads to upregulation of basal HIF1- α . This result would be consistent with previous reports that show that HIF1- α haploinsufficiency, contrary to what happens in heart, does not rescue brain defects in Cited2 KO embryos (Xu et al., 2007).

Figure 2.5. Cited2 deficiency does not affect Bmi-1 upregulation, p53 activity, or basal HIF1- α levels.

(A, B), Embryonic cortical neurons derived from heterozygous Cited2 crosses were independently cultured and treated with 10 μ M campto as indicated. Total RNA was extracted and subjected to RT-PCR for Bmi-1, PUMA, Noxa, and S12. **(A), Top**, RT-PCR results from representative embryos. Signals were analyzed by densitometry and Bmi-1 signal was normalized using S12. **Bottom**, Data are presented as fold increase relative to nontreated sample for each embryo. Each bar represents the mean \pm SEM for n embryos. **(B)**, RT-PCR results for Puma and Noxa transcripts from representative embryos. **C**, Quantitation of HIF1- α basal levels. The **top** panel shows mRNA levels in whole brains from littermates embryos from heterozygous Cited2 crosses. Data are presented as normalized values relative to S12 signal for each embryo. The **bottom** panel shows protein results from independently cultured cortical neurons from littermates embryos from heterozygous Cited2 crosses. Data are presented as normalized values relative to actin signal for each embryo. The bars represent the mean \pm SEM from n embryos of specified genotype. N.S., No significant difference.

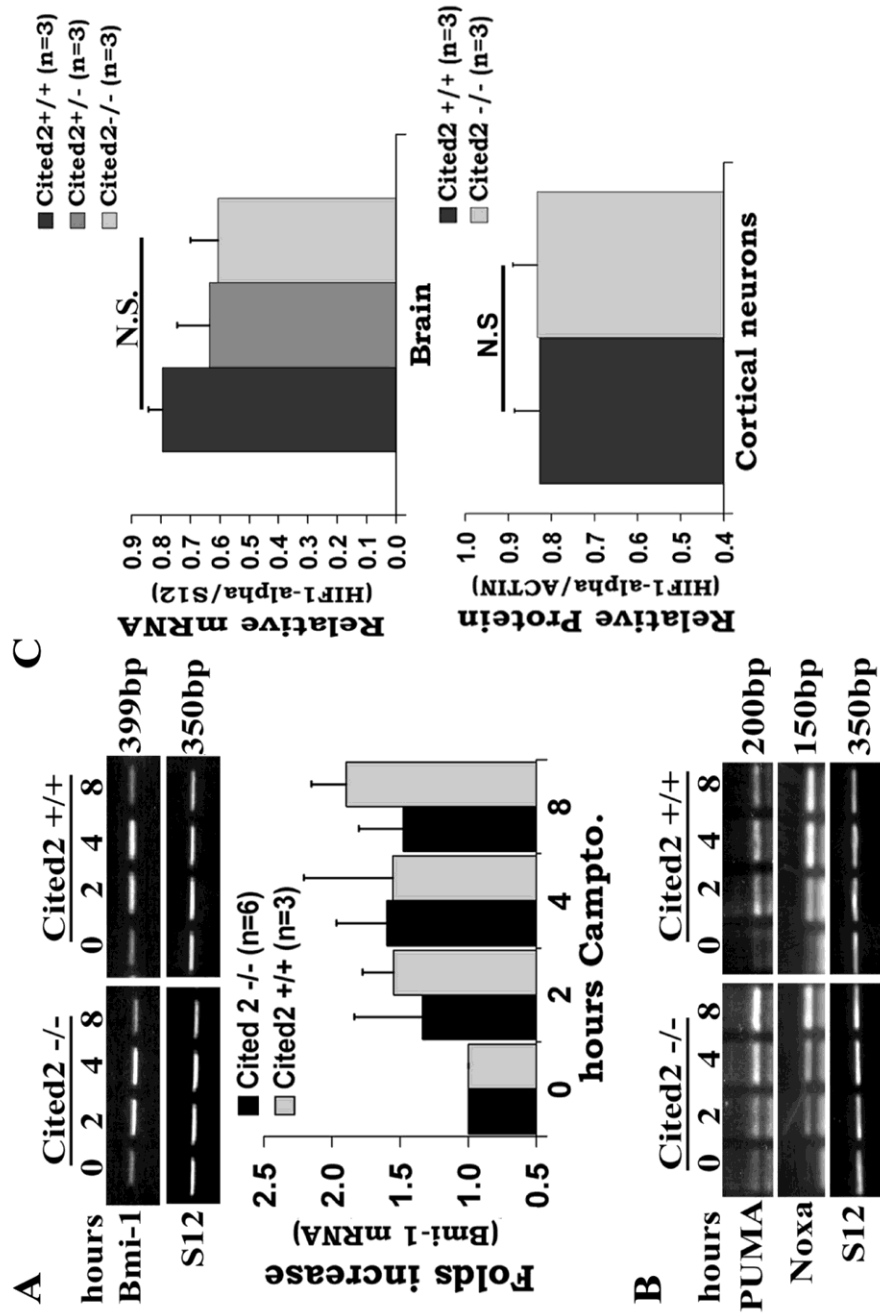


Figure 2.5. Cited2 deficiency does not affect Bmi-1 upregulation, p53 activity, or basal HIF1-alfa levels.

Cited2-mediated death occurs via PPAR γ activation

Interestingly, Cited2 is known to be able to act as a ligand-independent coactivator of PPAR γ (Tien et al., 2004). The role of PPAR γ in neuronal death induced by DNA damage is unclear. Accordingly, we first determined whether PPAR γ might play a role in our system of neuronal death. As shown in figure 2.6A, GW9662, a specific, potent, and irreversible antagonist of PPAR γ (Leesnitzer et al., 2002), significantly delayed death at 10–30 μ M concentrations. In addition, caspase 3 activation normally observed after DNA damage (Stefanis et al., 1999; Morris et al., 2001) was significantly decreased after treatment with the GW9662 inhibitor (Fig. 2.6B).

The above experiments indicate that PPAR γ blockers delay death induced by campto treatment. If our model that Cited2 signals death through PPAR γ were true, we would anticipate that death induced by direct Cited2 expression would also be blocked by GW9662. As shown in figure 2.6C, this antagonist significantly inhibited the death induced by both Cited2 expression alone as well as with campto/Cited2 co-treatment. Finally, consistent with the model that the Cited2-mediated signal acts upstream of the mitochondria, GW9662 treatment also blocked cyt c release (Fig. 2.6D).

Figure 2.6. Effects of PPAR γ inhibition with GW9662.

(A), GW9662 protects cortical neurons from DNA damage. After 4 DIV, cortical neurons were treated with 10 μ M campto alone or together with GW9662 at various concentrations (10 –30 μ M). Cells were lysed and intact nuclei were counted as described in Materials and Methods. The **top** panel quantifies neurons treated with campto and GW9662 when compared with untreated controls. Percentage survival was calculated as the number of remaining live nuclei in treated wells compared with completely untreated cultures. The **middle** panel quantifies neurons treated only with GW9662. Percentage survival was calculated as the number of remaining live nuclei in treated wells compared with completely untreated controls. The **bottom** panel shows values when neurons treated with campto and GW9662 are compared with those from neurons treated only with GW9662. The bars represent the mean \pm SEM from at least three independent experiments. *p<0.05; **p<0.01; ***p<0.001; N.S., no significant difference. **(B)**, GW9662 inhibits caspase activation induced by DNA damage. Cortical neurons were treated with campto alone or together with 20 μ M GW9662 for 16 h. Protein was extracted as described in Materials and Methods, and caspase activity was determined in triplicate as the slope of linear regression (fluorescence vs. time plot) for each replica. Each bar represents the mean \pm SEM from four independent experiments. **p<0.01. **(C)**, Cited2-induced death of cortical neurons is inhibited by GW9662. Seventy-two to 96 h after plating, cortical neurons were transiently cotransfected with empty pcDNA3 or pcDNA3-Cited2 and pEGFP using Lipofectamine. Twenty-four hours after, 10 μ M campto and/or 20 μ M GW9662 was added and incubated for 21 h followed by fixation and Hoechst 33258 staining. At least 100 GFP-positive nuclei per treatment were assessed. Percentage survival was calculated as percentage of live over total GFP-expressing neurons. Data represent the mean \pm SEM from three independent experiments. ***p<0.001. **(D)**, GW9662 blocks cytochrome c release from the mitochondria after DNA damage. Cortical neurons were cultured and treated with

campto and/or 20 μ M GW9662 for 12 h, followed by fixation and cyt c/Hoechst 33258 staining. Quantitation is presented as percentage of neurons positive for punctate cyt c. Data represent the mean \pm SEM from three independent experiments. *** p <0.001.

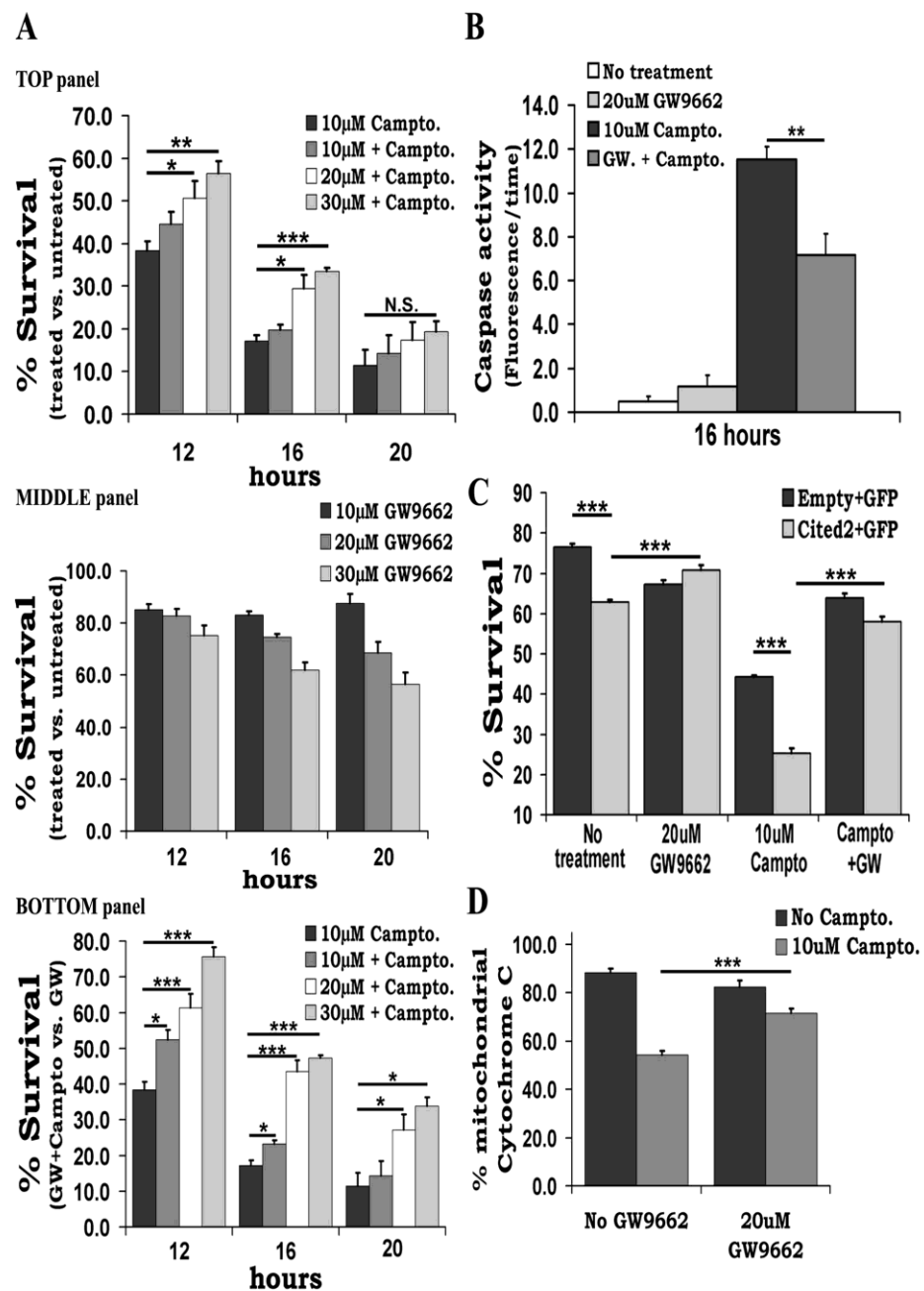


Figure 2.6. Effects of PPARgamma inhibition with GW9662.

We also explored the converse effects of PPAR γ activation by ciglitazone, a PPAR γ agonist (Willson et al., 1996), on death of cortical neurons. As shown in figure 2.7A, ciglitazone induces death of cortical neurons even in the absence of DNA damage, supporting the notion that PPAR γ activation can act as a death effector. Ciglitazone treatment also did not appear to further increase campto-induced death (Fig. 2.7B). Because ciglitazone may have other targets other than PPAR γ , we examined whether the death-promoting effect of Ciglitazone was dependent on PPAR γ . Expression of a double mutant (L468A/E471A) form of PPAR γ has been shown to be a potent dominant-negative inhibitor of wild-type PPAR γ activity (Gurnell et al., 2000). Accordingly, we first show that expression of DNPPAR γ blocks death of cortical neurons exposed to DNA damage (Fig. 2.7C). This supports the protection observed with pharmacological inhibition of PPAR γ . Second, DNPPAR γ expression also blocked death caused by Ciglitazone supporting the specificity of the agonist. Interestingly, we show that death induced by ciglitazone is actually dependent on Cited2 (Fig. 2.7D). This would be consistent with the notion that Cited2 is required coactivator of PPAR γ to induce death.

To further support the importance of PPAR γ , we next determined whether its activity is induced after DNA damage. To do this, we used a reporter system, which consisted of luciferase expressed under the regulation of a PPAR γ -responsive element (PPRE-luciferase). As shown in figure 2.8A, PPAR γ activity is upregulated after DNA damage, with a peak 3 h after initiation of campto exposure (Fig. 2.8D). This induction was inhibited by the Cdk inhibitor flavopiridol, consistent with the model that Cdks are upstream regulators of PPAR γ (Fig. 2.8A). The induction of PPAR γ activity could not be accounted for by upregulation of PPAR γ levels. In fact, PPAR γ mRNA levels appear to decrease after DNA damage (Fig. 2.8B). PPAR γ protein was also not significantly elevated after campto exposure (Fig. 2.8C). We also examined another PPAR family member with the potential to be activated by Cited2, PPAR α . Its protein level was not significantly

altered after DNA damage (our unpublished data).

To test whether PPAR γ activation after DNA damage is Cited2 dependent, we measured PPAR γ reporter activity after DNA damage in cultured neurons derived from Cited2-deficient cultures or WT littermate controls (Fig. 2.8D). We found that Cited2 deficiency significantly blocked the upregulation of PPAR γ activation. Importantly, the basal level of PPAR γ activity was not different between Cited2-deficient and WT neurons (Fig. 2.8E).

If Cited2 was critical for PPAR γ activation, we would predict that exogenous expression of Cited2 in neurons would induce PPAR γ activity, similar to DNA damage. Indeed, we found this to be the case. As shown in figure 2.8F, Cited2 expression alone caused an induction of PPAR γ reporter activity similar to that of campto treatment (Fig. 2.8A).

Finally, if Cited2 acts as a coactivator of PPAR γ (Tien et al., 2004), we would anticipate that they should interact. We examined this possibility in neurons untreated and treated with campto by IP/Western blot analyses. In this experiment, Cited2 was immunoprecipitated from cell extracts untreated or treated with campto followed by Western blot analyses for PPAR γ . As shown in figure 2.8G, we detected very little basal interaction between Cited2 and PPAR γ . However, significant interaction was observed after treatment with campto. The above data suggest that Cited2 is required for DNA damage-induced PPAR γ activation and subsequent cell death.

Together, these results present a model by which Cdk4-mediated induction of Cited2 leads to activation of PPAR γ , caspase activation, and consequent neuronal death.

Figure 2.7. Ciglitazone, a PPAR γ agonist, induces death of cortical neurons in a manner dependent on Cited2 activity.

(A), After 4 DIV, cortical neurons were treated with various concentrations (20–50 μ M) of ciglitazone. Cells were lysed at the specified time points and intact nuclei were counted as described in Materials and Methods. Percentage survival was calculated as the number of remaining live nuclei in treated wells compared with untreated control. The bars represent the mean \pm SEM from at least three independent experiments. *** p <0.001. **(B)**, Ciglitazone does not enhance campto-induced death. Cortical neurons were treated for 16 h with 1 or 10 μ M campto alone or together with ciglitazone (20–50 μ M). Cells were lysed and processed as described above. The bars represent the mean \pm SEM from at least three independent experiments. N.S., No significant difference. **(C)**, Expression of dominant-negative PPAR γ protects neurons from ciglitazone-induced and campto-induced death. Seventy-two to 96 h after plating, cortical neurons were transiently cotransfected with empty pcDNA3 or pcDNA3-DNPPAR γ and pEGFP using Lipofectamine. Twenty-four hours after, 10 μ M campto or 40 μ M ciglitazone was added and incubated for 21 h followed by fixation and Hoechst 33258 staining. At least 100 GFP-positive nuclei per treatment were assessed. Percentage survival was calculated as percentage of live over total GFP-expressing neurons. Data represent the mean \pm SEM from three independent experiments. *** p <0.001. **(D)**, Cited2 deficiency completely inhibits ciglitazone-induced death. Cortical neurons from littermate embryos derived from heterozygous Cited2 mice crosses were cultured as independent experiments and treated with 40 μ M ciglitazone for 16 h. Cells were lysed and number of surviving neurons was evaluated. Percentage survival was calculated as the number of live neurons after treatment compare with untreated control for each embryo. Each bar represents the mean \pm SEM from three embryos. *** p <0.001.

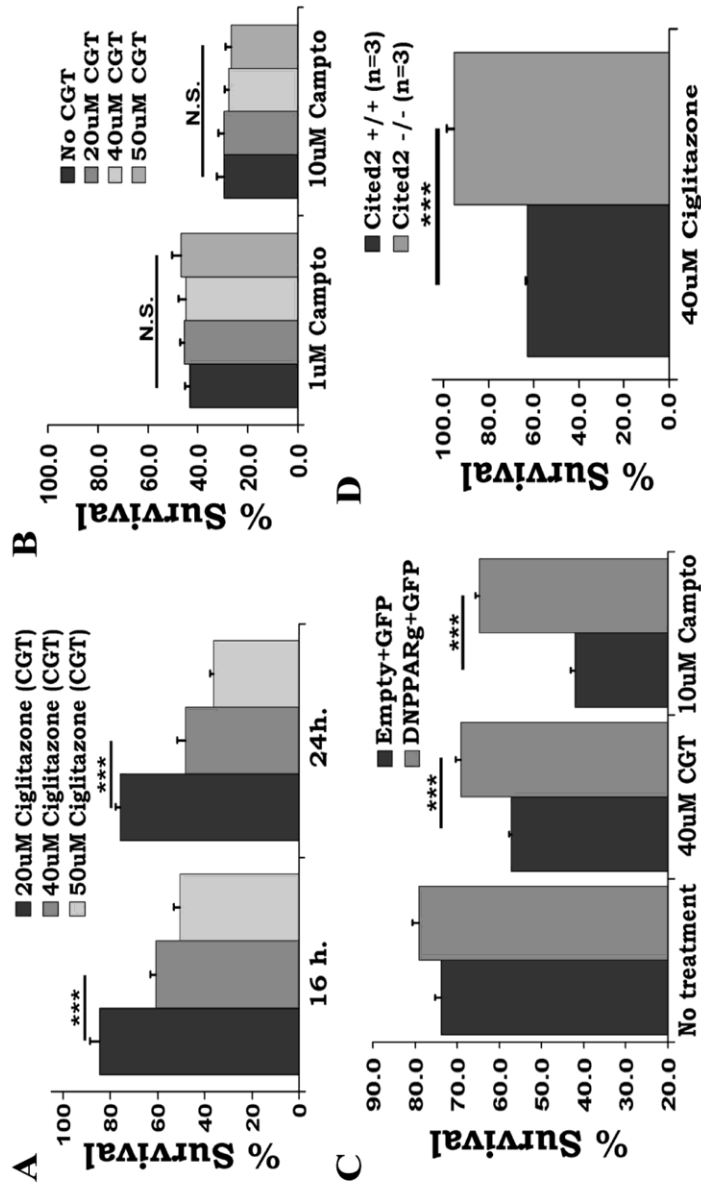


Figure 2.7. Ciglitazone, a PPARgamma agonist, induces death of cortical neurons in a manner dependent on Cited2 activity.

Figure 2.8. PPAR γ activity, but not levels, is upregulated after DNA damage in a Cdk-Cited2-dependent manner.

(A), PPAR γ activity increases after DNA damage and is blocked by the Cdk inhibitor flavopiridol. Seventy-two hours after plating, cortical neurons were transiently cotransfected with PPRE-luciferase- and CMV- β -galactosidase-expressing plasmids using Lipofectamine as described in Materials and Methods. Twenty-four hours after, cells were treated with 10 μ M campto and/or 1 μ M flavopiridol for specified times. Data represent values of luciferase/ β -galactosidase activity. Bars represent the mean \pm SEM from four independent experiments. ***p<0.001. **(B)**, PPAR γ message decreases after DNA damage. Cortical neurons were treated with campto at indicated times. Total RNA was extracted and RT-PCR result from one representative experiment is shown in inset. Normalized densitometry data are presented as fold change relative to nontreated sample. Each bar represents the mean \pm SEM from three independent experiments. **(C)**, Total protein was extracted from campto-treated cortical neurons and analyzed by Western blot as described. Results from one representative experiment are shown. **(D)**, Cited2 deficiency blocks PPAR γ activity upregulation by campto. Seventy-two hours after plating, cortical neurons from littermate embryos from heterozygous Cited2 crosses independently cultured were transfected and treated with 10 μ M campto to assess PPAR γ activity as described above. Values represent fold change of normalized luciferase activity (luciferase/ β -galactosidase) of correspondent sample relative to transfected nontreated sample. Each bar represents the mean \pm SEM from n embryos. *p<0.05. **(E)**, Basal PPAR γ activity does not differ between Cited2 KO and WT neurons. PPAR γ activity was measured as described in D and presented as normalized luciferase activity (luciferase/ β -galactosidase). The bars represent the mean \pm SEM from n independently cultured embryos. N.S., No significant difference. **(F)**, Cited2 overexpression induces PPAR γ activity. Seventy-two to 96 h after plating, cortical neurons were transiently cotransfected with empty pcDNA3 or pcDNA3-Cited2-, PPRE-

luciferase-, and CMV- β -galactosidase-expressing plasmids using Lipofectamine. Twenty-four hours after, luciferase activity was evaluated as described in Materials and Methods. Data are presented as normalized luciferase activity (luciferase/ β -galactosidase). Each bar represents the mean \pm SEM from three independent experiments. *** $p < 0.001$. **(G)**, Cited2 and PPAR γ interact after DNA damage. Cortical neurons were treated with campto at indicated times and total protein was extracted as indicated in Materials and Methods. Control IgG or anti-Cited2 antibodies were incubated with total cell lysates ($\sim 2 \times 10^7$ neurons). Antibodies were isolated by IP beads and resolved by SDS-PAGE followed by anti-PPAR γ Western blot. Results from two independent experiments are shown. Inp., Input, TCA precipitate from total cell lysate ($\sim 10^7$ untreated neurons).

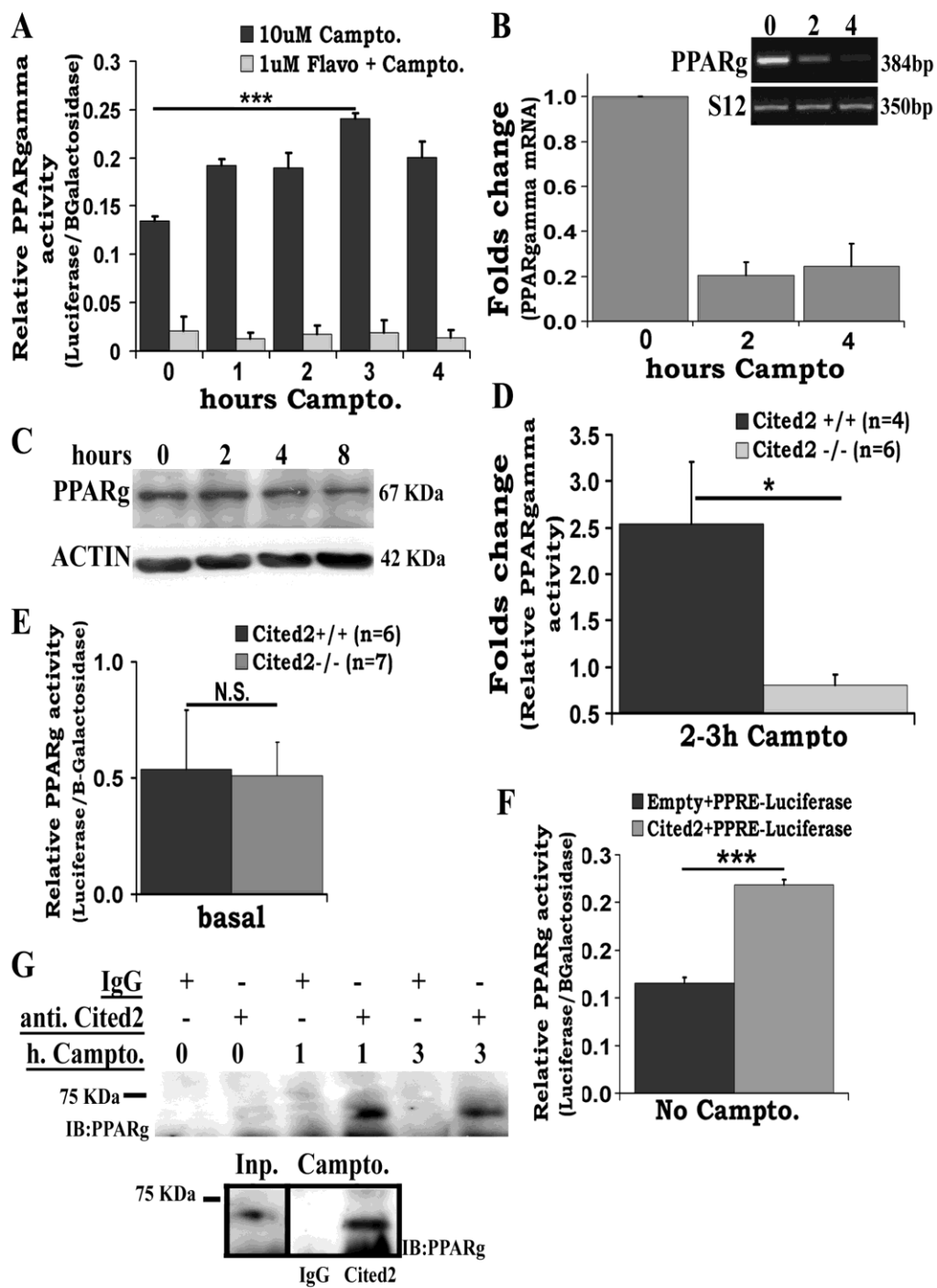


Figure 2.8. PPARg activity, but not levels, is upregulated after DNA damage in a Cdk-Cited2-dependent manner.

Discussion

Integrity and fidelity of DNA is essential for the proper function and survival of neurons. However, our understanding of the complex signals that govern neuronal death after DNA damage is incomplete. We previously showed that cell cycle Cdks as well as p53 regulate the mitochondrial pathway of death (Morris et al., 2001). We presently provide additional critical insight into signals that participate in DNA damage-induced death. Our results indicate that Cited2 is upregulated in a manner dependent on the cell cycle Cdk4 pathway, upstream of the mitochondrial pathway. Importantly, we also provide data that indicate a proapoptotic role for Cited2 and that Cited2 promotes death through direct activation of PPAR γ . Together, our results define a unique Cdk4–Cited2–PPAR γ -mediated pathway that regulates DNA damage-induced neuronal death.

Prodeath role of Cited2

The role of Cited2 in neuronal death had never before been examined. Nevertheless, a number of correlational observations have suggested that Cited2 might be somehow linked to neuronal survival (Sun et al., 2007). For example, 57% of Cited2 KO embryos exhibit enhanced apoptosis of midbrain cells (Bamforth et al., 2001). Cited2 upregulation could be detected by in situ hybridization in the hippocampus after global ischemia, but predominately, in areas which are spared (dentate gyrus) rather than injured (CA1) (Sun et al., 2006). These observations suggested that Cited2 might be functioning as a prosurvival gene to inhibit neuronal death. However, alternative explanations for these observations could be provided. As one example, death of midbrain cells in Cited2 KO embryos could be a non-cell-autonomous event caused by other defects.

Presently, we confirm an increase in Cited2 levels in cultured neurons after DNA damage. More importantly, we present direct evidence that define, for the first time, the

role of Cited2 in neuronal death/survival. Unlike previous suggestions, we show that Cited2 plays a proapoptotic role after DNA damage. Our evidence is as follows: (1) Cited2 message and protein are increased at early stages after insult, (2) exogenous expression of Cited2 promotes neuronal death, and (3) Cited2 deficiency significantly delays death after DNA damage. Whether Cited2 functions similarly in other neuronal injury paradigms is unknown. However, we observed similar increases of Cited2 message in the CA1 region of the hippocampus of rats after global ischemia (our unpublished data). This suggests the possibility that Cited2 may also play a role in neuronal death under other contexts. It is important to emphasize that Cited2 deficiency is only partially protective. This is consistent with the notion that multiple signals act in concert to regulate neuronal death after DNA damage (Morris et al., 2001; Ghahremani et al., 2002; Keramaris et al., 2003; Aleyasin et al., 2004).

Mechanism for Cited2 upregulation

Our present studies also begin to define the pathway by which Cited2 is induced. Our results indicate that the early increase in Cited2 message is not attributable to several critical signals we previously established to be important in this paradigm of death. These include (1) the mitochondrial pathway of death (Putcha et al., 1999; Keramaris et al., 2000; Cregan et al., 2002), (2) p53 (Morris et al., 2001; Vaughn and Deshmukh, 2007), and (3) JNKs (Ghahremani et al., 2002; Besirli and Johnson, 2003). Instead, our studies reveal that Cdk4 activity is critical for early Cited2 induction. We previously showed that cyclin D1-associated Cdks (Cdk4/6) are aberrantly activated in neurons after DNA damage (Park et al., 1998) and various different in vitro neuronal death paradigms (Park et al., 1996; Park et al., 1997b; Giovanni et al., 1999; Padmanabhan et al., 1999; Rideout et al., 2003). This evidence also extends to in vivo injury and neurodegenerative contexts including after stroke/ischemia (Osuga et al., 2000), Alzheimer's disease (McShea et al., 1997; Biswas et al., 2007), and amyotrophic lateral sclerosis (Nguyen et al., 2003). We and others

have shown that Cdk4/6 activity is essential to signal death in many paradigms of neuronal apoptosis, including after DNA damage in vitro (Park et al., 1998; Morris et al., 2001; Zhang et al., 2006) and stroke in vivo (Rashidian et al., 2005). Moreover, classic downstream targets of Cdk4, Rb, and E2F, have also been shown to be critical for neuronal death. We and others showed that Cdk4 regulates the activity of Rb by phosphorylation and its subsequent inactivation, and so releasing E2F transcription factors from inactivity (Giovanni et al., 2000; Park et al., 2000b; Gendron et al., 2001). E2F activation in turn induces the upregulation of proapoptotic genes, such as B- and C-myb (Liu et al., 2004; Biswas et al., 2005; Greene et al., 2007).

What is the link between cell cycle activation and Cited2? Cited2 has been linked to cell transformation (Sun et al., 1998; Chou and Yang, 2006; Haase et al., 2007). Additionally, Cited2 overexpression enhances fibroblast proliferation and Cited2 deficiency prematurely stops proliferation. It was proposed that Cited2 stimulates proliferation by induction of Bmi-1 that in turn represses the INK4/ARF locus of cell cycle inhibitors (Kranc et al., 2003). In the context of our present work, this suggested the intriguing connection that cell cycle may be activated by Cited2 expression through induction of Bmi-1. However, our results show that, although Bmi-1 is induced after DNA damage, it is not regulated by Cited2. This indicates that it is unlikely that Cited2 acts upstream of Cdk4 activation.

In contrast, our present data point out that inhibition of Cdk4 blocks Cited2 induction suggesting that Cited2 is a downstream target of Cdk4. Whether Cdk4 regulation of Cited2 is through the classic Rb/E2F pathway described above is unknown. A search of mouse Cited2 promoter using TRANSFAC software (version4.0-database7-publicI) and Genomatix/ Gene2Promoter analysis both revealed no clear consensus binding sites for E2F, C-myb, or B-myb. However, the same search exposed an AP-1 putative interacting site (1110 to 1101). In this regard, c-Jun has been shown to interact with the Cited2 promoter in human proliferating cells (Hayakawa, 2004). Intriguingly, our previous work in our model of DNA damage-induced neuronal death

indicated that c-Jun activation was dependent on Cdk4 activation (Ghahremani et al., 2002) as well as the classic upstream JNK family members. Our present results indicate that JNKs only partially regulate Cited2 induction and at late times, whereas Cdk4 more robustly regulated Cited2 levels. This is identical with that we previously reported for regulation of c-Jun. This suggests the possibility of Cited2 being a c-Jun target after DNA damage. However, more careful analyses will have to be performed to clarify this issue.

Mechanism for Cited2-mediated death

Our present results indicate that one important mechanism by which Cited2 promotes death is through regulation of PPAR γ . PPAR γ is a complex signal with both proapoptotic (Rohn et al., 2001) as well as protective functions (Arnold and Konig, 2006; Collino et al., 2006). The role of PPAR γ likely depends on the context of the cell or model system. For example, PPAR γ is known to regulate neuroinflammation by inhibiting microglial activation (Bernardo and Minghetti, 2006). In this context, PPAR γ activation strategies would be expected to promote survival under contexts in which inflammation is a critical component of injury. However, PPAR γ activation is also able to promote death of multiple cell types including neurons (Zhang et al., 2005).

Our findings indicate that PPAR γ participates in the death of neurons after DNA damage. This is supported by the following: (1) PPAR γ activity is increased after DNA damage, (2) inhibition of PPAR γ activation through multiple means promotes survival, and (3) PPAR γ agonist can induce death. Importantly, we also show that the upregulation of PPAR γ activity is dependent on Cited2 and that PPAR γ is a critical mediator of Cited2-induced death. This notion is substantiated by the observations that (1) Cited2 expression is sufficient to activate PPAR γ and Cited2 deficiency inhibits DNA damage-induced PPAR γ activation, (2) PPAR γ inhibition blocks Cited2-mediated death, and (3) Cited2 deficiency inhibits death induced by PPAR γ agonist. The latter two points

also suggest that both Cited2 and PPAR γ require each other to induce death. It must be noted that we do not know whether Cited2-mediated activation of PPAR γ is direct. However, our results show that PPAR γ activation could be accounted for by increase in Cited2 levels, because Cited2 upregulation at 2 h after campto is already significant. Moreover, we see an early increase in an interaction between PPAR γ and Cited2 as revealed by IP/Western blot, suggesting a more direct mechanism of action.

Our work defines, for the first time, the circumstances of Cited2 as a proapoptotic signal in neurons (Fig. 2.9). It is not known whether Cited2 may also play similar roles in adult contexts of neuronal injury such as with stroke. However, it is important to point out that similar players such as cell cycle signals are also critical in adult models of injury (Rashidian et al., 2005). Whether Cited2, then, mediates the prodeath function of cell cycle signals in ischemic death will be an intriguing area of future study.

Figure 2.9. Proposed model of the Cdk4–Cited2–PPAR γ pathway of neuronal death.

This model is based on previous and present results. Links that have been inferred but need to be tested for directness are identified with question marks. In this scheme, Cdk4 activation by DNA damage induces the upregulation of Cited2 levels. Cited2 in turn recruits PPAR γ and by this interaction causes PPAR γ activation. The Cited2:PPAR γ complex leads to cytochrome c release and subsequent caspase activation, followed by neuronal death.

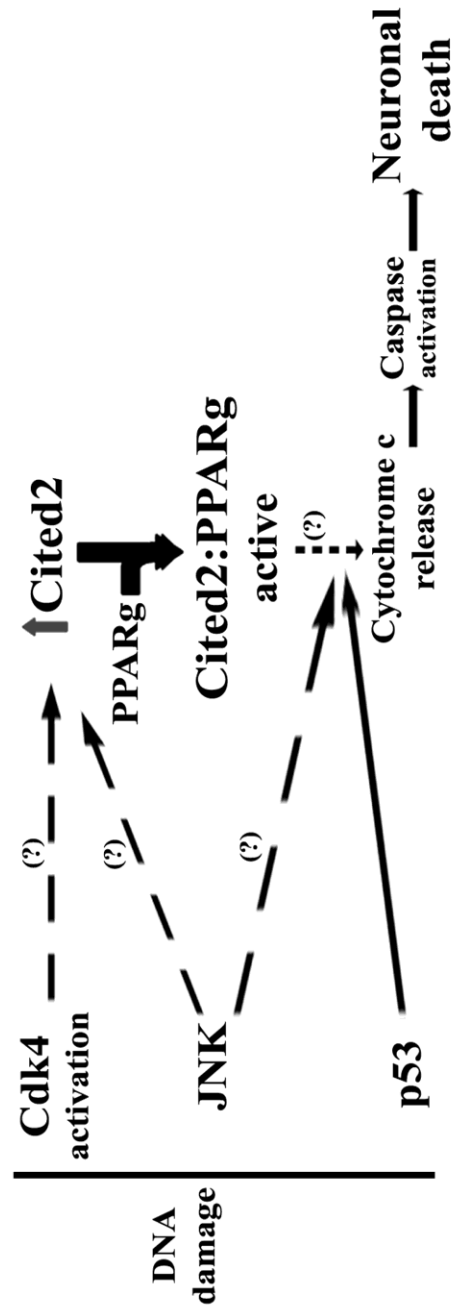


Figure 2.9. Proposed model of the Cdk4-Cited2-PPARg pathway of neuronal death.

Eip63E, the Drosophila PFTAIRE, is required for the proper development of the Central Nervous System.

Manuscript in preparation

Gonzalez YR, Jafarnejad P., Hawari D. Slack RS, Sonnenfeld M., Park DS.

Statement of author contribution

This manuscript examines the potential role of Eip63E, the *Drosophila* homolog of the PFTAIRE Cdk, in the development of the Central Nervous System of *Drosophila melanogaster* via examination of the embryonic Ventral Nerve Cord as a model.

The experiments presented were predominantly performed by Y. Rodriguez Gonzalez, with the assistance of D. Hawari for the embryo dissection experiments. All initial experiments pertaining to the screening of stage 14 mutant embryos using axonal markers BP102 and fasciclin II (1D4), including penetrance calculations for the defects (Fig 3.1), were performed by Dr. P. Jafarnejad. Initial training, guidance and flies facilities were provided by Dr. M. Sonnenfeld. All figures and text for the manuscript were prepared by Y. Rodriguez Gonzalez with the direction and assistance of Dr. David S. Park.

Eip63E, the Drosophila PFTAIRE, is required for the proper development of the Central Nervous System.

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Key words: PFTAIRE; Eip63E; Cyclin dependent kinases; axogenesis; neuronal development.

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Abstract

PFTAIRE is a novel member of the family of Cyclin Dependent Kinases (Cdks), a growing group of kinases with diverse function beyond cell cycle regulation. Recently, PFTAIRE was determined to be expressed in neurons. It is also highly similar in sequence to Cdk5, a kinase known to play a critical role in brain development and death. Very little is known about PFTAIRE biological function and regulation. However, its expression in neurons and similarity to Cdk5 suggest a potentially important role in the CNS. Using two different *Drosophila melanogaster* PFTAIRE mutant lines, we show here that mutant flies display defects as early as stage 11 of embryonic development, as revealed by immunostaining for CNS markers such as neuroglian (BP-104), reverse polarity protein (Repo, 8D12) and BP102 antigen. We also demonstrate that PFTAIRE deficiency leads to axon misguidance and defasciculation accompanied by disorganization of neuronal and glial cell bodies as well as abnormal arrangements of both commissural and longitudinal axons of the Ventral Nerve Cord (VNC). Our data corroborates for the first time the notion that PFTAIRE function is essential for proper development of the Central Nervous System (CNS).

Introduction

The proper development of the Central Nervous System (CNS) requires tight coordination of proliferation, cell cycle exit, differentiation, migration, axon targeting, synaptogenesis, and programmed cell death of progenitor cells. The characterization of these processes is critical for expanding our basic knowledge of how the brain functions and for a better understanding of the potential role of different CNS cell types during neurodegenerative diseases. Additionally, the success of therapeutic strategies that rely on accurate neuronal differentiation and targeting events, such as stem cells-based therapies (Lindvall and Kokaia, 2006) depend on our understanding of these events.

There is a growing list of cdc2-related kinases with no identified cyclin partners that do not appear to be cell cycle related. These are highly expressed/active in neurons. Included in this group are Cdk5, PCTAIRE 1-3 and PFTAIRE, among others. The best characterized of these Cdks is Cdk5. It is mainly active in neurons due to the tissue-specific expression of its activators p35/25 and p39. Cdk5 deficiency in mice is embryonic lethal (E18), with defective neuronal migration, differentiation and survival (Ohshima et al., 1996; Gilmore et al., 1998). Since its identification, Cdk5 has been shown to be involved in a variety of functions including intracellular trafficking and transport (Smith and Tsai, 2002), cytoskeletal dynamics (Xie et al., 2006) and dendrite and synapse development (Cheung and Ip, 2007). It has a crucial role in CNS development and maintenance: neuronal migration, synaptogenesis, learning and synaptic plasticity (Hawasli and Bibb, 2007; Ohshima et al., 2007). Furthermore, we and others have shown that Cdk5 plays a role as well in neurodegenerative conditions such as Parkinson's and Stroke (Rashidian et al., 2005; Qu et al., 2007). Almost nothing is known about the functions of the rest of the neuronal Cdks. PCTAIRE, PFTAIRE and Cdk5 are the three major subbranches from a same evolutionary ancestor (Liu and Kipreos, 2000) which would suggest they share the same functional relevance in neurons.

PFTAIRE was first identified in *Drosophila melanogaster* (Sauer et al., 1996), and soon after in mice (Lazzaro and Julien, 1997) and humans. It is highly expressed in the CNS (Lazzaro et al., 1997; Besset et al., 1998), predominantly in the brain, and it appears to be required for development, since PFTAIRE deficient flies die mostly at early larval stages (Stowers et al., 2000). These two features, together with the fact that PFTAIRE and Cdk5 share a high amino acid sequence similarity (~ 50-52%), had led scientists to believe that PFTAIRE could be as relevant as Cdk5 to CNS development.

We decided to explore the role of PFTAIRE in the CNS using the *Drosophila melanogaster* (fruit fly) model for several reasons. *D. melanogaster* has rapid generation time (~10 days at 25°C), inexpensive culture requirements and large progeny number from a single cross. This model provides a vast battery of genetic tools: rapidly expanding collection of mutants, transposon-based methods for gene manipulation, systems that allow controlled mosaic-like ectopic gene expression and balancer chromosomes. Moreover, Eip63E, the fly PFTAIRE, is 70% identical (AA) to the PFTAIRE kinase 1 (pftk1) mouse homolog and two different Eip63E null mutant fly lines are readily available (Stowers et al., 2000). Importantly, flies do not express other neuronal Cdks closely related to PFTAIRE (PCTAIREs) that could add a level of complication to our studies, due to possible compensation.

The Ventral Nerve Cord (VNC) of *Drosophila melanogaster* embryos provide for an excellent model to study developmental processes of the CNS and have been used so for decades (Garbe and Bashaw, 2004; Sanchez-Soriano et al., 2007). To test the hypothesis that PFTAIRE is required for CNS development, we decided to characterize the embryonic morphology of the VNC of Eip63E mutant flies.

Materials and Methods

Drosophila strains: Flies were kept under standard conditions. The following stocks were obtained from the Bloomington stock centre: W¹¹¹⁸, Eip63⁸¹/TM6tb, Df(3L)E1/TM6, green balancer flies (L²Pin¹/CyO_{krGFP}, D*gl³/TM3_{krGFP}, In(2LR)nocSco,b¹/CyO_{ActGFP} and Sb/TM3_{ActGFP,Ser}) and Gal4 driver lines (elav::Gal4, Repo::Gal4/TM3,Sb, elav::Gal4;UAS::Dcr2.D and UAS::Dcr2.D;Act5::Gal4/Cyo). Three independent RNAi *Drosophila* lines were obtained from the Vienna Drosophila RNAi Center (UAS::RNAiL63/Cyo, UAS::RNAiL63 and UAS::RNAiL63/TM3,Sb).

For the purpose of mutant embryos characterization, the TM6 balancer in both Eip63E deficient lines (Stowers et al., 2000) was replaced with the TM3.Kruppell-GFP balancer chromosome (Casso et al., 2000). This allows distinguishing KO Eip63E embryos from the rest of the population by the absence of GFP expression.

To perform Eip63E specific downregulation, the Gal4:UAS system was used (Duffy, 2002). Balancers in all UAS and Gal4 lines involved in the crosses strategies were changed for the Actin5c-GFP balancer chromosomes (Cyo or TM3 as needed) (Reichhart and Ferrandon, 1998). This would avoid the non-specific ectopic expression of the UAS constructs that the Kruppell-GFP chromosomes would drive (Casso et al., 2000).

Drosophila genetics: As suggested by Dietzl and collaborators (Dietzl et al., 2007), ubiquitous and neuronal Eip63E downregulation assays were performed using virgins from lines expressing, both, the intended Gal4 driver (Act5c or elav) and WT Dicer2 in the desired tissues. This would enhance the effects of the Eip63E RNAi (L63RNAi) (Dietzl et al., 2007). For Eip63E downregulation in glial cells, this was not possible, since a line expressing both Repo::Gal4 and UAS::Dcr2.D was not available. In that case, the F2 generation was analyzed, since homozygosity for the driver would enhance the expression levels of the UAS::L63RNAi construct in glial cells.

Immunohistochemistry: *Drosophila* embryos from Eip63E mutant lines were collected for 2 hours on grape juice-agar medium (Ashburner, 1989), and allowed to carry on development to embryonic stages 6-15 (Campos-Ortega and Hartenstein, 1985), in batches including only 2-3 stages each, by further incubation at 25°C. This developmental window includes all events of embryonic CNS development, from anlage formation to differentiation and axogenesis (Weigmann et al., 2003).

Whole mount embryos were prepared following standard procedures (Harlow and Lane, 2006). In general, embryos were dechorionated with 50% bleach, followed by fixation for 20 minutes in heptane saturated with 4% Paraformaldehyde in 136 mM NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4, (PBS). Vitelline membrane was removed by vigorously shaking the fixed embryos in methanol, followed by further methanol washes. Embryos were re-hydrated using PBS-0.1% Triton X-100 (PBT) and then blocked for 20 minutes using 20% Normal Goat Serum (NGS) (GE Healthcare, Amersham RPN410) in PBT. Fluorescence immuno co-staining was performed against GFP (to amplify the presence of the balancer embryonic marker), HRP and a CNS marker. Primary antibodies were as follows: mouse anti-GFP (JL-8, Living colors, 1/100), Cy3-anti-HRP (1/25), mouse BP102 (1/10), mouse anti-En/Inv (4D9, 1/10), mouse anti-single-minded (1/5), mouse anti-Futsch (22C10, 1/10), mouse anti-Neuroglian (BP-104, 1/10), mouse anti-elav (9F8A9, 1/14) and mouse anti-Repo (8D12, 1/10). All monoclonal antibodies against CNS markers were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Such antibodies were developed by C. Goodman, S. Crews, S. Benzer and G.M. Rubin. Since the anti-HRP antibody was Cy3-conjugated, only Alexa 488-Goat anti-mouse (1/100) was used as secondary antibody, after a second blocking step. Stained embryos were then directly mounted in Vectashield (Vector laboratories, H-1000) and visualized under a fluorescent microscope.

Embryo dissections were performed essentially as described by Benveniste and

collaborators (Benveniste et al., 1998). 7-13 hours timed embryos were transferred to a piece of double-sided tape mounted on a polylysine-covered microscope slide, and then rolled using forceps to mechanically remove the chorions. Embryos were then covered with PBS and examined under fluorescence light for GFP expression. Absence of GFP expression would indicate an Eip63E KO. After genotyping and fine determination of developmental stage based on morphological features, a dorsal incision was made in the vitelline membrane of each with a sharpened tungsten needle (Small Parts Inc TW-006-60). Each embryo was then removed from the membrane and placed ventral side down directly on the polylysine-covered surface of the slide, while still submerged in the PBS. Using the tungsten needle, an incision was made longitudinally along the dorsal surface of the embryo and the body walls were pressed down onto the glass. The gut and remaining yolk sac were then removed, resulting in a flat embryonic preparation. Dissected embryos were then fixed for 30 minutes at room temperature in 4% paraformaldehyde in PBT. One hour blocking was performed using 10% NGS in PBT, followed by antibody staining. Fluorescence immuno co-staining was performed against HRP and a CNS marker. Same antibodies mentioned above were used but at different concentrations: 1/50 for primary antibodies and 1/300 for secondary. Preparations were mounted using Fluoromount™ mounting media (Sigma F4680), sealed with clear nail polish and visualized under a fluorescent microscope.

Results

Eip63E deficient embryos show general defasciculation and misguidance of longitudinal and commissural axons of the Ventral Nerve Cord.

Preliminary observations of embryos from Eip63E mutant lines, both Df(3L)E1 and Eip63E⁸¹, revealed defects in axonal structures of the VNC potentially related to the deficiency. To confirm if these defects were indeed linked to Eip63E deficiency, an embryonic marker needed to be introduced to enable for the differentiation of homozygous mutant embryos from the rest of the progeny. For that purpose, both mutant lines were balanced with the TM3krGFP,Sb chromosome which provides for GFP expression under the kruppel promoter from embryonic stage 9-10 to adulthood (Casso et al., 2000). GFP expression was then amplified for all experiments with the use of fluorescent immunostaining against GFP.

Primarily, two different markers were used to assess VNC structures of stage 14-15 embryos from both mutant lines: BP102 and fasciclin-II (1D4). Both antigens are expressed in the axon bundles that define the commissural and longitudinal connectives of *Drosophila melanogaster's* Ventral Nerve Cord (VNC) (Fambrough et al., 1996; Hummel et al., 1999). Results for both markers confirmed that the axonal defects were indeed linked to Eip63E deficiency (Fig. 3.1). KO embryos showed a diffused appearance of all axonal pathways (Fig. 3.1A and 3.1C) suggesting defasciculation of the axon bundles. Additionally, axons appear not to follow the appropriate pathways, leading to malformation of the ladder-like normal axonal structure of the VNC, with rupture of both commissural and longitudinal pathways (Fig. 3.1A and 3.1C). In all the cases and for both markers, only GFP-negative embryos were found defective, pointing to the idea that half of Eip63E normal levels are sufficient for proper axonal structures to be formed.

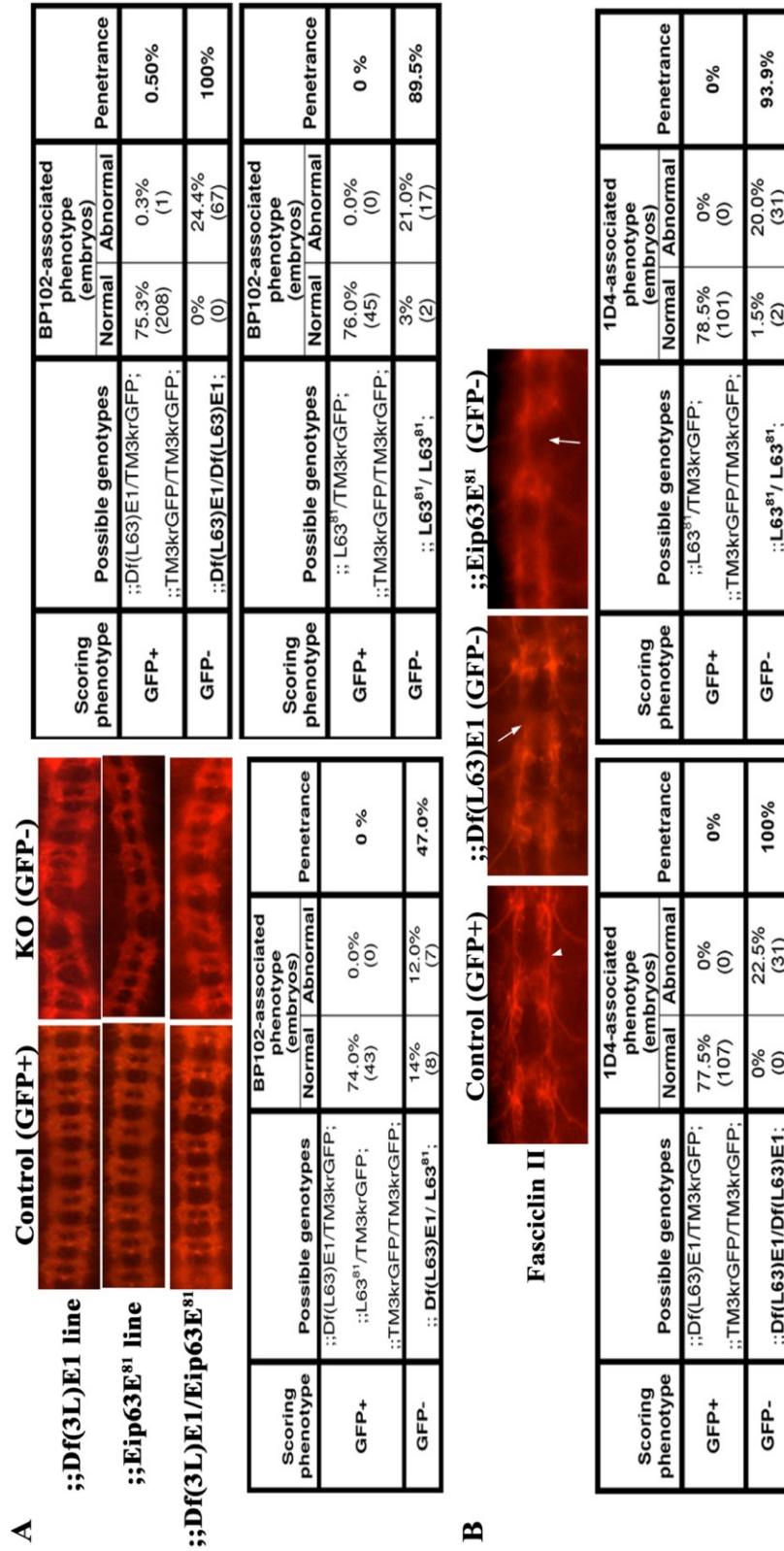
The nature of both Eip63E mutant alleles is different. The Df(3L)E1 allele is the

result of a large deletion that contains the transcription initiation point and almost the entire conserved kinase domain of the Eip63E gene. This deletion affects two other genes upstream the one of our interest: SDE3/armitage (Cook et al., 2004) and l(3)63D (Stowers et al., 2000). The Eip63E⁸¹ allele consists of a smaller 48bp in-frame deletion that removes 16 codons within the conserved kinase domain (213-228). Accordingly, we needed to test the possible influence of the genetic background on the VNC axonal defects. To do so, a cross was performed to generate transheterozygous embryos containing one copy of each of the mutant alleles. BP102 staining of this progeny revealed axon defects on the transheterozygous mutants similar to those found in the single mutant embryos (Fig. 3.1A, last panel). This, together with the facts that no GFP-positive embryo was ever found to be defective (Fig. 3.1) and that the only gene homozygously deficient in the trans mutants was Eip63E, suggested that the axonal defects were in fact caused at least in part by Eip63E deficiency and not by the secondary mutations.

The penetrance associated to the defects revealed by both axonal markers was determined for both mutant alleles and the transheterozygous mutants (Fig. 3.1B and 3.1D). The quantification showed a very high incidence of the defects (100% for Df(3L)E1 and 89.5% - 93.9% for Eip63E⁸¹), strengthening the notion of Eip63E having an essential role in axon development. It was interesting to see that such incidence was diminished by half in the transheterozygous mutants (47%), suggesting a degree of influence from the genetic background. Further experiments needed to be done to confirm the specificity of our findings (see ahead).

Figure 3.1. Eip63E deficiency leads to defasciculation and misguidance of commissural and longitudinal axons of the Ventral Nerve Cord.

Ventral views of whole mount stage 14-15 embryos from both Eip63E mutant lines were co-stained for GFP and BP102 **(A)** or Fasciclin-II (1D4 mAb) **(B)**. Penetrance of axon abnormalities was calculated as the % of embryos of a given scoring phenotype that show any defect from the total population of such phenotype (GFP+ or GFP- embryos). Arrowhead points to normal looking longitudinal connectives and arrows to disrupted structures in KO embryos.



Axon outgrowth appears to occur prematurely in Eip63E deficient embryos.

In *Drosophila* embryos, axogenesis starts at ~7:30h into development (early stage 12) after specification of the midline cells that will guide the pioneer axons contralaterally to cross the midline (Jacobs and Goodman, 1989b; Hummel et al., 1999).

To elucidate how early during CNS development the axon defects started to appear, we decided to look at CNS features using staining with anti-horseradish peroxidase (anti-HRP). This antibody recognizes a carbohydrate epitope (called HRP from now on) present in several cell-surface molecules expressed by neuronal cell bodies and axons (Snow et al., 1987), including fasciclin II (Desai et al., 1994). In our experiments, HRP staining in WT embryos is detectable along the VNC from late stage 10 on.

Our data revealed that 10% of stage 8-9 embryos (n=48) from the Eip63E⁸¹/TM3_{krGFP},Sb line showed what appears to be a higher expression of HRP, when compared to WT embryos (0%, n=65) (Fig. 3.2A). This abnormality became more evident when embryos were looked at laterally. WT embryos from stages 9-10 show very faint, background staining along the ventral region of the embryo where VNC axons would appear later. In contrast, Eip63E KO embryos (GFP-) show clear HRP-positive structures at stages when none would be expected (Fig. 3.2A and 3.2B). At later stages, an additional phenotype was detected. Late stage 11 KO embryos would show premature, and in some cases misguided, axon outgrowth affecting several segments of the VNC (Fig. 3.2C). The combined penetrance of defects for stages 10-11 Eip63E deficient embryos was 36.5% (n=52). These results show that axon-related defects appear quite early from the time axon structures tend to emerge and that the incidence of axon defects in KO embryos increases during development.

Figure 3.2. Eip63E deficiency appears to lead to premature HRP-positive structures and axon outgrowth.

Whole mount embryos stained with anti-HRP are shown oriented head to the left. In each section, a ventral and a lateral view of the same embryo from the specified line are shown. **(A)** Stage 9. Embryos from mutant line are of unknown genotype. 10% (n=48) of them show premature HRP staining, a defect that appears at stage 10-11 KO embryos as well **(B and C)** (examples pointed at by arrowheads). **(C)** At late stage 11, precocious axon outgrowth can be seen in 36% of KO embryos (n=52) (pointed by arrows).

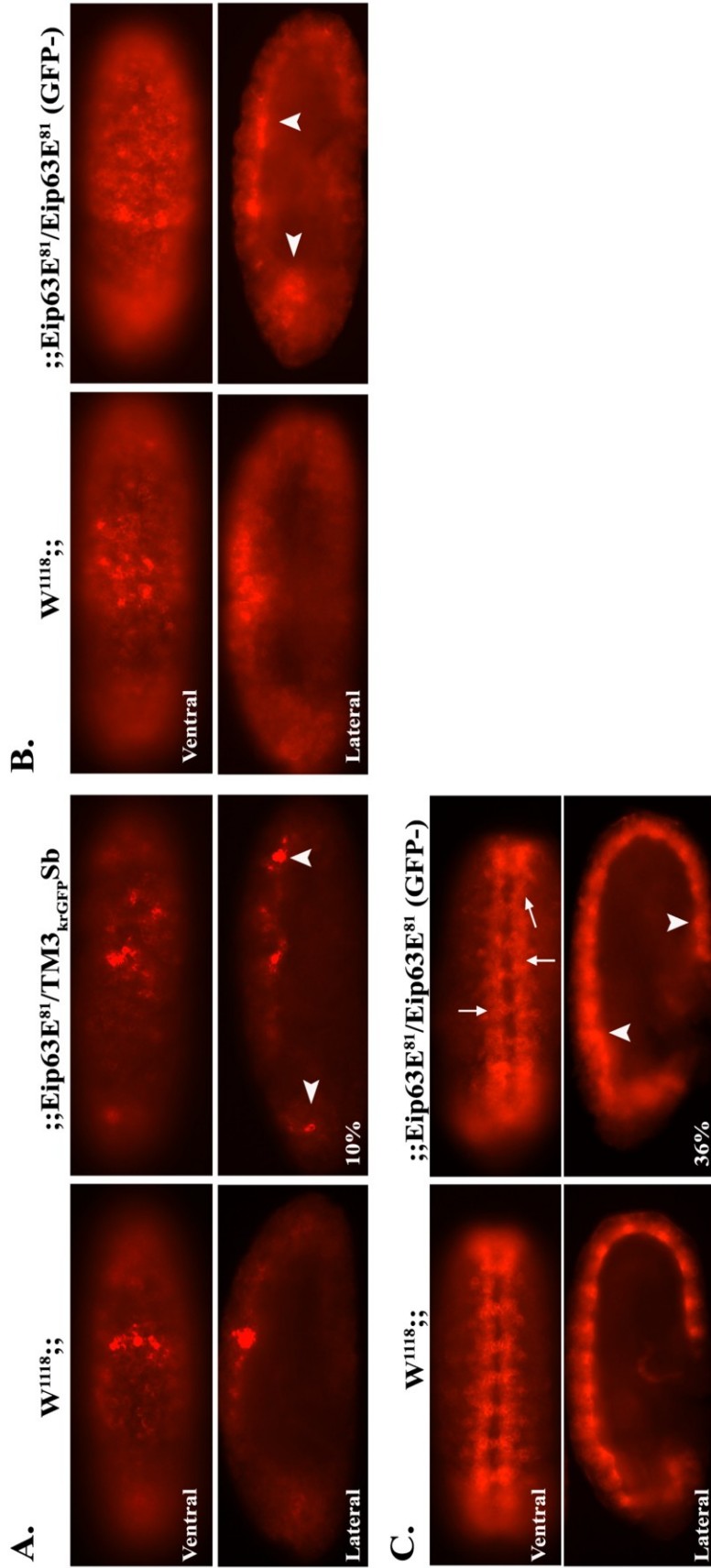


Figure 3.2. Eip63E deficiency appears to lead to premature evidence of HRP-positive structures and axon outgrowth.

Early neurogenesis events appear to be defective in Eip63E mutants.

The first event in *Drosophila* CNS development is the formation of the ventral midline. This early occurrence has an essential impact in axon outgrowth/guidance and the proper ladder-like scaffold of axons of the VNC (Klambt et al., 1991; Hummel et al., 1999). We decided to assess midline formation in Eip63E mutant lines using immunostaining for single-minded (sim), a protein which is exclusively expressed in midline precursors and their progeny and is a master regulator of the differentiation of midline neurons and glial cells (Nambu et al., 1990).

Results are presented in figure 3.3. It was clear for both mutant lines that Eip63E deficiency does not appear to affect midline formation, since no defects were ever found in any embryos (n=60-65, for both mutant lines, stages 5-12). This suggested that Eip63E might not be necessary for midline formation (Fig. 3.3A, stages 5-10). Moreover, the HRP co-staining done on these embryos revealed that even for KO embryos with abnormal HRP expression and axon defects, the ventral midline appeared to be normal (Fig. 3.3B, late stage 11). This suggested that the axon defects may be due to early events, but ones independent of midline formation.

Segmentation of the *Drosophila* embryo is another developmental process that is triggered very early (first 3 hours) and leads to the formation of stripe-like segments along the anterior-posterior axis. Segment polarity genes are part of the expression cascade that regulates this process and start being expressed, in 14 stripes, at the onset of gastrulation (~3h, stage 6-7) (Weigmann et al., 2003). Evidence shows that these genes: hedgehog (hh) and wingless (wg), patched (ptc), gooseberry (gsb), engrailed (en) and invected (inv) are major players in neurogenesis (Bhat and Schedl, 1997; Bhat, 1999). They are expressed in the neuroectoderm preceding neuroblast delamination and continue to be expressed in neuroblasts (Joly et al., 2007; Colomb et al., 2008). Based on this, we decided to look at the expression pattern of engrailed/invected proteins to evaluate in one step if segmentation and/or neuroblasts delamination/segregation was somehow impaired in Eip63E deficient embryos.

Immunostaining with the 4D9 antibody, that recognizes both engrailed and invected proteins, revealed that 18% of stage 8-9 embryos (n=48) from the Eip63E⁸¹/TM3_{krGFP},Sb line showed abnormal patterning (Fig. 3.4A). Since genotyping is not feasible at these stages, it is not possible to link this phenotype to Eip63E deficiency. Nevertheless, comparable defects were found in similarly timed embryos from the Df(3L)E1/TM3_{krGFP},Sb line (data not shown). Furthermore, only 3% of WT embryos of similar stages show very mild defects (n=71). Interestingly, although abnormalities were present as well in older KO embryos (stages 10), their incidence tended to decrease (8% Eip63E⁸¹ allele, n=25). This could be explained by the probable death of the early defective embryos. Nevertheless, ~7-8h embryos (stages late 11-early 12) showed a different anomaly, where cell bodies appeared to be disorganized and of varied shape and size (Fig. 3.4B). This could suggest that the boundary normally existing between the neuroectoderm (generally small cells) and neuroblasts (larger and rounder cells) regions is disrupted or absent in Eip63E deficient embryos. Taken together, these results raise the possibility of Eip63E having a role in neuroblasts' delamination and/or segregation, but further and mechanistic experiments need to be done to explore this hypothesis.

Figure 3.3. Eip63E deficiency does not appear to be linked to midline defects.

Ventral views of whole mount embryos stained for single-minded (sim mAb) and HRP are shown head to the left. **(A)** Early embryonic stages (st 5-10). Stage 6-8 embryos from mutant line are of unknown genotype. No differences between mutant and control embryos were detected. **(B)** Single-minded (sim) and HRP staining for the same late stage 11 embryos of specified lines are shown. Mutant embryos appear to have normal midline even when having abnormal HRP positive morphology.

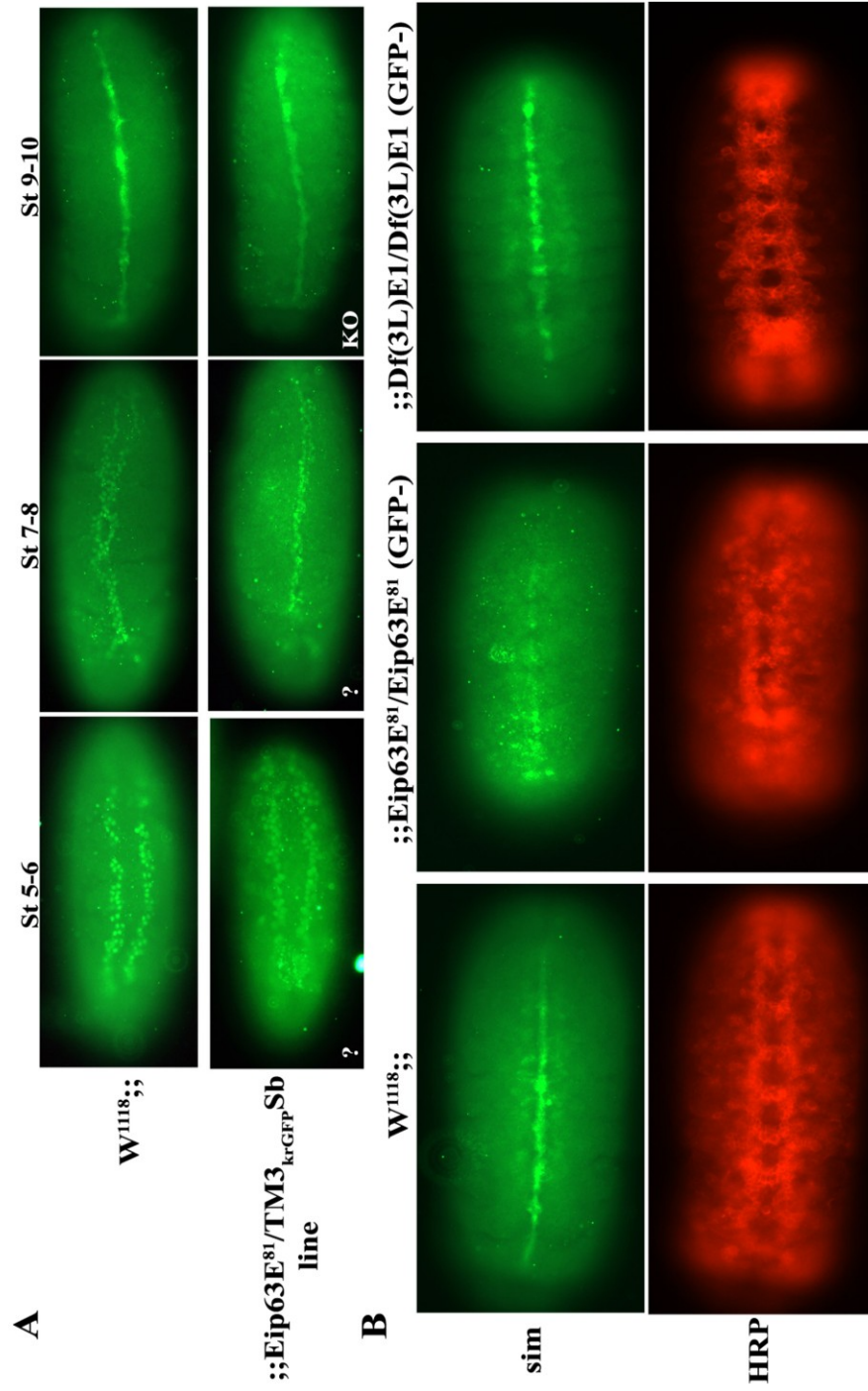


Figure 3.3. Eip63E deficiency does not appear to be linked to midline defects.

Figure 3.4. Eip63E deficiency might lead to early segmentation defects and later disorganization of neuroblasts along the dorso/ventral axis.

Ventral views of whole mount embryos stained for engrailed/invented (4D9 mAb) are shown head to the left. **(A)** Stage 8-9 embryos from Eip63E81/TM3krGFP,Sb line were of unknown genotype. 18% of these embryos show segmentation defects as illustrated. Panels for stages 10-11 show Eip63E81 homozygous mutant embryos. Cell bodies appear disorganized (examples pointed by arrowheads) to the point of lack of hemisegments division at the midline (wavy arrow for KO embryos vs straight arrow for midline of control W1118 embryos). **(B)** Stage 12 embryos. Two different focal planes for the same embryo are included to show neuroectoderm region (ventral) and neuroblasts' region (dorsal). Neuroectoderm and neuroblast layers are almost undistinguishable from one another in Eip63E null embryos, suggesting that cell disorganization along the dorso/ventral axis.

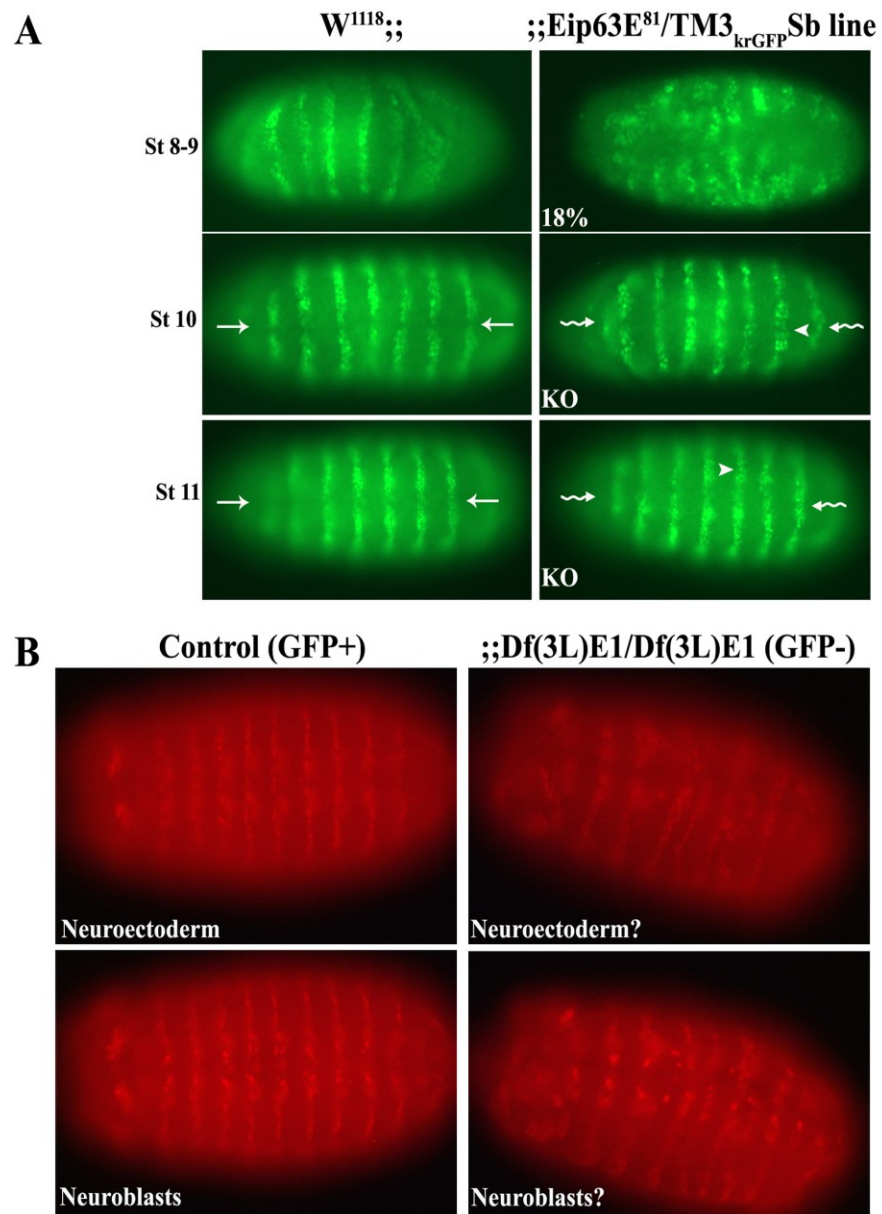


Figure 3.4. Eip63E deficiency might lead to early segmentation defects and later disorganization of neuroblasts along the dorso/ventral axis.

PFTAIRE deficiency leads to other morphology defects in CNS that includes disorganization and/or absence of neurons and glial cells.

At this point, it was important to explore if the cell bodies of neurons and glia were present and normally located in Eip63E mutants.

The embryonic lethal abnormal visual system (*elav*) gene of *Drosophila melanogaster* codifies for a RNA-binding protein that is required for the development and maintenance of the nervous system (Jimenez and Campos-Ortega, 1987). It is a generally accepted belief that, during development, the ELAV protein is expressed exclusively in neurons shortly after their birth (Robinow and White, 1991). This has made ELAV a widely used marker for differentiated neurons. Immunostaining of Eip63E mutant embryos for ELAV expression revealed defects from early stages (Fig. 3.5). It was apparent from stage 11 KO embryos that ELAV expression seems to be diffused when compared to WT embryos of similar stages, where most neuronal cell bodies can be easily differentiated one from another (Fig. 3.5A). Additionally, it was clear that the morphology of neuromeres and hemisegments along the VNC of mutant embryos was disrupted and abnormal. This made it very hard to distinguish midline neurons from the rest in several VNC segments of mutant embryos of stage 12-13 (Fig. 3.5A). When *elav* and HRP images were merged and embryos were looked at sideways (Fig. 3.5B) it became obvious that Eip63E deficiency was linked to a disorganized neuronal cortex, since *elav*-positive cells can be detected dorsally deeper than normal, underneath the axon scaffold. This could be the reason why *elav* expression seems to be lower in mutant embryos than in WT ones of equal developmental stage, but this observation needs to be explored further.

Figure 3.5. Eip63E deficiency leads to early neuronal defects that include diffused elav expression and cell bodies disorganization.

Whole mount embryos were co-stained for elav (9F8A9 mAb) and HRP. **(A)** Ventral view of elav-stained embryos, stage 11-13 oriented head to the left. Abnormalities were apparent from stage 11 on in KO embryos. Elav expression appears diffused, neuromeres are poorly defined and neuronal cell bodies highly disorganized, so that midline neurons are undistinguishable from the rest in several VNC segments at stage 13 (midline indicated by arrow). **(B)** Lateral view of stage 12 embryos showed in panel A. Merged images of elav and HRP staining are shown. KO embryos not only have abnormal HRP profile, but elav-positive cells appear to be dorsally deeper than normal (arrowheads), under the axon bundles, which suggests that Eip63E deficiency affects the dorso-ventral organization of the cell cortex.

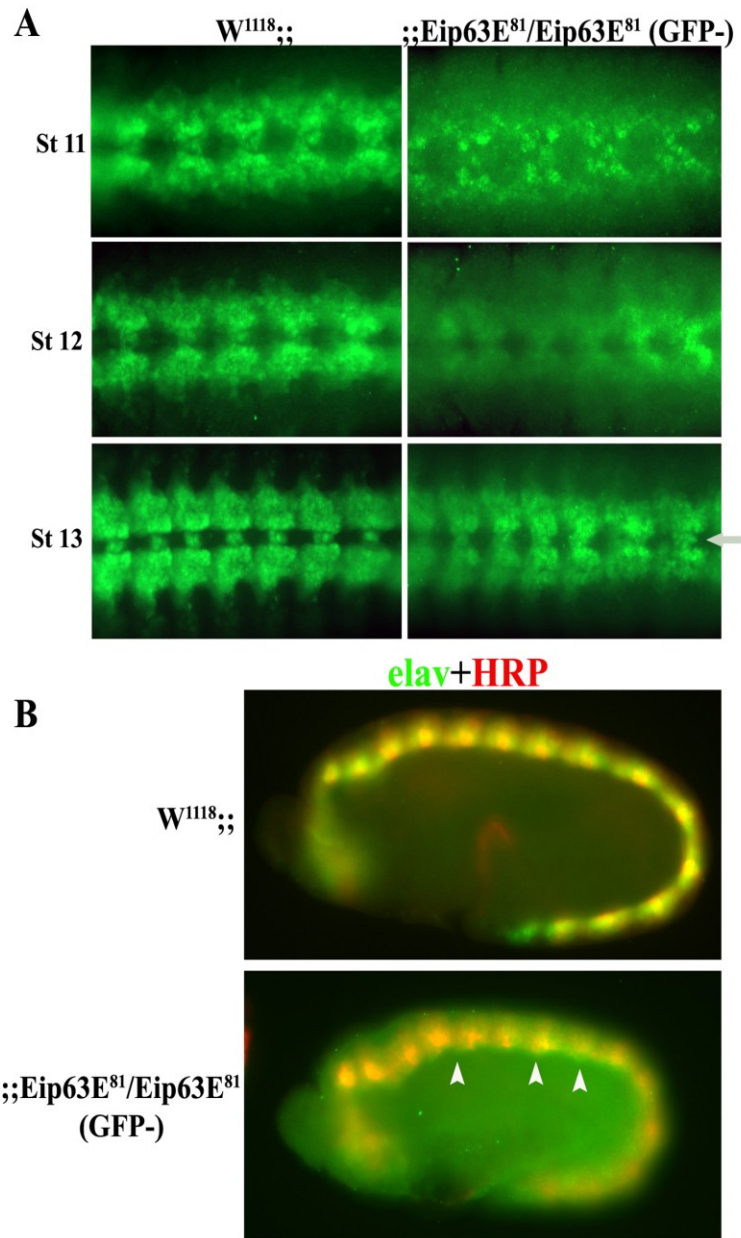


Figure 3.5. Eip63E deficiency leads to early neuronal defects that include diffused elav expression and cell bodies disorganization.

To test if these results were reflective of the neuronal morphology and not only related to the *elav* expression pattern in mutants, a second neuronal marker was used. Neuroglian (long isoform, BP-104 mAb) is a cell-adhesion molecule expressed in part of the population of neuronal cell bodies and axons (Hortsch et al., 1990). When our immunostaining analysis for this marker was performed (Fig. 3.6), it showed that *Eip63E* mutant embryos were indeed abnormal. At early stages (10-11) KO embryos show an irregular pattern of neuroglian expression, with either less or more than expected amount of cells being lighted up. Interestingly, it has been suggested that in WT embryos the structures expressing neuroglian at stage 11 coincide with the area and pattern where longitudinal axon connectives will be formed at later developmental stages (Hortsch et al., 1990). In KO embryos, the most common defect was a less restricted neuroglian expression in this area, with a 44% penetrance for the *Eip63E*⁸¹ allele (n=25). Later, at stages 12-13, there is a clear malformation of neuromeres and hemisegments linked axon pathfinding defects. The lack of symmetry between the hemisegments and the midline pattern of different neuromeres suggest a disorganization of neuronal cell bodies consistent with the observations for *elav* immunostaining.

Similar disarray can be observed when neurons and axons were stained by Futsch staining using the 22C10 mAb and anti-HRP (Fig. 3.7). Futsch is a microtubule-associated protein, necessary for axon and dendrite development in *Drosophila* (Hummel et al., 2000). From the images on figure 3.7 it is clear that the axonal bundles in *Eip63E* KO embryos are highly defasciculated when compared with controls (HRP staining) and that defective hemisegments are paired to misplaced neurons (22C10 staining). Additionally, Futsch/HRP merged images made evident that, in KO embryos, neuronal cell bodies appear to be abnormally localized dorsally to the axon scaffold, as suggested before by *elav* staining (Fig. 3.5B).

Taken together, the results associated to neuronal markers strongly suggest that the axon defects in *Eip63E* deficient embryos are accompanied by defects in neuronal

cell body localization. This appears to happen from early stages of CNS development.

Figure 3.6. Staining for Neuroglial confirms irregular neuromeres morphology and abnormal axon outgrowth/guidance in Eip63E mutants.

Ventral views of whole mount embryos stained with mAb BP104 are shown oriented head to the left. Eip63E deficient embryos have an irregular, less restricted pattern of neuroglial expression by odd-shaped cells, different to those on WT embryos (44% penetrance for stage 11 Eip63E⁸¹ homozygous embryos). Additionally, premature axon outgrowth can be detected from stage 11 (examples indicated by arrowheads) and misguided axons can be seen at stages 12-13 (examples indicated by arrows).

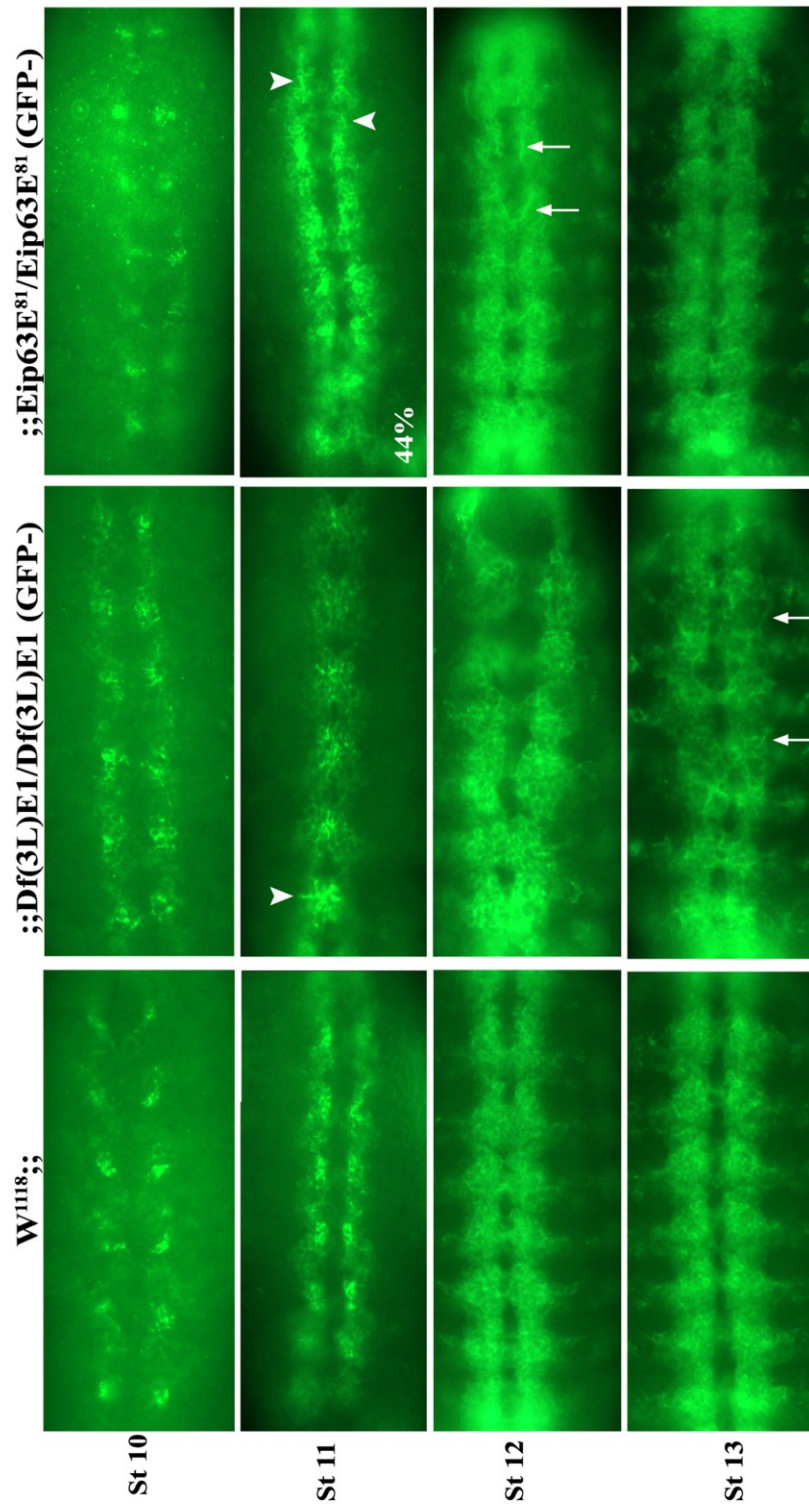


Figure 3.6. Staining for Neuroglian confirms irregular neuromeres morphology and abnormal axon outgrowth/guidance in Eip63E mutants.

Figure 3.7. Eip63E deficient embryos show spatial disarray of neuronal cell bodies.

VNC flat preparations of timed embryos (10-11h, stage 14) co-stained for futsch (22C10 mAb) and HRPare shown dorsal up, head to the left. Neuronal cell bodies disorganization and axon misguidance and defasciculation are acute for the Df(3L)E1 homozygous embryos. Arroheads point to some of the estructures where neuronal organization is different from control embryos. Arrows point to examples of axon growth abnormalities.

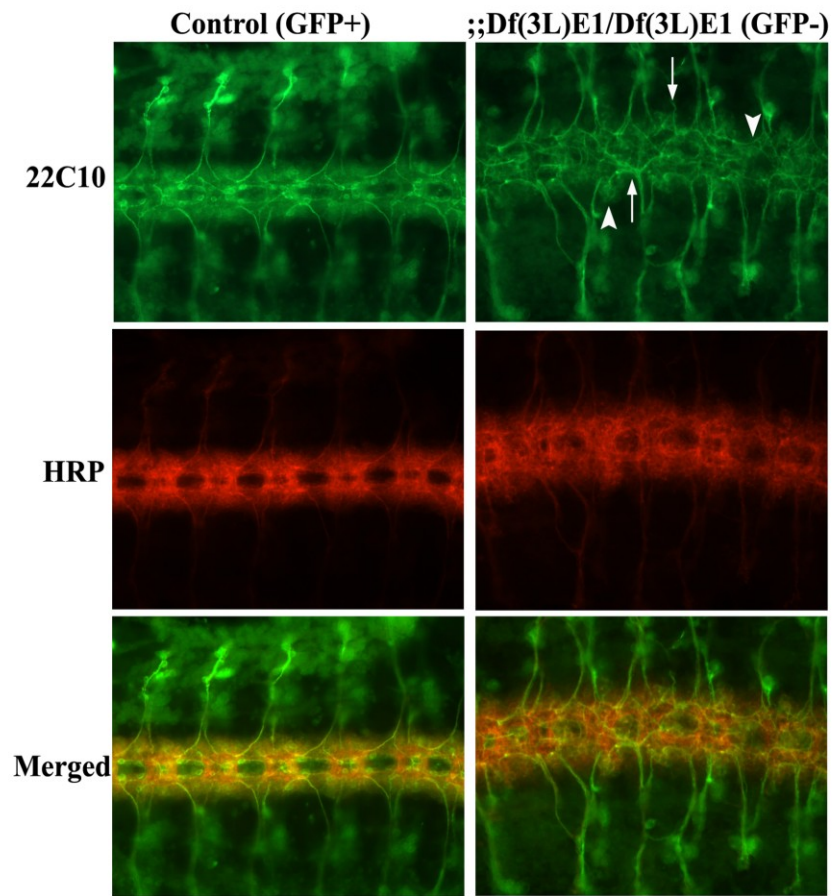


Figure 3.7. Eip63E deficient embryos show spatial disarray of neuronal cell bodies.

In *Drosophila*, as in vertebrates, glial cells in the CNS have a key role in neuronal and axon development, particularly in axon guidance (Chotard and Salecker, 2004; Parker and Auld, 2004; Freeman, 2006). Since the axon defects linked to Eip63E deficiency appear as early as stage 10 and it has been shown before that a glial scaffold of cells precedes axon outgrowth during VNC development (Jacobs and Goodman, 1989a), we explored glial-related morphology in mutant embryos.

Glial population in *Drosophila* is complex and varied, with groups of cells originating from different progenitor tissues: midline glial from the mesectoderm (ventral midline) (Klamt et al., 1991) and the rest from the neuroectoderm (Jones, 2001). The reverse-polarity (Repo) gene codifies for a homeodomain protein that is expressed in all embryonic glial populations, except midline glial, from stage 9 glioblasts to mature glial cells (Xiong et al., 1994).

Trying to avoid the visual complexity added by the autofluorescence of embryos internal organs, we initially decided to perform repo staining (8D12 mAb) on flat preparations instead of whole mount embryos. Although the dissection process was successful for most embryos from the Df(3L)E1/TM3_{krGFP},Sb line, including KOs, this was not the case for the Eip63E⁸¹/TM3_{krGFP},Sb line. Control, GFP+ embryos from this second mutant line would be easily dissected, while very few of KOs would withstand the process. Only older than stage 13 KO embryos, and never the defective ones, would bear up the dissection process. This difficulty made us think that embryos homozygous for the Eip63E⁸¹ allele were somehow more fragile than those for the Df(3L)E1 allele. Therefore, we decided to perform the repo immunostaining for the second mutant line on whole mount embryo preparations. Results for this assay are presented in figure 3.8. For the Df(3L)E1 allele, abnormalities were varied and clear from stage 11 on. Only one of the phenotypes detected in KO embryos is shown in figure 3.8 (Fig. 3.8A). In the presented cases, it was evident that some of the glial cell bodies were missing. For the rest of the KO embryos, there would be a less dramatic absence, an obvious disarray of cell bodies, or a mix of these two situations with different degrees of severity.

Nevertheless, the same was not true for the Eip63E⁸¹ allele. Although disorganization of cell bodies was observed as well from stage 11 (data not shown) and later (Fig. 3.8B), this defect was mild in comparison to what was observed for the Df(3L)E1 allele. Additionally, absence of glia cell bodies was never detected in KO embryos for the Eip63E⁸¹ allele.

We can not explain the above discussed differences between the mutant alleles. Nevertheless, if glial-related defects would be behind the very consistent and highly incidental abnormal axon phenotype of Eip63E KO embryos, it would be expected that such glial defects would be as consistent as well. The lack thereof and the varied nature of glial-associated abnormalities points to the possibility of this defects being secondary to whatever may be happening with neuroblasts and neurons earlier during development. Although intriguing, this is a hypothesis that would need further testing.

Figure 3.8. Glial cells are either missing or misplaced in Eip63E deficient embryos.

(A) VNC flat preparations of dissected embryos from the Df(3L)E1 line stained for repo (8D12 mAb) and HRP are presented dorsal up, head to the left. Defects for the Df(3L)E1 allele included absence and disorganization of glial cell bodies with varied severity spectrum. Examples of KO embryos with acute glial cell bodies absence are shown.

(B) Whole mount embryos from the Eip63E⁸¹ line stained for repo (8D12 mAb) and HRP, are shown ventral up, head to the left. Absence of glial cell bodies was not apparent for Eip63E⁸¹ homozygous embryos. Instead, disorganization of cell bodies was detected associated to axon defects. Arroheads point to some of the estructures where glial organization is different from control embryos.

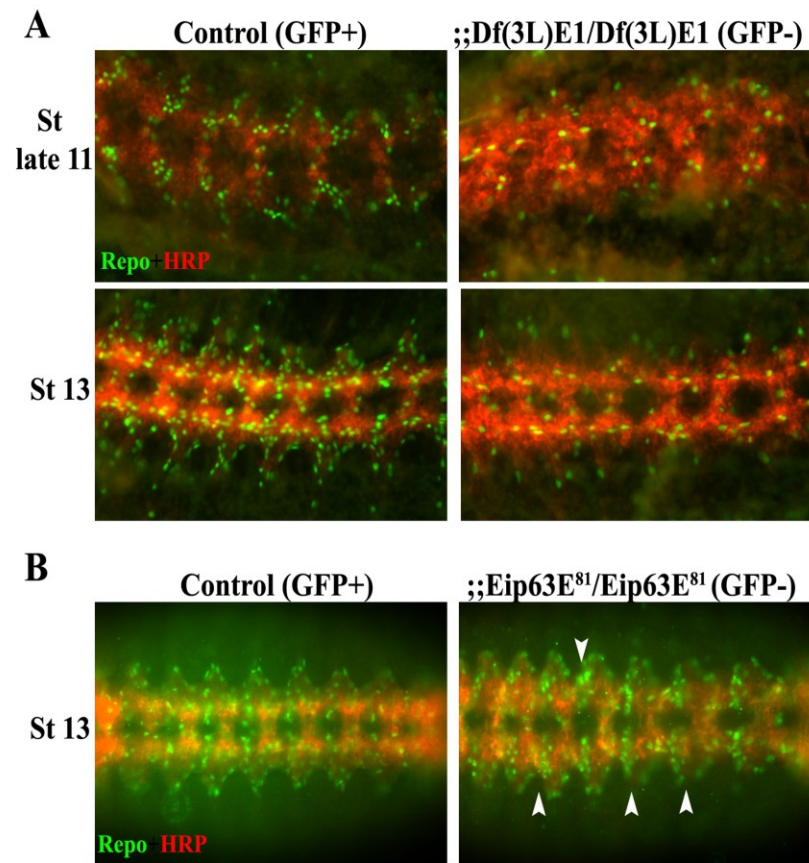


Figure 3.8. Glial cells are either missing or misplaced in Eip63E deficient embryos.

Specific Eip63E downregulation by RNAi in neurons, but not glial cells, leads to axon defects consistent with those observed in mutant lines.

Although our immunochemistry results strongly suggest an essential role for Eip63E in CNS development, two main issues remained to be answered. One was relevant to the specificity of the CNS defects observed in the Eip63E germline mutant lines. Were the defects due to Eip63E deficiency? Or are the secondary mutation plus genetic background players as well? Second, where and when in the CNS is Eip63E necessary for proper development? One of the approaches that would begin to address these questions would be to perform time/tissue specific downregulation of Eip63E taking advantage of the Gal4-UAS system (Duffy, 2002) for manipulation of ectopic gene expression.

To do this, we utilized RNAi fly lines for Eip63E developed by the Vienna Drosophila RNAi Centre. We followed a strategy consistent with that suggested by Dietzl and colleagues in 2007 (Dietzl et al., 2007). Since the efficiency of gene downregulation would be increased by co-expression with Dicer-2 (Dietzl et al., 2007), one of the regulatory enzymes for RNAi mechanism of action (Agrawal et al., 2003), we also received lines that expressed the UAS-Dicer2 construct, either under an ubiquitously expressed driver (Act5-Gal4) or a neuronal driver (elav-Gal4). Our strategy was to obtain, by crosses, embryos that would express the UAS-RNAiL63 (short for Eip63E-RNAi) together with the UAS-Dicer2 in the desired tissues. For Eip63E downregulation in glial cells, the strategy would not include the Dicer-2 approach for lack of availability of lines, and so F2 progeny of a repo-Gal4 x UAS-RNAiL63 cross would be examined, looking for embryos homozygous for at least one of them.

To test the effects of ubiquitous downregulation of Eip63E on VNC axon features, a cross was performed between virgins of a *UAS-Dcr.2.D; Act5-Gal4/Cyo* line and *;;UAS-RNAiL63* males. 50% of the F1 progeny of this cross would be embryos expressing in heterozygosity all three constructs: *UAS-Dcr2.D/+;Act5-Gal4/+;UAS-RNAiL63/+*. The other half of the progeny would be lacking the driver: *UAS-*

Dcr2.D/+;Cyo/+;UAS-RNAiL63/+, and so function as an internal control. BP102 and HRP staining of the F1 revealed that 41.2% of embryos had VNC defects (n=35), for an equivalent penetrance of 82.4%. Two of the defective embryos are shown in figure 3.9A. Additionally, a noticeable number of embryos under stage 11 appeared to be unhealthy. Furthermore, when embryos were left to complete the life cycle, all the flies that eclosed were positive for the *Cyo* marker (n=57), suggesting that all those expressing *Dicer2* and *RNAiL63* died during the developmental stages. It is clear that other controls should be introduced and that *Eip63E* levels should be object of biochemical analysis. Nevertheless, our observations suggest that the downregulation strategy was working properly, since our results are consistent with what happens in mutant lines, where *Eip63E* deficient embryos are not only defective for VNC structures, but do not survive to adulthood.

To achieve *Eip63E* downregulation in neurons, an *elav-Gal4* driver was introduced in the preliminary experiments. Since its identification, *elav* has been considered an exclusively neuronal protein, and so this driver line was created for genomic studies purposes (Yao and White, 1994). A more recent report argues that *elav* is transiently expressed in a reduced population of neuroblasts and glioblast during early neurogenesis (Berger et al., 2007). Since this finding does not contradict the fact of *elav* being exclusively expressed in the CNS, we still consider our approach appropriate.

Two parallel crosses were started to discard the possibility of defects being caused by *Dicer2* overexpression, one using *Dicer2* co-expression and one without. For the first one, virgins from an *elav-Gal4;UAS-Dcr2.D*; line were crossed to *;;UAS-RNAiL63* males. 100 % of the F1 progeny would be heterozygous for the three constructs: *elav-Gal4/+;UAS-Dcr2.D/+;UAS-RNAiL63/+*. Out of this homogeneous progeny, 67% of embryos younger than stage 11 appeared dead (n=161) and 9% (n=23) of embryos older than stage 11 showed axonal defects (Fig. 3.9B). This suggested an acute lethal effect associated to *Eip63E* neuronal downregulation mediated by *Dicer2* overexpression. The

second preliminary cross was performed between virgins of an *elav-Gal4*; line and ;;UAS-RNAiL63 males. In this case, the F1 generation would be homogeneously heterozygous for both the *elav* driver and the UAS-RNAiL63 transgene. None of the embryos collected and immunostained from this generation showed any VNC defects (n=35), probably due to insufficient Eip63E downregulation. F1 flies were then crossed to obtain and analyze the F2 generation. Only the ~16% of the embryos of this generation would be homozygous both for the driver and the RNAiL63 constructs. Immunostaining with the axonal markers revealed that 11.5% of stages 11-15 embryos were defective (n=26), which would be equivalent to a 71.9% penetrance for the defects (Fig. 3.9C). These results not only complement the previous ones, but are consistent with the notion of a neuronal Eip63E requirement for proper CNS development.

We next tested the effects of Eip63E glial downregulation over axon features. For this purpose, the lines to be used were balanced with chromosomes that provided for an embryonic marker and so allowed for identification of homozygosity for the constructs. This was necessary, since the lack of a line expressing both the driver and the UAS-Dcr2.D constructs would push the strategy into the screening of the F2 generation. The *kruppel-GFP* balancers used before would not be appropriate, since they possess an internal Gal4-UAS system that would interfere with our experiments (Casso et al., 2000). Therefore, lines were introduced to the *Cyo_{ACT5}GFP* or *TM3_{ACT5}GFP,Ser* balancers as needed, which provided for GFP expression under the Actin A5c promoter (Reichhart and Ferrandon, 1998). GFP expression was then amplified for all experiments with the use of fluorescent immunostaining against GFP.

To induce Eip63E downregulation in glial cells, we decided to use a ;;repo-Gal4/*TM3_{ACT5}GFP,Ser* line for the crosses. To test the basal morphology of these embryos as control for future experiments, immunostaining for axons (BP-102 and HRP) was performed. None of the embryos that were screened from this line showed to be defective in any way (n=46 for GFP+ embryos, n=14 for GFP- embryos). This suggested that there was no CNS defect associated with the repo-Gal4 driver

homozygous expression.

With the goal of getting embryos that would be homozygous for at least one of the constructs (the repo driver and/or the UAS-RNAiL63) and so maximize Eip63E downregulation in glial, a crossing strategy was started. First, *;;repo-Gal4/TM3^{ACT5}GFP,Ser* and *;UAS-RNAiL63/CyO^{ACT5}GFP;* flies were crossed. This F1 generation would be heterozygous for both constructs and were used for two parallel crosses. The first one consisted in back-crossing this F1 flies to the driver line, looking for homozygosity for the driver: *;UAS-RNAiL63/+;repo-Gal4/+* crossed to *;;repo-Gal4/TM3^{ACT5}GFP,Ser* flies. About 12.5% of the progeny from this cross would be *;UAS-RNAiL63/+;repo-Gal4/repo-Gal4*, as desired, and they would constitute one half of the GFP negative embryos of the population. Axon immunostaining of this progeny revealed no defective embryos (n=73 for GFP+ embryos, n=46 for GFP- embryos). These data pointed to the possibility of Eip63E-dependent axon development not being linked to Eip63E glial function. Nevertheless, an alternative explanation would be that these embryos did not sufficiently downregulate Eip63E expression to cause a VNC defect. To test that last possibility, an alternative F1 cross was performed to obtain the F2 generation. 11% of that progeny would be homozygous for both UAS-RNAiL63 and repo-Gal4 and an additional 22% would be homozygous for one of them and heterozygous for the second one. None of the embryos stained for axons (BP102 and HRP) showed any degree of defect in the VNC (n=36). These results reinforced the idea that Eip63E is not required in glial cells for proper axon development in the *Drosophila* embryos.

Although additional experiments and controls are needed for a full understanding of Eip63E requirements, this set of experiments strongly suggests that the functions of Eip63E in neurons, and possibly neuroblasts, are key to axon development in *Drosophila*, especially to the regulation of axon outgrowth and guidance.

Figure 3.9. Specific downregulation of Eip63E leads to early embryonic axon defects.

Ventral view of whole mount embryos co-stained for BP-102 and HRP are presented oriented head to the left. **(A)** The F1 embryos from a cross between *UAS-Dcr.2.D;Act5-Gal4/Cyo* and *;;UAS-RNAiL63* flies were processed. 41.2% of them presented defects as those shown, for an equivalent penetrance of 82.4%. The presumed genotype is specified on the BP-102 image: *UAS-Dcr.2.D/+;Act5-Gal4/+;UAS-RNAiL63/+* (see text for details). **(B)** F1 embryos from a cross between *elav-Gal4;UAS-Dcr.2.D*; and *;;UAS-RNAiL63* flies were analyzed. 9% of embryos over stage 11 (n=23) were defective. Known genotype is specified on the BP-102 image: *elav-Gal4/+;UAS-Dcr.2.D/+;UAS-RNAiL63/+*. **(C)** Progeny from a cross between *elav-Gal4/+;;UAS-RNAiL63/+* flies were processed. 11.5% of stages 11-15 embryos were defective (n=26) which would be consistent with 71.9% penetrance of defects. The presumed genotype is specified on the BP-102 image: *elav-Gal4;;UAS-RNAiL63* (see text for details). These results suggest that ubiquitous and neuronal downregulation of Eip63E leads to CNS defects similar to those observed in germline Eip63E mutant lines.

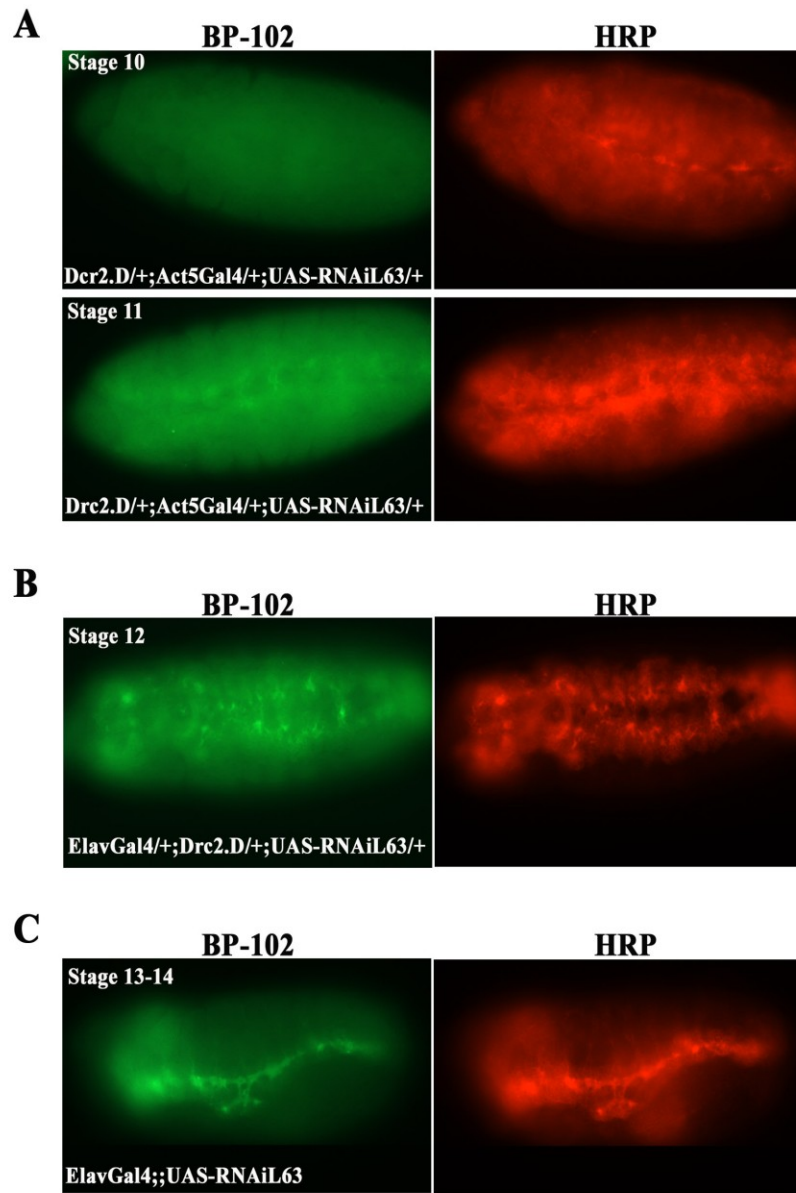


Figure 3.9. Specific downregulation of Eip63E leads to early embryonic axon defects.

Discussion

Eip63E is required for proper development of the *Drosophila* Ventral Nerve Cord.

Screening of Eip63E deficient embryos from two different and independent mutant alleles consistently show to be defective for VNC structures. The earliest to show and the most robust of CNS-related defects are those relevant to axon development. Eip63E KO embryos appear to have premature outgrowth, followed by defasciculation and misguidance of the axons of the VNC ladder-like scaffold. These axon defects appear along neuronal and, to a lesser extent, glial cell body disorganization (Fig. 3.10). It is apparent from stage 10 on that axon and neuron-related defects are present in a concerted manner in KO embryos, with comparable penetrance values. This is likely not true for glial defects. No consistent glial perturbations were noticed between the two Eip63E mutant alleles. Taken together, these observations suggest that the axon defects may be related to neuronal rather than glial-related Eip63E functions. Furthermore, we show that neuronal downregulation of Eip63E, in contrast to downregulation in glia, leads to VNC defective embryos (Fig. 3.9B and 3.9C). These results also reinforce the notion that Eip63E may function in neurons to regulate axon development in *Drosophila*.

Results associated to preliminary experiments using cultures of murine cortical neurons are also consistent with this idea (unpublished data). In these experiments, we performed the downregulation of pftk1 activity by viral infection with a dominant-negative form of the kinase. Data showed that pftk1 downregulation in neurons leads to elongated average axon length (33%) when compared with control. Additionally, the number of neurons with longer axons was much higher in this population than for those expressing GFP control.

Future experiments should be done to further reinforce our conclusions. For example, ectopic expression in neurons of wild type Eip63E in the KO background should rescue the axon defects detected in our experiments.

What are the potential roles of Eip63E in the CNS?

More than one of the CNS markers used to characterize the VNC phenotype of Eip63E deficient embryos: engrailed/invented, elav and neuroglian, point to a potential defect in biological processes that regulate or mediate cell identity specification and/or neuronal differentiation processes. Figures 3.4, 3.5 and 3.6 show that a number of the marker-positive cells in KO embryos appear to be morphologically different than those in control or WT embryos. Additionally, those cells often seem to be positioned in the wrong locations (Fig. 3.4B and 3.5B). These defects could be described in general as an abnormal cell cortex laminar organization along the VNC of mutant embryos, a process that is highly dependent on the timing and pattern of progenitors birth and divisions (Isshiki et al., 2001). Could Eip63E mutants' defects be related to temporal control on neuronal birth/differentiation mechanisms? It has been shown before for *Drosophila* that the absence of cell cycle progression or cytokinesis in neuroblasts leads to abnormal expression pattern of hunchback (Hb) (Grosskortenhaus et al., 2005), one of the progenitor temporal transcription factors that regulate first-born cell fates in *Drosophila* (Isshiki et al., 2001). Furthermore, it has been previously reported that Eip63E has a role in cell cycle regulation, since its downregulation in embryonic cell cultures by RNAi leads to low mitotic index and so larger G2 cells (Bettencourt-Dias et al., 2004). Additionally, there is evidence that Eip63E interacts with cyclinY to phosphorylate the low density lipoprotein receptor-related 6 (LRP6; 'arrow' in *Drosophila*) and mediate Wnt signaling (Davidson et al., 2009); a pathway broadly recognized as essential for regulating progenitors proliferation, cell lineage decisions and neuronal differentiation in insects and vertebrates (Zechner et al., 2003; Ille et al., 2007; Grigoryan et al., 2008).

In addition to this early potential function for Eip63E, our data strongly support a later role for this kinase in neurons to regulate axon development. It is evident that early deregulation of differentiation timing would set a precedent for axon defects, since for example, pioneer neurons and axons are needed for correct pathfinding and

fasciculation of more mature axon pathways (Sanchez-Soriano and Prokop, 2005). Nevertheless, the fact that Eip63E elav-driven downregulation on a WT background leads to axon defects (Fig. 3.9B and 3.9C) suggest the direct involvement of Eip63E in mechanisms relevant to axon guidance. Taking into account the potentially similar relevance of PFTAIRE and Cdk5 in the CNS, cues could be gathered from what is already known about Cdk5, 1) Cdk5 inactivation in cultured neurons by a dominant negative approach leads to shorter neurites when compared to controls (Nikolic et al., 1996); 2) In vivo Cdk5 deficiency causes defects in axon patterning, with abnormal trajectories and defasciculation (Ohshima et al., 2007); and 3) Cdk5 appears to promote neurite outgrowth through the regulation of the Rac-Pak1 pathway (Nikolic et al., 1998). In the case of Eip63E, its deficiency appears to have similar effects over axon guidance as those associated to Cdk5 deficiency/downregulation, although may be with opposite in-vitro outcome based on our pftk1 unpublished data mentioned before. Since Rho/Rac GTPases have such a central role in *Drosophila* axon development (reviewed in (Sanchez-Soriano et al., 2007)), it would be interesting to explore if genetic interaction studies *in vivo* would reveal these GTPases as potential Eip63E targets.

In conclusion, our work show for the first time, *in vivo*, that Eip63E has an essential role in CNS developmental processes, especially in axon development. Our data constitute a very good platform for future mechanistic studies for elucidating Eip63E targets and neuronal cellular functions.

Figure 3.10. Eip63E deficiency leads to concerted defects in axons and neurons of the *Drosophila* VNC.

The combined penetrances of markers relevant to the same VNC structures are presented as one. Axon penetrance reflects those calculated from HRP, BP-102 and 1D4 antibodies. Neurons-related values include BP-104, elav and 22C10 assessed penetrance. Penetrance was calculated as the % of defective KO embryos from the total KO screened embryos. It seems that defects associated to the Df(3L)E1 allele appeared later in development, but get to be more severe, than the same defects when linked to the Eip63E⁸¹ allele. **ND**: Not determined.

Stages	Eip63E ^{s1}	Eip63E ^{s1}	Df(3L)E1	Df(3L)E1
	Axons	neurons	Axons	neurons
8-9	10.00	ND	ND	ND
10-11	35.84	36.10	7.14	28.60
12-13	50.00	52.86	88.00	85.70
14-15	85.71	44.50	96.67	100.00

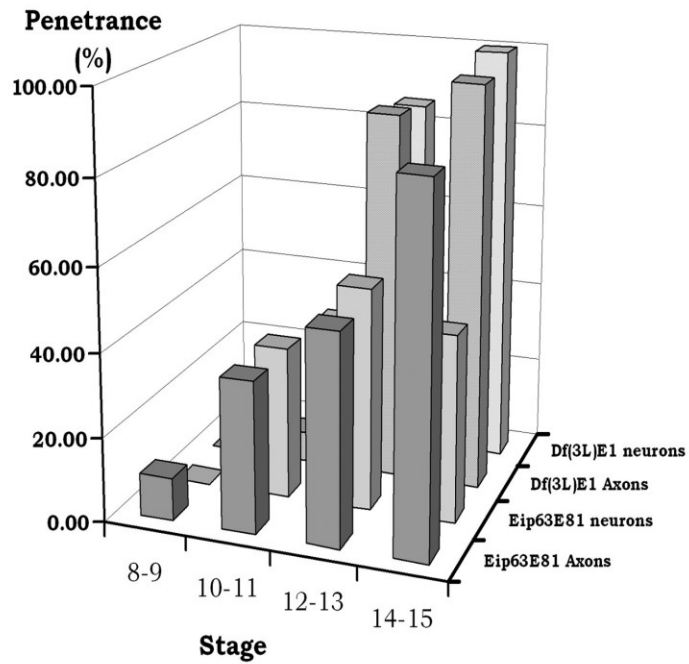


Figure 3.10. Eip63E deficiency leads to concerted defects in axons and neurons of the *Drosophila* VNC.

General Discussion.

Summary

In the present, much effort is dedicated to the development of strategies to treat and/or prevent the progression of pathological events in neurodegenerative conditions. The success of these approaches, such as the use of pharmacological inhibitors of pro-apoptotic signals or stem cells-based therapies depend upon a profound understanding of CNS function. It is already clear that Cdks are essential to neuronal development, maintenance and survival. Nevertheless, their regulation and targets in cells of the CNS are only now beginning to emerge. The work presented in this thesis is part of the endeavour to elucidate how Cdk4 and Cdk14/15 (PFTAIRE kinases) regulate death and development of the CNS.

Two different projects were then developed. First, research was concentrated on identifying molecular mechanisms that may mediate neuronal degeneration in conditions such as Parkinson's disease and Stroke, triggered by DNA damage. Specifically, work was directed to discover how Cited2, a signal up-regulated by Cdk4, induces neuronal death after DNA damage. The results associated to this research project were published on the year 2008 (Gonzalez et al., 2008).

For the second project, a *Drosophila* model was used to explore the effects of PFTAIRE deficiency during CNS development evaluating the nature and penetrance of defects regarding neurogenesis and axogenesis at the VNC. This work is part of the body of a second manuscript still under preparation.

This general discussion will then be divided accordingly into two sections relevant to the results presented in chapters two and three of this thesis. They both will include a brief description of the major findings as well as my assessment of the possible future directions these two projects may take. The latter will be based both on the results presented here as well as on preliminary observations not included in previous chapters.

The cell cycle related Cdk4–Cited2–PPAR γ pro-apoptotic pathway.

Overview of major findings.

In chapter two, critical insight was provided into signals that participate in DNA damage-induced neuronal death. Our results indicate that Cited2, a transcriptional cofactor, is upregulated in a manner dependent on the cell cycle Cdk4 signal and upstream of the mitochondrial pathway. Importantly, we also provide data that indicate a novel pro-apoptotic role for Cited2 and that Cited2 promotes death through direct activation of PPAR γ .

Relevant to the apoptotic nature of the Cited2 signal, it was shown that exogenous expression of Cited2 promotes neuronal death, and that Cited2 deficiency significantly delays death after DNA damage. Data showed as well that inhibition of Cdk4 activity by the use of a pharmacological agent as well as expression of a kinase dead Cdk4 blocks Cited2 induction, signifying that Cited2 is a downstream target of Cdk4. Several potential Cited2 targets were considered. However, only PPAR γ appeared to be involved. On that note, we showed that the DNA damage-induced upregulation of PPAR γ activity is dependent on Cited2, since Cited2 expression is sufficient to activate PPAR γ and Cited2 deficiency inhibits DNA damage-induced PPAR γ activation. The notion of PPAR γ being a critical mediator of Cited2-induced death was substantiated by the observations that PPAR γ inhibition blocks Cited2-mediated death and Cited2 deficiency inhibits death induced by PPAR γ agonist. The latter two points also suggest that both Cited2 and PPAR γ require each other to induce death, a perception supported as well by the direct interaction between these two molecules induced by DNA damage as detected by co-immunoprecipitation.

In conclusion, a model is presented (Fig. 2.9), in which Cdk4 activation by DNA damage induces the upregulation of Cited2 levels. Cited2 in turn recruits PPAR γ and by this interaction causes PPAR γ activation. The Cited2:PPAR γ complex leads to

cytochrome c release and subsequent caspase activation, followed by neuronal death. Together, our results define, for the first time, a unique Cdk4–Cited2–PPAR γ -mediated pathway that regulates DNA damage-induced neuronal death (Gonzalez et al., 2008).

Future directions...

One of the most intriguing aspects of the Cdk4-Cited2 link is the nature of the way in which Cdk4 might be inducing Cited2 expression. Presently, the only identified Cdk4 target during neuronal apoptosis is Rb which hyperphosphorylation leads to des-inhibition of E2F transcription factors (Park et al., 2000b; Rashidian et al., 2007). During our studies, we noticed at least one conserved E2F consensus site in the promoter region of Cited2. Additionally, Chromatine-Immunoprecipitation followed by microarray analysis (ChIP-chip) from Dr. Ruth S. Slack's laboratory (unpublished data) suggests that Cited2 strongly binds E2F in six other sites located in exon1 and intron1-2. It is then clear that mechanistic experiments need to be done to further elucidate the Cdk4-Cited2 link. One of the approaches could be to explore the consequences of E2F inhibition, either by deficiency (E2Fs KO neurons) or downregulation (E2Fs RNAi approach), over Cited2 induction.

In the year 2004, Park KS and collaborators reported that JNK activation appears to be an event downstream PPAR γ activity during neuronal differentiation (Park et al., 2004). In addition, we detected that upregulated levels of Cited2 after DNA damage are somehow dependent on JNK activity (Gonzalez et al., 2008). Furthermore, we and others have reported that the Cited2 promoter have several c-jun interacting sites (Lee, 2005; Gonzalez et al., 2008). Since one of the still unresolved links of the DNA damage apoptotic events is the dependence of c-jun phosphorylation levels on Cdk4 activity (Ghahremani et al., 2002), it would be interesting to explore if the Cited2:PPAR γ complex would be part of the missing link between these two signals. If proven true, this could be a feedback mechanism by which Cited2 itself would ensure

its own levels of expression by inducing c-jun phosphorylation through PPAR γ activity. A quick way to test this hypothesis would be to study the effects of Cited2 deficiency and/or PPAR γ inhibition over c-jun phosphorylation levels after DNA damage.

Another point that could be explored further is the potential involvement of the Cdk4-Cited2 pathway in alternative neuronal death paradigms, such as hypoxia. The relevance of Cdk4 activation during hypoxia-induced neuronal death has already been reported (Rashidian et al., 2005). Furthermore, the Cited2-HIF1- α link (Bhattacharya et al., 1999; Bhattacharya and Ratcliffe, 2003) is suggestive of a Cited2 role in hypoxic events during development but no evidence has been reported yet on the importance of this link in neurons. Therefore, it could be investigated if Cited2 deficiency confers any neuronal protection against hypoxia and, if so, if such a protection is dependent on Cdk4 activation. This line of research would also clarify if Cited2 is a universal target for Cdk4 in all those neuronal death paradigms in which this cell cycle Cdk has proven to be relevant.

Finally, the significance of the Cdk4-Cited2:PPAR γ pathway *in-vivo* should be assessed. It was already mentioned in chapter two that preliminary RT-PCR data on CA1-enriched samples from global ischemia mouse brains (courtesy of Iyirhiaro, G.) revealed Cited2 upregulation 6-12 hours after insult. The most effective way to test if Cited2 is indeed a critical pro-apoptotic signal in this model would be to assess if the deficiency would confer any level of neuronal protection. This approach is not possible with the germline KO mice, since they die at embryonic stages. Fortunately, a conditional CRE-Cited2 mouse strain has been developed and already been successfully used for research (Preis et al., 2006; Chen et al., 2009). In the event that Cited2 deficiency in the CNS proves not to be embryonic lethal, these mice would provide for the possibility of testing the effects of Cited2 deficiency during neurodegeneration, in cases such as after global ischemia. This line of research would ultimately challenge the relevance of the mechanistic model proposed in chapter two in more complex, *in-vivo*

circumstances.

The post-mitotic PFTAIRE in the development of the *Drosophila* VNC.

Overview of major findings.

Chapter three of this thesis describes, for the first time, the effects of the deficiency of Eip63E (the *Drosophila* PFTAIRE), a post-mitotic Cdk, in the development of CNS structures. Different embryonic developmental stages of the VNC of two independent mutant fly lines were characterized using glial, neuronal and axon markers. The anomalies detected included glial and neuronal cell bodies disorganization along with significant axon defects. The latter appeared as early as stage 11 of embryonic development. Axons in KO embryos appeared to have premature outgrowth, followed by defasciculation and misguidance of the VNC ladder-like scaffold. It was apparent that axon and neuron-related defects were present in a concerted manner in KO embryos, with similar penetrance values. Alternatively, glial perturbations were not consistent between the two Eip63E mutant alleles. These observations suggested that the axon defects may be related to neuronal rather than glial-based Eip63E functions. This idea was further supported by the fact that neuronal downregulation of Eip63E, in contrast to downregulation in glia, leads to VNC defective embryos. Our results reinforce the concept that Eip63E may function in neurons to regulate axon development in *Drosophila*.

Future directions...

Considering that our work is just the beginning of the functional characterization of this post-mitotic Cdk, much remains to be done. The results that are

accumulating suggest that PFTAIRE is not only essential for CNS development, but that it might be so at different stages and/or at different CNS-related structures. At this moment, the data provided in chapter three answers affirmatively to the question of PFTAIRE involvement in CNS development and supply leads as to when and where this Cdk is being required. Nevertheless, the answers to which isoforms, when, where and how Eip63E is functioning and regulated are still vastly incomplete. I will then discuss some of the experiments that could be done to further our knowledge in these matters using the *Drosophila* model.

Primarily, data should be expanded regarding the question as to the specificity of the effects of Eip63E deficiency over VNC structures. Rescue experiments driving the ubiquitous expression of wild type Eip63E on a KO background would reinforce the results obtained by the specific ubiquitous down-regulation experiments (RNAi experiments, Fig. 3.9). Equally, cell-specific expression of wild type Eip63E on a KO background would challenge further the notion of Eip63E being required in neurons vs glial cells to regulate axon development. Additionally, differential temporal downregulation of Eip63E in wild type embryos would add to the clarification as to when during development Eip63E starts to be required for CNS development. All these experiments would relay on the use of the Gal4-UAS system as described in chapter one.

Similarly, strategies to identify the possible targets of this Cdk that might be involved in axon development would add relevance to this body of work. A general approach would be to perform a functional screening using the well described *Drosophila* eye model. Alternatively, a more specific approach would be to use the VNC Eip63E mutant phenotype as a starting reference to assess the potential suppressor/enhancer effects that other candidate genes would have. Preliminary data from our own lab (I.P. by Dr. Qu., unpublished data) suggest that the Rho/Rac GTPases should be at the top of the candidate genes list, since the mouse Pftk1 seems to directly interact with both Cdc42 and Rac molecules *in-vitro*. Another gene that would be

interesting to test would be cyclinY and so determine if it is indeed this Eip63E partner the one that mediates PFTAIRE involvement in axon development.

Most of the data reported on PFTAIRE argues for its relation to the cell cycle machinery. It is my opinion that the argument is far from over and that PFTAIRE will prove to be as versatile as Cdk5. So far it is clear that Pftk1 can be localized to the membrane, the cytoplasm and the nucleus (just like Cdk5), which suggest that this Cdk may have targets in all these cellular compartments and mediate multiple pathways. *In-vitro* experiments using primary neurons, either from *Drosophila* or mice, deficient or not for PFTAIRE would be a useful way to explore the mechanistic details of the role of this post-mitotic Cdk in axogenesis. Relevant to this line of work, a mouse strain deficient for Pftk1 is already available at our laboratory, which opens many possibilities to the future of this project.

The work presented in this thesis has included an ambitious variety of investigative neuroscience fields. Nevertheless, the results reported here represent accurate and novel contributions to the current knowledge about the roles of Cdks in the CNS. My main goal as a scientist has been to be objective when producing and interpreting data while remaining passionate about the potential outcomes and future approaches of my projects. It has been my intention to see that goal reflected in the writing of this document.

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Gonzalez YR, Zhang Y, Behzadpoor D, Cregan S, Bamforth S, Slack RS, Park DS. (2008) CITED2 signals through peroxisome proliferator-activated receptor-gamma to regulate death of cortical neurons after DNA damage. Journal of Neuroscience, 28(21):5559-69.

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Appendix 2: Additional Publications

Sertad1 plays an essential role in developmental and pathological neuron death. Journal of Neuroscience (2010), 30(11):3973-82.

Sertad1 Plays an Essential Role in Developmental and Pathological Neuron Death

Subhas C. Biswas,^{1*} Yi Zhang,^{3*} Grace Iyirhiaro,³ Ryan T. Willett,² Yasmilde Rodriguez Gonzalez,³ Sean P. Cregan,⁴ Ruth S. Slack,³ David S. Park,^{3,5} and Lloyd A. Greene¹

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Developmental and pathological death of neurons requires activation of a defined pathway of cell cycle proteins. However, it is unclear how this pathway is regulated and whether it is relevant *in vivo*. A screen for transcripts robustly induced in cultured neurons by DNA damage identified Sertad1, a Cdk4 (cyclin-dependent kinase 4) activator. Sertad1 is also induced in neurons by nerve growth factor (NGF) deprivation and A β (β -amyloid). RNA interference-mediated downregulation of Sertad1 protects neurons in all three death models. Studies of NGF withdrawal indicate that Sertad1 is required to initiate the apoptotic cell cycle pathway since its knockdown blocks subsequent pathway events. Finally, we find that Sertad1 expression is required for developmental neuronal death in the cerebral cortex. Sertad1 thus appears to be essential for neuron death in trophic support deprivation *in vitro* and *in vivo* and in models of DNA damage and Alzheimer's disease. It may therefore be a suitable target for therapeutic intervention.

Introduction

Neuronal loss by apoptosis is a physiological process during development (Oppenheim, 1991) and a pathological hallmark of many neurodegenerative disorders such as Alzheimer's disease (AD) and of additional insults to the nervous system such as DNA damage (Park et al., 1997a). There are striking and mutually informative similarities between the molecular mechanisms that govern neuron death under these various conditions (Greene et al., 2004, 2007). However, the molecular events, particularly those that initiate death of neurons during development and disease/injury, are incompletely understood.

One major focus regarding the mechanisms of developmental and disease-associated neuron death has been the aberrant activation of cell cycle-related proteins (Becker and Bonni, 2004; Greene et al., 2004, 2007; Herrup et al., 2004). Past studies have indicated a sequential and multistep pathway that is activated by various apoptotic insults including nerve growth factor (NGF) deprivation, DNA damage, and β -amyloid (A β) exposure and

that is required for neuron death. The first described step is rapid activation of the G₁/S kinase cyclin-dependent kinase 4 (Cdk4). This in turn hyperphosphorylates members of the Rb family, leading to dissociation of complexes comprised of Rb family members and E2F transcription factors. Ultimately, these events lead to induction of proapoptotic genes such as Bim and to activation of the core apoptotic machinery (Greene et al., 2007).

An important and currently unresolved issue about the apoptotic cell cycle pathway is how Cdk4 is activated in neurons by apoptotic stimuli. Understanding this will not only further elaborate how apoptotic stimuli lead to neuron death but may also identify additional molecular targets for therapeutic intervention. In addition, it must be recognized that the majority of evidence that causally links the steps in the apoptotic cell cycle pathway has been generated by *in vitro* studies. Thus, it remains important to demonstrate that the elements that make up this pathway are relevant *in vivo*.

The protein Sertad1, also known as p34(SEI-1) or Trip-Br1, has been implicated as a regulator of Cdk4 activity. Sertad1 was first identified as an antagonist of p16 INK4a that facilitates the formation and activation of cyclin D–Cdk4 complexes (Sugimoto et al., 1999). Additional studies revealed that it directly binds and activates Cdk4 in a concentration-dependent manner (Li et al., 2004). Functions in addition to regulation of Cdk4 have been described for Sertad1 including stimulation of the transcriptional activities of E2F1 (Hsu et al., 2001) and p53 (Watanabe-Fukunaga et al., 2005). Sertad1 was also additionally reported to exhibit antiapoptotic activity by stabilizing X-linked inhibitor of apoptosis protein (XIAP) in cancer cells (Hong et al., 2009).

In a screen for genes regulated in neurons after DNA damage, we identified Sertad1 transcripts as being robustly induced. Ac-

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*S.C.B. and Y.Z. contributed equally to this work.

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cordingly, we examined the potential role of Sertad1 in neuron death induced by apoptotic stimuli relevant both to normal development and to neurodegeneration. We find that Sertad1 is required for neuronal apoptosis both *in vitro* and *in vivo*. Furthermore, our findings indicate that Sertad1 is essential for initiating the Cdk4-dependent cascade of cell cycle events in neuronal cells that follow from trophic factor deprivation.

Materials and Methods

Materials. Platinum TaqDNA polymerase, V5 antibody, green fluorescent protein (GFP) antibody, and Lipofectamine 2000 were from Invitrogen; anti-human NGF antiserum and anti- β -actin antibody were from Sigma-Aldrich; anti-Sertad1 antibody and Bim antibody were from Abcam; anti-ERK1, phospho-Rb, and C-Myb antibodies were from Santa Cruz Biotechnology; ZsGreen antibody was from Clontech; and the *In Situ* Cell Death Detection kit, TMR red was from Roche Applied Science. pSIREN vector was from BD Biosciences. Human recombinant NGF was a kind gift from Genentech. Camptothecin was obtained from Sigma-Aldrich. Bis-isopropylthiomethyl-K-252a (CEP11004) was obtained from Cephalon. E2F-1 and control short interfering RNAs (siRNAs) and E2F-1 antibody were purchased from Santa Cruz Biotechnology. p53 mice were genotyped according to published protocols (Aleyasin et al., 2004).

Cell culture. PC12 cells were cultured and neuronally differentiated as previously described (Greene and Tischler, 1976). For NGF deprivation, after a week of NGF treatment, the cultures were washed with NGF-free medium twice, and anti-NGF antibody (1:100) was added. Control cells were washed with serum-free medium and maintained in RPMI 1640 medium supplied with NGF without serum. Neonatal rat superior cervical ganglion sympathetic (SCG) neurons were cultured as previously described (Park et al., 1998). HEK293 cells were cultured in DMEM with 10% fetal bovine serum. Embryonic rat and mouse cortical neurons were cultured as previously described (Park et al., 1998).

Microarray. Total RNA was extracted from cortical neuron cultures using Trizol reagent according to the manufacturer's instructions (Invitrogen). RNA was sent to the Ottawa Genomics Innovation Centre Microarray Facility for processing and expression analysis using the Affymetrix Mouse 430 array (Affymetrix). Probe signals were scaled and normalized according to standard facility procedures.

Semiquantitative reverse transcriptase-PCR. Total RNA was extracted using TriPure isolation reagent (Roche Applied Science). Fifty nanograms of total RNA were used for cDNA synthesis and gene amplification reactions using SuperScript One-Step RT-PCR kit (Invitrogen). cDNA synthesis was performed at 48°C for 45 min, followed by a 2 min initial denaturation step at 94°C. This was followed by 30 cycles (Sertad1) or 25 cycles (S12) at 94°C for 30 s, melting temperature (T_m) 60°C for 30 s, and 72°C for 1 min. Targeting primers were as follows: 5'-CGCAAGCGGGAGGAGGAGAC-3' and 5'-AGGGGCTGGGGCTGGATGG-3' for Sertad1, 5'-GGAAGGCATAGCTGCTGG-3' and 5'-CCTCGATGACATCCTGG-3' for S12. Transcript levels were normalized against S12 signals, and results were reported as times fold increase in reference to untreated control values. Data are presented as mean \pm SEM of three independent experiments.

Reverse transcription-quantitative PCR. Each sample of total RNA was isolated from cultured neurons by using TRI reagent (Molecular Research Center). cDNA was transcribed from total RNA with Superscript RT II (Invitrogen). The primers used for PCR amplification of rat Sertad1 were 5'-GCCTCTGGAAGATCTCAGTC-3' and 5'-CATTCTCAGGGACAGGTTTGA-3'. The primers for α -tubulin were 5'-ATGAGGCATCTATGACATC-3' and 5'-TCCACAACTGGATGTAC-3'. Equal amounts of cDNA template were used for each PCR analysis of Sertad1 or α -tubulin. Quantitative PCR was performed using a Cepheid SmartCycler following the manufacturer's specifications. α -Tubulin was used for Sertad1 transcript normalization. cDNA was added to a 25 μ l volume reaction mix containing OmniMix HS master mix (Cepheid) and SYBR Green I (Invitrogen) together with appropriate

Table 1. Sertad1 upregulation in gene chip analysis

	Untreated	Camptothecin ^a	Camptothecin/untreated signal ratio	p value
Sertad1	135.8	6264.2	46.1	0.000167
Noxa	542.8	2141.6	3.95	0.000273
Puma	1124.2	3640.7	3.24	0.00013
Tubb3 ^b	22,718.3	21,207.1	0.93	NS ^c

Microarray analysis of RNA extracted from cortical neurons after camptothecin (10 μ M) treatment for 8 h or with vehicle (0.1% DMSO). Fold change represents the ratio of signal in camptothecin-treated neurons relative to vehicle-treated neurons for each probe set.

^aCortical neurons were treated with camptothecin for 8 h.

^bTubb3 is β -tubulin.

^cNot significant.

primers at 0.2 μ M each. Analyses of growth curves of real-time fluorescence and of melting curves were performed as described previously (Troy et al., 2000).

Western immunoblotting. Neuronal PC12 cells were lysed and protein was analyzed by Western immunoblotting as described previously (Biswas and Greene, 2002). For mouse cortical neurons, Sertad1 was detected using a chicken IgY antibody against Sertad1 (1:1000; Genway). Goat anti-chicken HRP (1:3000) was used as secondary antibody.

Plasmids. Rat Sertad1 was generated by reverse transcription (RT)-PCR of PC12 cDNA. The primers for the amplification were 5'-AGGATGCTGAGCAAAGGTCT-3' and 5'-GCGCCAGGTCTGGTGGCC-3'. The PCR product was gel purified and cloned into pCDNA3.1 vector (Invitrogen), and then verified by sequencing. Sertad1 was also subcloned into pCMS-EGFP vector (Clontech) by using primers 5'-GATCTCGA-GACCATGCTGAGCAAAGGTCTG-3' and 5'-CTAGTCGACCTAGC-GCCAGGTCTGGTGG-3'.

Preparation of short hairpin RNA. Sertad1 short hairpin RNAs (shRNAs) were prepared in the pSIREN vector by using BD Knockout RNAi Systems according to the manufacturer's instructions (BD Biosciences) based on the following sequences: 5'-CCGTGGCTTCTAGCTCTCT-3' (#2), 5'-GCTCCACCACAGCCTTCGG-3' (#3), 5'-CCAGACCTCCGACACCTGG-3' (#4), 5'-GATCTCAGTCATATTGAGG-3' (#5). pSIREN-shRNA-RAND-ZsGreen was as described previously (Sproul et al., 2009). For *in utero* electroporation (see below), GFP constructs of Sertad1 shRNA and control shRNA were prepared by subcloning the shRNA expression cassette from pSIREN vector into pCMS-EGFP backbone sequence. The (CMV promoter)-MCS sequence in pCMS-EGFP was substituted with the (U6 promoter-shRNA) sequence from pSIREN-RetroQ-zsGreen by subcloning with BglII and EcoRI restriction enzymes. The control shRNA is an inactive mutant of the primary siRNA knockdown construct for GATA2: 5'-GCACCTGATGTCTTCTTCAACC-3'.

Transfections. DNA was prepared with Plasmid Maxi kits (QIAGEN). Neuronal PC12 cells were cotransfected with 0.5 μ g of plasmid pCDNA-V5, pCDNA-Sertad1-V5, pCMS-EGFP, pCMS-Sertad1-EGFP, pSIREN-shRNA-Sertad1-ZsGreen (#2, #3, #4, or #5), pSIREN-shRNA-Rand-ZsGreen, or pSIREN-shRNA-Luc-ZsGreen in 500 μ l of serum-free medium per well in 24-well dishes using Lipofectamine 2000. Six hours later, medium with Lipofectamine 2000 was replaced with fresh complete medium. HEK293 cells were transfected as previously described (Xu et al., 2001). E2F-1 siRNA were transfected as previously described (Zhang et al., 2006).

Sertad1 shRNAs and viruses. Mouse Sertad1-specific shRNA oligos (Applied Biosystems) were cloned into pSilencer3.0-H1 vector (Applied Biosystems). The shRNA fragments containing the H1 promoter were subcloned into the pAdTrack vector. shRNA adenoviruses and DN-c-Jun adenoviruses were constructed as previously described (He et al., 1998).

Preparation of amyloid. Lyophilized, HPLC-purified A β ₁₋₄₂ was purchased from American Peptide, and dodecamer A β ₁₋₄₂ was prepared as described previously (Barghorn et al., 2005). Briefly, A β ₁₋₄₂ was reconstituted in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to 1 mM, and HFIP was removed by evaporation in a Speed Vac, and then resuspended to 5 mM in anhydrous DMSO. This stock was then diluted with PBS to a final concentration of 400 μ M. SDS was added to a final concentration of

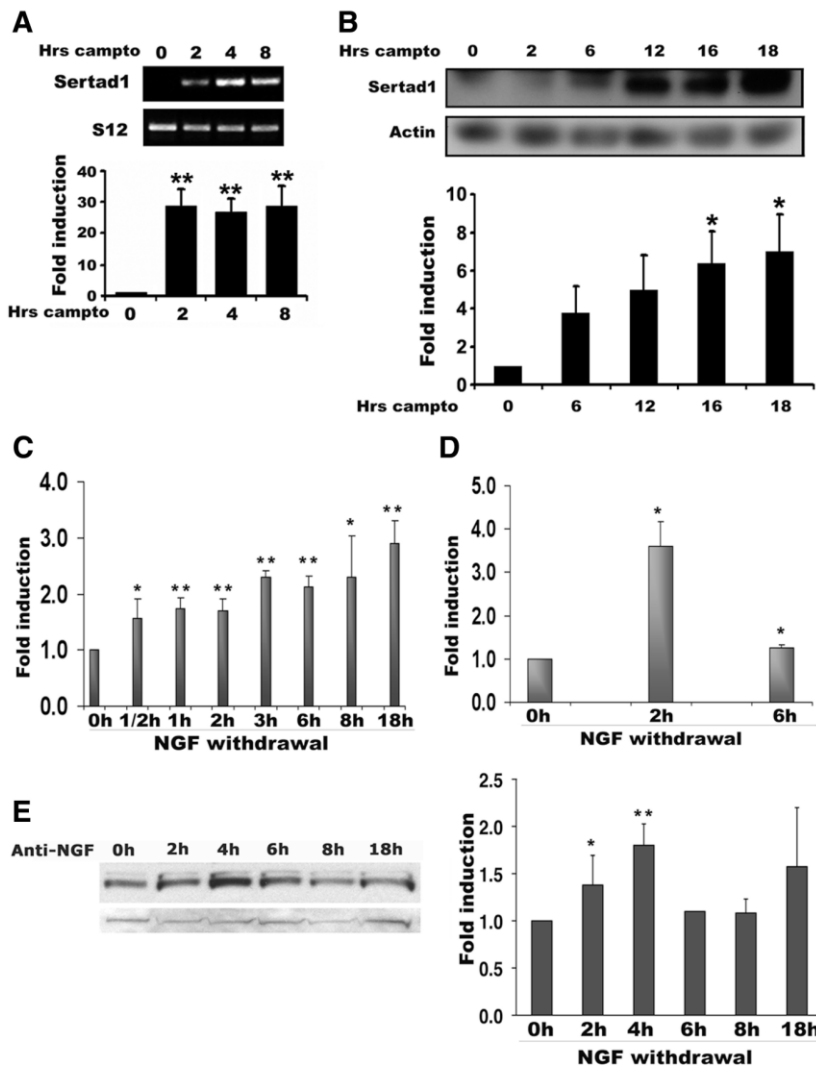


Figure 1. Sertad1 mRNA and protein levels are upregulated after DNA damage and NGF deprivation. **A, B.** Total mRNA or whole-cell lysates were extracted from cortical neurons treated with camptothecin (10 μ M) for the indicated times and subjected to RT-PCR (**A**, top) or Western blot (**B**, top), respectively. Densitometry of all signals was performed using NIH ImageJ software and Sertad1 signals were normalized to S12 (**A**, bottom) or actin (**B**, bottom). Data are presented as fold increase relative to untreated control. Each bar represents the mean \pm SEM from four independent experiments (one-way ANOVA/Tukey's least significant difference test; significance comparisons with untreated controls, * $p < 0.05$, ** $p < 0.01$). **C, D.** Sertad1 mRNA levels are increased in response to NGF deprivation. Neuronal PC12 cells (**C**) and sympathetic neurons (**D**) were subjected to NGF deprivation for the indicated times, and total RNA was isolated, reverse transcribed, and amplified by PCR using specific primers for Sertad1 and α -tubulin. Data represent means \pm SEM of three experiments (neuronal PC12 cells) or two experiments (SCG neurons), each performed with three replicate cultures. The asterisks denote statistically significant differences from 0 h control: * $p < 0.05$; ** $p < 0.001$. **E.** NGF withdrawal elevates Sertad1 protein levels. Neuronal PC12 cells were subjected to Western immunoblotting using enhanced chemiluminescence for the detection of Sertad1 and ERK 1 (loading control). The right panel shows quantification of Sertad1 signals, normalized against ERK1 expression. Data represent means \pm SEM of five experiments (except 6 h which is a single experiment). The asterisks denote statistically significant differences from 0 h control: * $p < 0.05$; ** $p < 0.001$.

0.2% and the resulting solution was incubated at 37°C for 18–24 h. The preparation was diluted again with PBS to a final concentration of 100 μ M and incubated at 37°C for 18–24 h.

In utero electroporation. Sprague Dawley rats (Charles River Laboratories) were housed, cared for, and electroporated under the guidelines established by Columbia University Medical Center Institutional Animal Care and Use Committee. Timed pregnant rats [embryonic day 16 (E16)] were anesthetized with ketamine/xylazine (100/10 mixture; 0.1

mg/g body weight, i.p.). The uterine horns were exposed, and the left lateral ventricles of embryos were injected with DNA constructs of a control shRNA or Sertad1 shRNA (1–5 μ g/ μ l) and Fast Green (2 mg/ml; Sigma-Aldrich) by using pulled glass capillaries (Sutter Instrument). Electroporation was accomplished with a BTX electro square electroporator, model ECM830 (BTX). The head of each embryo was held between tweezer-type circular electrodes (Harvard Apparatus) across the uterus wall, and five electrical pulses (amplitude, 50 V; duration, 50 ms; intervals, 100 ms) were delivered. Brains from postnatal pups of 5 d of age were fixed in 4% paraformaldehyde (PFA) by cardiac perfusion.

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling assay and immunohistochemistry. Rat pups were anesthetized and perfused transcardially with 4% PFA in PBS, pH 7.4. After perfusion, brains were dissected out from the skull and postfixed overnight in fresh fixative. Then brains were washed with PBS, pH 7.4, and cryoprotected in 30% sucrose phosphate buffer. They were frozen with OCT and dry ice and sectioned (20 μ m thick) in the coronal plane with a cryostat. To visualize nuclei with DNA cleavage, residues of fluorescein-labeled nucleotides were catalytically added to DNA fragments by terminal deoxynucleotidyl transferase (TdT). Briefly, sections were fixed in fresh 4% PFA/PBS at room temperature for 20 min, washed in PBS three times for 5 min, permeabilized with proteinase K for 5 min on ice, and incubated with nucleotide mix and TdT (*In Situ* Cell Death Detection kit, TMR red) at 37°C for 1 h. Apoptotic cells exhibit strong, nuclear red fluorescence. To visualize GFP-positive cells, sections were then immunostained with rabbit anti-GFP antibody (1:1000; Invitrogen) in 3% nonimmune goat serum overnight at 4°C, followed by secondary labeling with goat anti-rabbit antibody (1:4000; Alexa Fluor 488; Invitrogen) for 1 h.

Survival assays. Neuronal PC12 cells, sympathetic neurons, or cortical neurons were transfected with either pCMS-Sertad1-EGFP, pCMS-EGFP, pSIREN-Sertad1-shRNA, pSIREN-Luc-shRNA, or a Random pSIREN-ZsGreen, and then 48 h later deprived of NGF (in case of neuronal PC12 cells and sympathetic neurons) or treated with 1.25 μ M dodecamer A β (in case of cortical neurons). The numbers of surviving transfected (green) cells per well were assessed just after treatment and at 24 and 48 h after NGF deprivation or A β exposure as described previously (Biswas et al., 2007). Data represent means \pm SEM of three experiments performed in triplicate.

Immunostaining. Neuronal PC12 cells were transfected as described above with appropriate constructs of shRNA. Forty-eight hours later, cells were subjected to NGF withdrawal for 18 h and then immunostained as described by Angelastro et al. (2003). Briefly, PC12 cells were fixed with 4% paraformaldehyde for 10 min. After three washes with PBS, cells were blocked in 3% nonimmune goat serum for 2 h. The cultures were immunolabeled with rabbit anti-Bim (1:1000; Abcam) antibody, rabbit C-Myb antibody (1:500; Santa Cruz Biotechnology), or rabbit p-Rb antibody (1:100; Santa

Cruz Biotechnology) in 3% nonimmune goat serum overnight at 4°C, followed by secondary labeling with goat anti-rabbit antibody (1:1000; Alexa Fluor 568; Invitrogen) for 1 h. For Sertad1 knockdown experiment, the cultures were immunostained with anti-Sertad1 antibody (1:200; Genway) followed by secondary labeling with goat anti-chicken antibody (1:500; Invitrogen) for 1 h.

Results

Sertad1 is rapidly induced in response to DNA damage and NGF deprivation

In an initial microarray screen for genes induced in cortical neurons by treatment with the DNA-damaging agent camptothecin (8 h), we identified Sertad1 as a candidate regulated gene. Induction was 46-fold over controls (Table 1). In the same screen, we detected increases in the Puma and Noxa genes as expected and previously reported (Aleyasin et al., 2004; Cregan et al., 2004). In contrast, β -tubulin did not change (Table 1). To verify the array data, we performed a semiquantitative reverse transcriptase-PCR assay. Consistent with the array data, the RT-PCR results demonstrated a robust (~27-fold) increase of Sertad1 message as early as 2 h after camptothecin exposure. This increase persisted even at 8 h after camptothecin treatment (Fig. 1A). A similar magnitude of increase was obtained by quantitative PCR (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Next, we asked whether the DNA damage-induced elevation in Sertad1 message is associated with an increase in protein level. As revealed by Western blotting (Fig. 1B), Sertad1 protein expression was significantly induced by 6 h (~3.2-fold) of camptothecin exposure and gradually increased until 18 h (~7.2-fold). Under the conditions of our experiments, neuronal death first becomes apparent by 8–10 h of camptothecin exposure and 50% of neurons die within 16–20 h. Thus, Sertad1 induction is observed early and before overt signs of death.

To identify upstream signals that mediate upregulation of Sertad1 mRNA in response to DNA damage, we tested three potential candidates: p53, E2F1, and c-Jun N-terminal kinases (JNKs). As shown in Figure 2A, germ line deficiency of p53 does not affect camptothecin-induced upregulation of Sertad1 message in cultured cortical neurons. In contrast, siRNA-mediated knockdown of E2F-1 blocks Sertad1 mRNA upregulation by ~50% (Fig. 2B). Finally, neither JNK inhibitor CEP11004 nor dominant-negative c-Jun expression affect camptothecin-induced Sertad1 upregulation (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Together, these findings indicate that E2F1, but not p53 or the JNK pathway at least partially regulates Sertad1 mRNA expression after DNA damage.

Because neuronal death caused by DNA damage shares with death evoked by trophic factor deprivation a reliance on cell cycle proteins (Park et al., 1996, 1997a,b; Liu and Greene, 2001; Zhang et al., 2006; Gonzalez et al., 2008), we also investigated whether Sertad1 expression is regulated in response to NGF withdrawal. For this purpose, we used neuronally differentiated PC12 cells and primary cultures of SCG neurons. Both cell types undergo

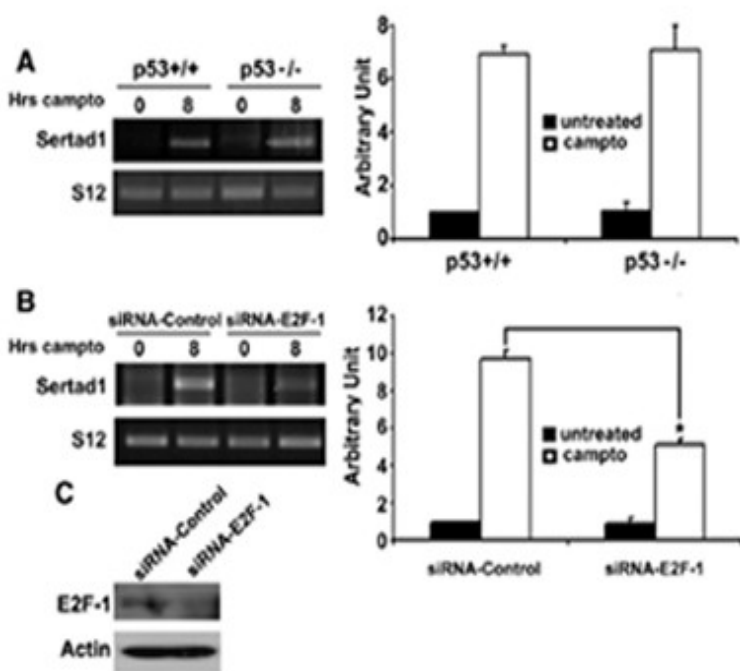


Figure 2. A, p53 deficiency does not inhibit camptothecin-induced upregulation of Sertad1. Cortical neurons from p53^{+/+} and p53^{-/-} mice were treated with and without camptothecin for 8 h. Left, Sertad1 levels were then analyzed by RT-PCR. S12 was shown as a control for equal input. Right, Densitometric analysis of Sertad1 signals normalized against S12 signals. B, E2F-1 downregulation partially blocks camptothecin-induced upregulation of Sertad1. Cortical neurons were transfected with E2F-1 and control siRNA and were treated with and without camptothecin for 8 h. Left, Sertad1 levels were then analyzed by RT-PCR. S12 was shown as a control for equal input. Right, Densitometric analysis of Sertad1 signals normalized against S12 signals. Significance comparisons with untreated controls: * $p < 0.05$. Error bars indicate SEM. C, Western blot analyses showing downregulation of E2F-1 in cells treated with E2F-1 siRNA compared with control siRNA. Actin is provided as loading control.

apoptosis (evident starting at ~18 h) in response to NGF deprivation (Rukenstein et al., 1991; Xu et al., 2001). Similar to DNA damage, a time course performed by quantitative PCR revealed that Sertad1 mRNA levels were elevated in neuronal PC12 cells as early as one-half an hour after NGF deprivation and were consistently and significantly increased in SCG neurons after 2 h of such treatment (Fig. 1C,D). Sertad1 protein expression was also elevated by 2–4 h in response to NGF deprivation, and this change was similar in magnitude (by approximately twofold) to that of mRNA (Fig. 1E). Thus, both Sertad1 mRNA and protein expression are induced in response to DNA damage and trophic factor withdrawal, although this response is more modest in the case of NGF deprivation.

Sertad1 overexpression is not sufficient to trigger neuron death but enhances death in response to NGF deprivation

Next, we examined whether elevated expression of Sertad1 is sufficient in itself to trigger neuron death in the presence of trophic support. Expression of Sertad1 alone did not induce death of neuronal PC12 cells (Fig. 3A), cortical neurons (supplemental Fig. S3, available at www.jneurosci.org as supplemental material), or cerebellar granule neurons (data not shown). However, overexpression of Sertad1 significantly enhanced the level of neuronal cell death that occurs in response to NGF deprivation (Fig. 3B).

Sertad1 is required for neuron death induced by NGF deprivation and DNA damage

Because of the observed upregulation of Sertad1 in multiple neuronal death paradigms and the sensitization to death with Sertad1

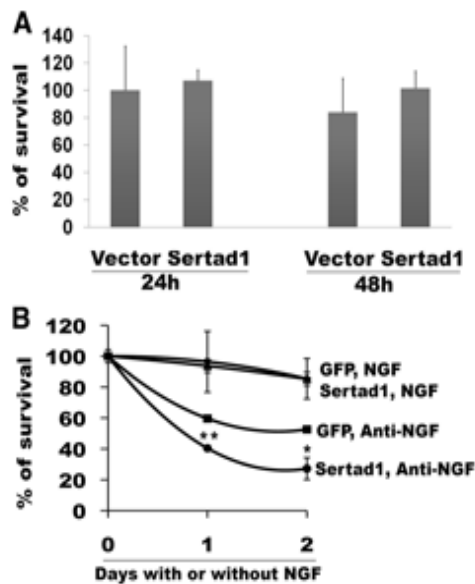


Figure 3. Overexpression of Sertad1 is not sufficient to cause death but enhances death in response to NGF withdrawal. **A**, Neuronal PC12 cells were transfected with pCMS-EGFP or pCMS-Sertad1-EGFP. At 24 and 48 h after transfection, surviving transfected (green) cells were counted. Data represent mean of a representative experiment performed with three replicate cultures per point. Similar results were obtained in an independent experiment. **B**, Neuronal PC12 cells were transfected with pCMS-EGFP or pCMS-Sertad1-EGFP. After 48 h, they were maintained with or deprived of NGF and surviving transfected cells were counted at 24 and 48 h. Data represent mean \pm SEM of a representative experiment performed with three replicate cultures per point. Similar results were obtained in an independent experiment. The asterisks denote statistically significant differences from control (GFP) in absence of NGF: * $p < 0.05$; ** $p < 0.01$.

overexpression, we next examined whether neuron death in our systems requires Sertad1. We first studied the role of Sertad1 in NGF deprivation. To achieve this, we prepared several shRNAs specifically targeted to rat Sertad1 and identified three that substantially reduced expression of the overexpressed protein (Fig. 4A). Each of the effective shRNAs significantly protected neuronal PC12 cells from death induced by NGF withdrawal (Fig. 4B,C). Moreover, most of the Sertad1 shRNA-expressing cells preserved overall neuron morphology even after 2 d of NGF deprivation (Fig. 4B). This is reminiscent of the preservation of neuronal morphology after NGF deprivation that is achieved with small molecule Cdk inhibitors (Park et al., 1996, 1997b) or Cdk4 shRNAs (Biswas et al., 2005). Similar experiments with SCG neurons also showed that downregulation of Sertad1 by two independent shRNAs significantly blocks death and preserves overall neuron morphology after NGF deprivation (Fig. 4D) (data not shown).

Similarly, we used a shRNA knockdown strategy to assess whether Sertad1 also plays a required role in neuronal death after DNA damage. In this case, we used adenoviruses to deliver shRNAs to cultured mouse cortical neurons. We screened two potential shRNA sequences directed against mouse Sertad1 to knockdown endogenous Sertad1. As shown in Figure 5, A and B, infection with both Sertad1 shRNA viruses, but not a control virus, significantly reduced the Sertad1 immunostaining signal (54 and 33%, respectively) after camptothecin treatment. Survival assays in response to camptothecin treatment were performed using these two Sertad1 shRNAs along with a control shRNA virus. As shown in Figure 5C, expression of the two dif-

ferent Sertad1 shRNAs resulted in significant protection from camptothecin at times up to 48 h. This finding is consistent with our observations in the NGF deprivation model and indicates that Sertad1 plays a required role in the neuronal death process.

Sertad1 is induced and required for neuron death in a model of Alzheimer's disease

We additionally examined the importance of Sertad1 in a culture model directly relevant to neurodegeneration. A substantial body of evidence has implicated cell cycle molecules in neuron death associated with Alzheimer's disease (Herrup et al., 2004; Greene et al., 2007; Zhu et al., 2007; Copani et al., 2008). Moreover, there are striking similarities between the molecular mechanisms of neuron death induced by NGF deprivation, DNA damage, and A β exposure (Greene et al., 2007), and recent findings support a mechanism in which neuron death triggered by NGF deprivation is mediated by elevated production and release of A β (Matrone et al., 2008a,b). We therefore examined whether Sertad1 is induced and is necessary for death of cultured cortical neurons induced by exposure to aggregated A β . Sertad1 transcripts were increased by nearly fourfold within 3 h of A β treatment (Fig. 6A). Downregulation of Sertad1 by shRNA significantly protected the cortical neurons from A β induced death (Fig. 6B). Moreover, Sertad1 shRNA-expressing cells showed preservation of overall neuron morphology in presence of aggregated A β (Fig. 6C). Together, these findings indicate that Sertad1 is induced and plays a required role in *in vitro* paradigms of both development and pathological neuron death.

Sertad1 is required for developmental death of cortical neurons *in vivo*

The above evidence indicates the importance of Sertad1 in *in vitro* models of neuronal death. However, its involvement *in vivo* is unknown. Critically, this uncertainty extends generally to whether the cell cycle pathways may be of importance in developmental death *in vivo*. Because limiting levels of target-derived trophic support appear to regulate neuron death *in vivo*, our *in vitro* data led us to test whether Sertad1 is essential for developmental neuron death in the early postnatal cerebral cortex. We used *in utero* electroporation to deliver DNA encoding Sertad1 shRNA and GFP or a control DNA expressing random shRNA and GFP into the left lateral ventricles of E16 rat embryonic brains. DNA delivered in this manner is taken up by ventricular zone progenitor cells that subsequently differentiate and migrate toward the pial surface. Because maximum cortical neuron death occurs during the first week of development (Spreafico et al., 1995), we killed the DNA-electroporated rat pups on postnatal day 5 (P5) and then analyzed their fixed cortices by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assays coupled with immunohistochemistry for GFP to assess the proportions of transfected neurons undergoing death. Past studies have established that TUNEL staining faithfully reports apoptotic neurons in developing brain as judged by nuclear morphology and caspase 3 activation (Sophou et al., 2006). Transfected, morphologically identified neurons were mostly present in cortical layers II, III, and IV of the electroporated side of the brain (Fig. 7A; supplemental Fig. S4, available at www.jneurosci.org as supplemental material). Counts of TUNEL-positive cells indicated that ~14 per 1000 transfected neurons ($1.4 \pm 0.13\%$; $N = 3$) were apoptotic in these layers of the cerebral cortex for those animals electroporated with DNA expressing control shRNA (Fig. 7A,B). This is consistent with a past study that reported ~15 apoptotic (TUNEL-positive)

nuclei per 1000 neurons in the same cortical layers of normal P5 brains (Spreafico et al., 1995). In contrast, we found an average of only 6 apoptotic nuclei per 1000 Sertad1 shRNA-expressing neurons ($0.6 \pm 0.03\%$; $N = 3$) in the same three layers of the cerebral cortex (Fig. 7*B,C*). There were no evident effects of the Sertad1 shRNA on the migration or morphology of the transfected neurons. Together, our findings indicate that Sertad1 plays an essential role in neuron death during cortical development *in vivo*.

Sertad1 is required for Rb phosphorylation and consequent myb and Bim induction in response to NGF deprivation

Our past studies (Park et al., 1997b; Liu and Greene, 2001; Liu et al., 2004, 2005; Biswas et al., 2005, 2007; Greene et al., 2007) have established a sequential pathway for neuron death caused by NGF deprivation and A β exposure in which (1) activated Cdk4 phosphorylates members of Rb family of transcription-regulating proteins; (2) such phosphorylation leads to dissociation of repressor complexes containing Rb family members, E2F transcription factor proteins, and chromatin modifiers; (3) there is consequent depression and elevated expression of E2F-responsive genes including the transcription factors B- and C-Myb; and (4) the induced mybs activate transcription of the gene encoding the proapoptotic BH3-only protein Bim. If Sertad1 acts as anticipated at the proximal end of this pathway by binding and activating Cdk4, we would predict that the downstream responses such as Rb phosphorylation, Myb induction, and Bim induction should be blocked by Sertad1 downregulation. We first assessed the effects of shRNA-mediated Sertad1 knockdown on Rb phosphorylation after NGF deprivation. As shown in Figure 8*A* and consistent with our past findings, NGF deprivation (18 h) raised the proportion of cells with a high level of phospho-Rb immunostaining from ~8 to 65%. Transfection with Sertad1 shRNA reduced this to an average of 12.5%. Similar findings were achieved for expression of C-Myb and its downstream target Bim (Fig. 8*B,C*). In each case, shRNAs targeted to Sertad1 strongly suppressed the large increases in the proportions of transfected cells that showed high staining for C-Myb and Bim after NGF deprivation. Together, these results support the conclusion that Sertad1 is required for the activation of a neuronal apoptotic pathway in response to NGF deprivation that depends on Cdk-mediated Rb family phosphorylation and consequent induction of the E2F-responsive gene Myb and its proapoptotic target Bim.

Discussion

Cell cycle proteins have been implicated as required elements in the mechanisms of postmitotic neuron death, both during normal development and in response to injury, stroke, and a range of neurodegenerative diseases (Smith et al., 2004; Neve and McPhie, 2006; Greene et al., 2007; Nunomura et al., 2007; Rashidian et al.,

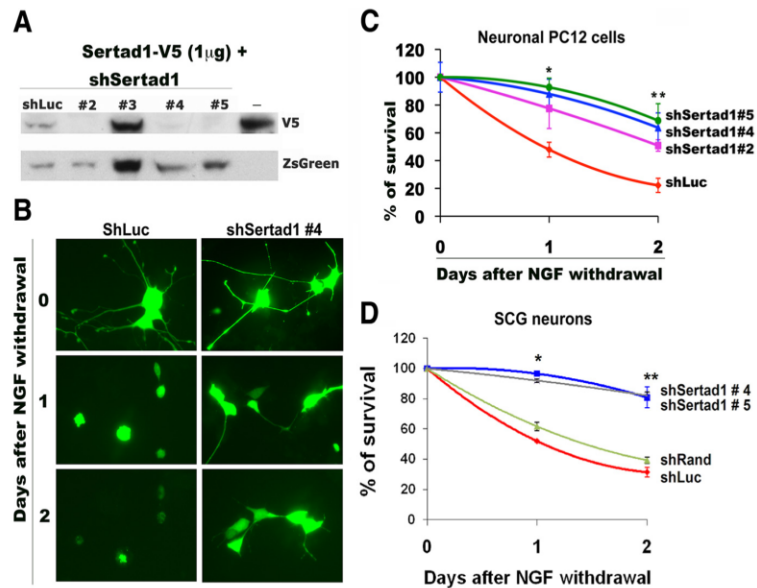


Figure 4. shRNAs targeted to Sertad1 specifically block gene expression and neuron death in response to NGF deprivation. *A*, Sertad1 shRNAs suppress Sertad1 expression. HEK293 cells were cotransfected with pCDNA-Sertad1-V5 along with pSIREN-Luc (Luciferase)-ZsGreen shRNA or pSIREN-Sertad1-ZsGreen shRNAs. After 48 h, cells were lysed and subjected to Western immunoblotting using enhanced chemiluminescence for the detection of V5 and ZsGreen. *B*, Sertad1 knockdown prevents neuronal degeneration and death after NGF deprivation. Neuronal PC12 cells were transfected with pSIREN-Random-ZsGreen shRNA (control shRNA) or pSIREN-Sertad1#5-ZsGreen shRNA (Sertad1 shRNA), maintained for 48 h, and then washed twice with RMPI 1640 medium and maintained with or without NGF. Representative images of transfected cells were taken in each case before and after 24 and 48 h of treatment. *C, D*, Sertad1 shRNAs provide protection against death evoked by NGF deprivation of neuronal PC12 cells or SCG neurons. Neuronal cells were transfected with pSIREN-Luc-ZsGreen shRNA (shLuc), pSIREN-Sertad1-ZsGreen shRNA (shRand), or pSIREN-Sertad1-ZsGreen shRNAs (shSertad1#2, shSertad1#4, and shSertad1#5), maintained for 48 h, and then deprived of NGF as indicated. Numbers of surviving transfected (green) cells were counted just before NGF deprivation and after 24 and 48 h of NGF deprivation. Data are from one of three independent experiments, each with comparable results and are shown as means \pm SEM, performed in triplicates. The asterisks denote statistically significant differences from control (shLuc or shRand): * $p < 0.05$; ** $p < 0.001$.

2007). However, the proximal events that initiate the involvement of cell cycle proteins, particularly the activation of Cdk4, have been unclear. Here, we performed experiments that now implicate the cell cycle regulatory protein Sertad1 in three different paradigms of neuron death: NGF deprivation, DNA damage, and A β exposure. We find that Sertad1 expression is rapidly elevated in cultured neurons in all three apoptotic paradigms and that downregulation of Sertad1 by shRNA is protective in each case. We further show that downregulation of Sertad1 inhibits the increase in Rb phosphorylation and consequent induction of Myb and Bim that mediate neuron death caused by NGF deprivation. These findings thus place Sertad1 proximal to the other defined events in the Cdk4-dependent apoptotic cell cycle pathway that is triggered by loss of trophic support.

The steps that comprise the apoptotic cell cycle pathway described here have been determined mostly by *in vitro* experimentation. We used *in vivo* electroporation to extend our studies to the developing cortex and found that, in this instance also, Sertad1 plays an essential role in normally occurring neuronal death. Such findings thus implicate not only Sertad1 but also events downstream of it in developmental neuron death.

Sertad1 has been found to directly bind and activate Cdk4 as well as to render active Cdk4–cyclinD1 complexes resistant to inhibition by p16(INK4a) (Sugimoto et al., 1999). Such actions are entirely consistent with our findings that knockdown of Sertad1 protects neurons in our three death paradigms, each of

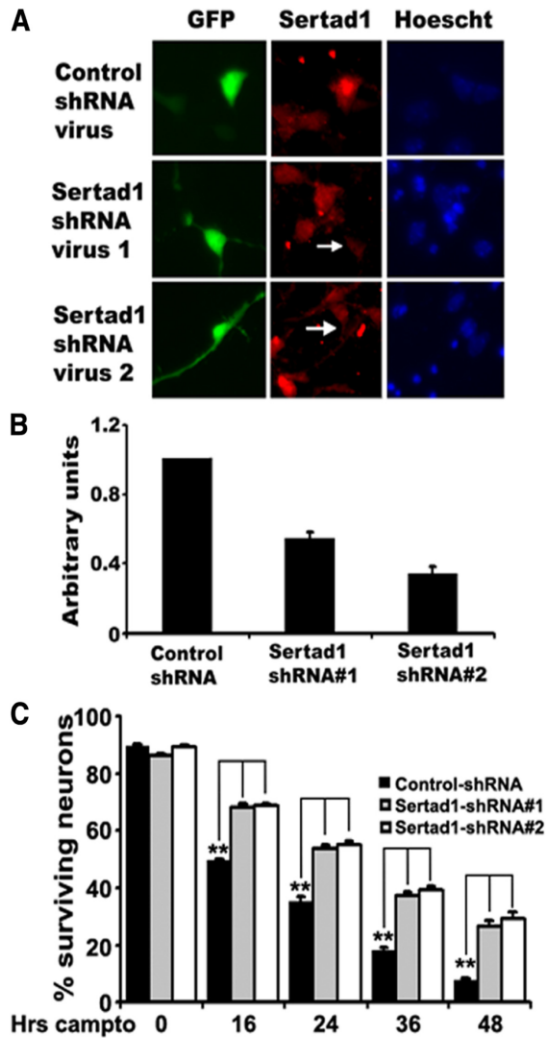


Figure 5. Sertad1 downregulation by adenoviral shRNAs protects cortical neurons from DNA damage-evoked cell death. *A, B*, shRNA-mediated knockdown of Sertad1 in cortical neurons. Cortical neurons were infected with two adenoviral shRNAs targeting mouse Sertad1 gene or a control virus at the time of plating, and 24 h after infection cells were treated with camptothecin for 16 h to induce Sertad1 expression. Cells were fixed, and immunostaining was performed using Sertad1 chicken IgY antibody. *A*, Both Sertad1 shRNA viruses repress the upregulation of Sertad1 in cortical neurons treated with camptothecin (arrows indicate Sertad1 shRNA-expressing cells). *B*, Quantification of fluorescence intensity of Sertad1-immunostained neurons infected with control and Sertad1 shRNA viruses. Values are mean \pm SEM ($n = 30-35$). *C*, Expression of two Sertad1 shRNAs diminishes death of primary cultured cortical neurons treated with camptothecin. Cortical neurons were infected with control and Sertad1 shRNA viruses (multiplicity of infection, 10) at the time of plating. Two days after plating, cells were treated with 10 μ M camptothecin for 16, 24, 36, and 48 h, fixed, and stained with Hoechst 33258 (0.25 μ g/ml). GFP-positive cells were counted based on nuclear integrity. Significance comparisons with camptothecin-treated controls: ** $p < 0.01$. Data represent the mean \pm SEM from three independent experiments.

which requires Cdk4 activation (Greene et al., 2004, 2007). Our studies of the effects of Sertad1 knockdown on cell cycle events triggered by NGF deprivation also support the idea that the capacity of Sertad1 to activate Cdk4 is crucial to its death-promoting actions in neurons. For example, this mechanism would explain why Sertad1 knockdown blocks hyperphosphorylation of Rb, a major cellular target of Cdk4 as well as its inhibition of the subsequent induction of Myb and of Bim. In contrast,

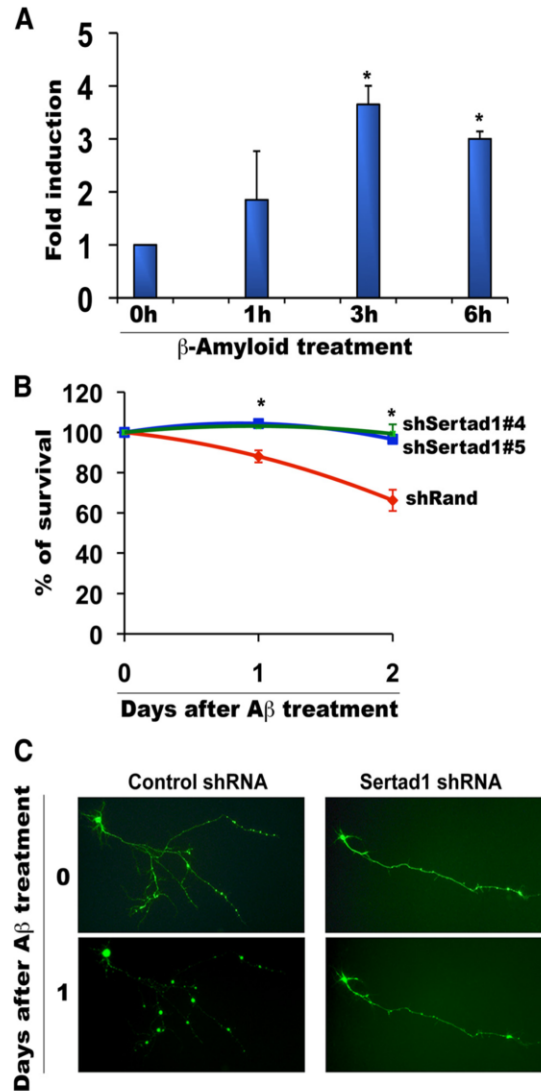


Figure 6. Sertad1 is induced and is required for degeneration of cultured cortical neurons in response to A β treatment. *A*, Sertad1 mRNA levels are increased in response to A β . Cortical neurons were treated with 1.25 μ M dodecamer A β for the indicated times and total RNA was isolated, reverse transcribed, and amplified by PCR using Sertad1- and α -tubulin-specific primers. Data represent means \pm SEM of three independent experiments. The asterisks denote statistically significant differences from control (0 h): * $p < 0.01$. *B, C*, Sertad1 knockdown prevents neuronal degeneration and death after A β treatment. Cortical neurons were transfected with pSIREN-Random-shRNA-ZsGreen (shRand) or pSIREN-Sertad1-shRNA-ZsGreen (shSertad1-#4, #5), maintained for 48 h and then treated with 1.25 μ M dodecamer A β . Numbers of surviving transfected (green) cells were counted just before treatment and after 24 and 48 h with or without treatment. Data are from one of three independent experiments, each with comparable results, and are shown as means \pm SEM, performed in triplicates. The asterisks denote statistically significant differences from control (shRand): * $p < 0.01$. Representative images of transfected neurons for pSIREN-Random-shRNA (control shRNA) and pSIREN-Sertad1#5-ZsGreen shRNA (Sertad1 shRNA) were taken before and after 24 h of treatment.

other known functions of Sertad1 seem less likely to account for its role in neuron death caused by NGF withdrawal. For instance, Sertad1 binds the E2F1 partner DP-1, thereby enhancing the transactivation of DP-1/E2F1 complexes (Hsu et al., 2001). However, our past findings showed that gene de-repression rather than transactivation is required for neuron death triggered by

NGF deprivation (Liu and Greene, 2001). Such a mechanism would also not account for our observations that Sertad1 knockdown blocks Rb hyperphosphorylation and induction of Myb (which is repressed by E2F complexes) and Bim. Another potentially relevant action of Sertad1 is its capacity to stimulate p53 transcriptional activity (Watanabe-Fukunaga et al., 2005). However, the major proapoptotic targets of p53, Noxa and Puma, do not appear to be either induced or required for death in response to NGF deprivation (S. C. Biswas and L. A. Greene, unpublished observations), and it is unclear that p53 plays a major role in death associated with this paradigm (Sadoul et al., 1996). In addition to activating Cdk4, Sertad1 was recently reported to stabilize XIAP and thus disinhibit caspases in cancer cells (Hong et al., 2009). Such an action of Sertad1, if occurred in neurons, would have an antiapoptotic effect and does not seem to be consistent with the role found here of being proapoptotic in response to NGF deprivation, DNA damage, and A β . However, in our death paradigms, either camptothecin or A β evokes a series of death pathways that are both caspase-dependent and caspase-independent (Stefanis et al., 1999; Selznick et al., 2000; Cregan et al., 2002). Even if Sertad1 confers antiapoptotic properties, delayed neuronal death still occurs in a caspase-independent manner in these paradigms.

Although Cdk4 activity is essential for neuron death induced by DNA damage, this paradigm of death differs from that caused by trophic factor withdrawal in that it requires both E2F1 and p53 as well as induction of Puma (Park et al., 2000; Wyttenbach and Tolkovsky, 2006). This raises the possibility that, in addition to its role in activating Cdk4, the capacities of Sertad to activate E2F1 and p53 may be relevant to neuronal death triggered by DNA damage. Additionally, we found that E2F1 at least partially mediates death induced by camptothecin.

Although Sertad1 overexpression enhanced neuronal death caused by NGF deprivation, it was not sufficient to promote neuron death when overexpressed in healthy neurons. This observation indicates that Sertad1 is necessary, but not sufficient for neuron death and that additional events are required to initiate the death pathway. This could include, for instance, modification of Sertad1 triggered by apoptotic stimuli, induction of additional proteins essential for Cdk4 activation, or promotion of Sertad1-independent events such as phosphorylation/dephosphorylation of Cdk4 that are needed for its full activation. A past study has shown that activation of Cdc25A is required for Cdk4 activation and neuron death in response to DNA damage (Zhang et al., 2006), raising the possibility for its involvement at least in the case of this death paradigm.

Past studies have described strong parallels between the mechanisms by which neurons die in response to NGF deprivation and A β treatment (Greene et al., 2007). This also appears to be the case for the vulnerable populations of neurons in AD that coexpress elevated levels of Cdk4, hyperphosphorylated Rb, and Bim

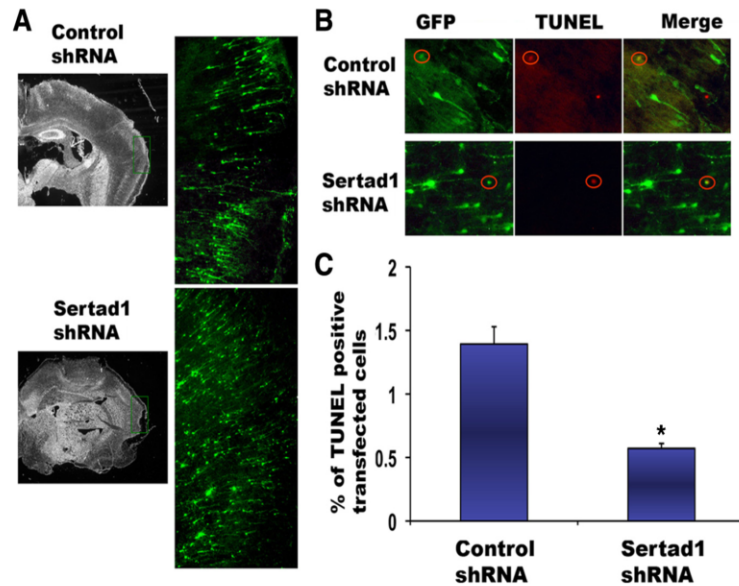


Figure 7. shRNAs targeted to Sertad1 block cortical neuron death *in vivo* during development. **A**, Distribution of transfected neurons in cortex of the electroporated brain. GFP-expressing DNA constructs of control shRNA or Sertad1 shRNA #4 were electroporated into the left lateral ventricles of E16 rat embryos, which were subsequently harvested at P5. The brains were fixed and subjected to staining for eGFP expression. Morphologically identified eGFP-expressing neurons are mostly present in cortical layers II, III, and IV of the electroporated side of the brain in each case. **B**, Location of TUNEL-positive eGFP-expressing neurons in cortices of brains electroporated with control and Sertad1 shRNA. Note that TUNEL-positive eGFP-expressing neurons are encircled in each case. **C**, Sertad1 shRNA inhibits developmental cell death of cortical neurons. The cortices of animals electroporated with control and Sertad1 shRNAs were evaluated for proportions of transfected cells (eGFP positive) that show TUNEL staining. Data represent means (SEM of 3 animals, each from an independent electroporation). Approximately 2000–3000 cells were evaluated per animal. The asterisks denote statistically significant differences between the two mean values: * $p < 0.01$.

protein (Biswas et al., 2007). A unifying explanation has recently been provided by the observations that neurotrophic factor deprivation causes the enhanced formation and release of A β , which then interacts with cells to trigger an apoptotic pathway (Matrone et al., 2008a,b). In this light, it is significant that Sertad1, as in the case of trophic deprivation, is also required for neuron death triggered by exposure to aggregated A β . Such findings further support a common pathway for the two apoptotic stimuli and raise the possibility that Sertad1 may be a therapeutic target for amelioration of neuron death and degeneration in AD. In addition to AD, activation of cell cycle proteins has also been implicated in a number of neurodegenerative diseases and nervous system insults associated with neuron death (Becker and Bonni, 2004; Greene et al., 2004, 2007; Herrup et al., 2004). For example, Sertad1 was among those genes that were induced in a cellular model of Parkinson's disease (Ryu et al., 2005), raising the possibility of its involvement at least in this disorder and perhaps in others.

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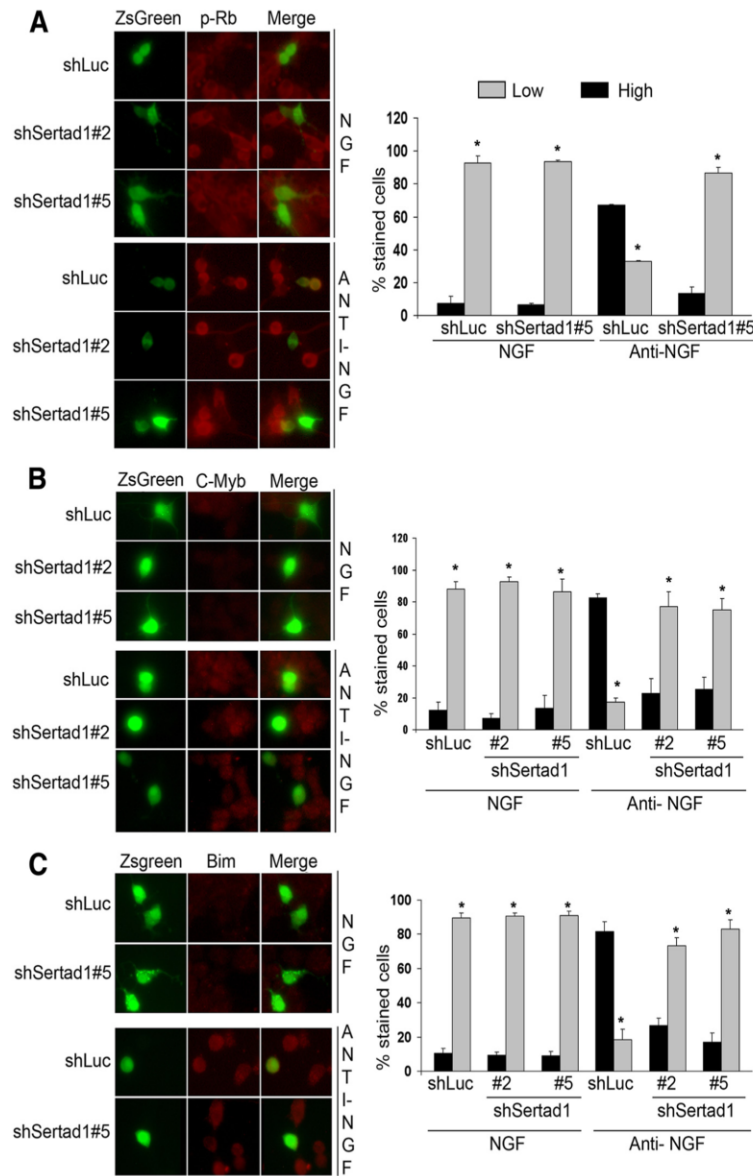


Figure 8. shRNAs targeted to Sertad1 block Rb phosphorylation and repress upregulation of endogenous Myb and Bim in PC12 cells subjected to NGF deprivation. **A–C**, Left panels show neuronal PC12 cells that were transfected with the indicated constructs, maintained for 48 h, and then deprived of NGF for 18 h after which they were immunostained with antibodies against phospho-Rb (p-Rb), C-Myb, or Bim. The percentage of stained cells shown in the right panels pertain to the proportions of transfected cells (as indicated by Zs-Green expression) that show p-Rb, C-Myb, or Bim staining at either high (more than that of vast majority of NGF-maintained cells) or low (equal or less than that of vast majority of NGF-maintained cells) levels. Data represent means (SEM of 3 independent experiments, each performed in triplicate). Approximately 50 cells were evaluated per culture. The asterisks denote statistically significant differences between low-staining cells and high-staining cells: * $p < 0.001$.

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Pim-1 kinase as activator of the cell cycle pathway in neuronal death induced by DNA damage. Journal of Neurochemistry (2010), 112(2):497-510.

Pim-1 kinase as activator of the cell cycle pathway in neuronal death induced by DNA damage

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Abstract

DNA damage is a critical component of neuronal death underlying neurodegenerative diseases and injury. Neuronal death evoked by DNA damage is characterized by inappropriate activation of multiple cell cycle components. However, the mechanism regulating this activation is not fully understood. We demonstrated previously that the cell division cycle (Cdc) 25A phosphatase mediates the activation of cyclin-dependent kinases and neuronal death evoked by the DNA damaging agent camptothecin. We also showed that Cdc25A activation is blocked by constitutive checkpoint kinase 1 activity under basal conditions in neurons. Presently, we report that an additional factor is central to regulation of Cdc25A phosphatase in neuronal death. In a gene array screen, we first identified Pim-1 as a potential factor up-regulated following DNA damage. We confirmed the up-regulation of Pim-1

transcript, protein and kinase activity following DNA damage. This induction of Pim-1 is regulated by the nuclear factor kappa beta (NF- κ B) pathway as Pim-1 expression and activity are significantly blocked by siRNA-mediated knockdown of NF- κ B or NF- κ B pharmacological inhibitors. Importantly, Pim-1 activity is critical for neuronal death in this paradigm and its deficiency blocks camptothecin-mediated neuronal death. It does so by activating Cdc25A with consequent activation of cyclin D1-associated kinases. Taken together, our results demonstrate that Pim-1 kinase plays a central role in DNA damage-evoked neuronal death by regulating aberrant neuronal cell cycle activation.

Keywords: Cdc25A, cell cycle, DNA damage, neuron, NF- κ B, Pim-1.

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Substantial evidence suggests that neuronal apoptosis evoked by DNA damage may be a critical event in neurodegenerative disease and stroke. DNA lesions have been widely reported in the pathogenesis of Parkinson's disease (Robison and Bradley 1984; Alam *et al.* 1997; Jenner 1998), Alzheimer's disease (Robison and Bradley 1984; Gabbita *et al.* 1998; Lovell and Markesbery 2001) and stroke (Tobita *et al.* 1995; Chen *et al.* 1997; Cui *et al.* 2000). Furthermore, defective response to DNA damage can lead to severe neurodegeneration (Deans *et al.* 2000; Frank *et al.* 2000; Culmsee *et al.* 2001; Vemuri *et al.* 2001). However, the mechanisms by which DNA damage triggers neuronal death in these neurological disorders are not fully understood.

Previous studies have shown that DNA damage in neurons induces death signals that involve aberrant activation of multiple cell cycle elements (Becker and Bonni 2004). For example, in cultured embryonic neurons treated with DNA

damaging agent camptothecin, cyclin D1-associated kinases (Cdk4/6) are rapidly activated (Park *et al.* 1996, 1998), which results in phosphorylation of Rb family members and subsequent release and activation of E2F transcription factors (Park *et al.* 2000). Over-expression of dominant-negative Cdk4/6, over-expression of Rb or loss of E2F activity

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Abbreviations used: ATM, ataxia telangiectasia mutated; CAPE, caffeic acid phenethyl ester; Cdc, cell division cycle; Cdk, cyclin-dependent kinase; Chk1, checkpoint kinase 1; IP, immunoprecipitation; JNK, c-Jun N-terminal kinase; NF- κ B, nuclear factor kappa beta; PP2A, protein phosphatase 2A; SDS, sodium dodecyl sulfate.

protects cortical neurons from death attributable to DNA damage (Park *et al.* 1998, 2000). These observations are quite intriguing and unexpected given that neurons are terminally differentiated and should not utilize any cell cycle signals.

The mechanism by which abnormal cell cycle signals are activated in neurons is not fully understood. Recent reports have implicated checkpoint kinase 1 (Chk1) and cell division cycle (Cdc) 25A phosphatase as mediators of Cdk/Rb/E2F pathway in this death paradigm (Zhang *et al.* 2006). Camptothecin exposure leads to rapid inactivation of Chk1 and subsequent activation of Cdc25A which in turn activates Cdk4/6. Moreover, over-expression of Cdc25A promotes neuronal death evoked by camptothecin while siRNA-mediated knockdown of Cdc25A or over-expression of Chk1 blocks death (Zhang *et al.* 2006).

Despite progress on elucidating this cell cycle signaling pathway, the mechanisms underlying Cdc25A activation is not completely understood. For example, in proliferating cells exposed to genotoxic stress, Chk1-mediated Cdc25A inactivation occurs through phosphorylation-dependent degradation of Cdc25A (Sanchez *et al.* 1997; Mailand *et al.* 2000; Sorensen *et al.* 2003). However, in post-mitotic neurons, Cdc25A protein level does not change significantly upon DNA damage. This suggests that there may be other regulators of Cdc25A activation in addition to Chk1 critical for its activation. To address this issue, we recently performed a preliminary gene array screen to examine for increased message in cortical neurons exposed to camptothecin. One potential candidate of interest that we identified was Pim-1.

Pim-1 is a proto-oncogene which encodes a serine/threonine protein kinase that regulates cell proliferation and growth. It was originally identified as a preferential proviral insertion site in Moloney Murine Leukemia Virus induced T-cell lymphomas (Cuyper *et al.* 1984). Pim-1 plays important roles in signal transduction in hematopoietic and lymphoid systems (Wang *et al.* 2001; Bachmann and Moroy 2005). In addition, dysregulated expression or function of Pim-1 is associated with hematopoietic neoplasia (Amson *et al.* 1989; Saris *et al.* 1991; Laird *et al.* 1993; Lilly *et al.* 1999). Critically, it promotes cell survival in dividing cells consistent with its oncogenic properties (Lilly *et al.* 1999). These findings led us to examine whether Pim-1 signals camptothecin-mediated neuronal death and cell cycle activation. Presently, we show that Pim-1 activity in neurons promotes death, unlike that in dividing cells. In addition, we show that Pim-1 is critical for Cdc25A activation in neurons following DNA damage. This finding provides crucial understanding to how abnormal cell cycle signals are inappropriately activated in neurons. Finally, we show that nuclear factor kappa beta (NF- κ B) is essential for the activation of Pim-1 in neurons exposed to DNA damage thereby providing a mechanism of how Pim-1 itself is activated.

Experimental procedures

Materials

Camptothecin and staurosporine were purchased from Sigma (St. Louis, MO, USA). Helenalin, BAY 11-7082 and CAPE (caffeic acid phenethyl ester) were obtained from Biomol (Plymouth Meeting, PA, USA). 4-Nitrophenol phosphate (pNPP) was purchased from Roche Applied Science (Indianapolis, IN, USA).

Neuronal cultures and transfection

Primary cortical neurons were cultured from embryonic day 15 (E15) CD1 mice (Charles River Laboratories, Wilmington, MA, USA) as previously described (O'Hare *et al.* 2005; Haque *et al.* 2008) and maintained in Neurobasal media (Invitrogen, Carlsbad, CA, USA) supplemented with B27 (Invitrogen), N2 (Invitrogen), and 0.5 mM glutamine (Sigma). 2 days after plating cells were treated with camptothecin (10 μ M) or NF- κ B inhibitors as indicated in the text and figures. Lipofectamine mediated transfection of siRNA for NF- κ B have been described previously (Aleyasin *et al.* 2004). siRNAs used in Fig. 7 to (siRNAp65, AAGAAGCACAGAUACCACCAA) and (control siRNA, GCGCGCUUUGUAGGAUUCG) were obtained from Ambion (Austin, TX, USA) and siRNAs used in the Fig. S2 was a mixture of three targeted siRNA from Santa Cruz Biotechnology (Santa Cruz, CA, USA) (Cat.#, sc-29411). Pim-1 plasmid was transfected using calcium phosphate method as previously mentioned (Zhang *et al.* 2006).

RT-PCR

Total RNA was extracted from cultured cortical neurons using TRIzol reagent (Invitrogen). 50 ng of total RNA was used for cDNA synthesis and targeted gene amplification using SuperScript One-Step RT-PCR kit (Invitrogen). After DNase treatment, cDNA synthesis was performed at 45°C for 45 min followed by a 2-min initial denaturation step at 94°C. This was followed by 29 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. The mouse-specific Pim-1 PCR primers (5'-GGCGCCGGGCAAAGAGAAGGAG-3' and 5'-ACCCGAAGTCGATGAGTTTGATTT-3') were used to generate a 491-bp product. Expression of ribosomal S12 mRNA was used as a standard to quantify the relative amount of expression of Pim-1 as described previously (Cregan *et al.* 2004). S12 cDNA was amplified using 5'-GGAAGGCATAGCTGCTGG-3' and 5'-CCTCGATGACATCCTTGG-3' as primers.

Western blot analysis

Western blot analysis was performed as previously described (O'Hare *et al.* 2005) using antibodies against Pim-1 (Cat.# sc-13513; 1 : 1000; Santa Cruz Biotechnology) and β -actin (Cat.# A5316; Sigma) as a loading control. For Pim-1/Cdc25A binding assay, a rabbit anti-Cdc25A (Cat.# 07-459; Upstate, Lake Placid, NY, USA) and mouse anti-Pim-1 antibody mentioned above were used for immunoprecipitation of Cdc25A and Pim-1, respectively. A normal rabbit IgG (Cat.# sc-2027; Santa Cruz Biotechnology) and a mouse anti-GST IgG (Cat.# sc-138; Santa Cruz Biotechnology) were used as controls. The anti-Pim-1 antibody mentioned above and a rabbit anti-Cdc25A antibody (Cat.# sc-97; Santa Cruz Biotechnology) were used to detect immunoprecipitated Pim-1 and Cdc25A, respectively. Anti-p65 antibody (Cat.# sc-372; Santa Cruz Biotechnology) was used for p65 knockdown experiments.

Protein kinase assay

As previously reported (Appendix S1).

Immunofluorescence

As previously reported (Appendix S1).

Knockout mice

Pim-1 knockout mice (Laird *et al.* 1993) were maintained on a mixed C57BL/6 × 129/Ola background and genotyped as previously reported (Appendix S1).

Cdc25A phosphatase assay

As previously reported (Appendix S1).

Chromatin immunoprecipitation

Embryonic day 15 mouse cortical neurons at 3 DIV were incubated with 1% formaldehyde with mild rocking for 10 min at 22°C. Cells were washed with phosphate-buffered saline and harvested and resuspended in 200 µL lysis buffer [1% sodium dodecyl sulfate (SDS), 10 mM EDTA, 50 mM Tris/HCl, pH 8.1] containing protease inhibitors and placed on ice for 10 min. Cell lysates were sheared by sonication for a total of 50–100 s at 10 s per interval and centrifuged at 15 000 g at 4°C for 10 min and the supernatant was diluted at 1 : 10 in dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris/HCl, pH 8.1, 150 mM NaCl). 20 µL of the sample were used as the input chromatin. Immunoprecipitation was performed overnight with p65 antibody and rabbit IgG mentioned above, then 50 µL protein A agarose beads was added for 1 h at 4°C. Beads were washed three times with wash buffer (100 mM Tris-HCl, pH 9.0, 500 mM LiCl, 0.1% NP-40 (Sigma, St. Louis, MO, USA), and 1% deoxycholic acid). Bead precipitates were eluted twice with elution buffer (1% SDS 0.1 M NaHCO₃). Formaldehyde cross-linking was reversed by heating overnight at 65°C, and incubating 1 h with RnaseA at 37°C, and incubating 1 h with proteinaseK at 37°C. DNA were isolated with phenol/chloroform and subjected to PCR reaction. The primers used for PCR amplification of mouse Pim-1 promoter region are 5'-AACCTAGGATTTCTTCTGT-3' and 5'-CATGCACCTGTACCA-3'. Human promoter sequences are also as follows: 5'-AGCCGACTTGGAGGTCTCGGT-3' and 5'-GCTGTAGGCTGGGGCGGATTCTG-3'. PCR products were resolved on an agarose gel and photographed.

Luciferase assay

HEK293 cells were transfected using Lipofactamine 2000 with a luciferase vector (pGL4.23; Promega, Madison, WI, USA) containing a minimal promoter with or without the Pim-1 promoter sequence (–1261 to –23) along with plasmid cytomegalovirus vector (pCMV) (empty or expressing p65) and plasmid cytomegalovirus vector (pCMV)-LacZ 24 h later, cells were washed with cold phosphate-buffered saline, triturated off the plates, and pelleted in microcentrifuge tubes. Cell pellets were lysed in buffer provided in the Promega Luciferase System (Promega). Luciferase assay was carried out according to the manufacturer's instructions. Relative luciferase activities were obtained by normalizing the luciferase activity against β-gal activity.

Recombinant adenovirus infection

As previously reported (Appendix S1).

Results

To examine the signaling pathways induced by DNA damage, we have used the *in vitro* model of cortical neuronal death evoked by the DNA topoisomerase I inhibitor, camptothecin. Camptothecin has been shown to initiate apoptotic death of embryonic cortical neurons in a manner dependent on Bax and the conserved mitochondrial pathway of death including cytochrome *c* release and apoptosome activation (Morris and Geller 1996). This conserved mitochondrial pathway of death is only active when both the cell cycle and p53 upstream regulatory pathways are activated. Inhibition of either pathway blocks the conserved mitochondrial death signal (Morris *et al.* 2001; Sedarous *et al.* 2003). Based upon our initial gene array screen, we focused on the role of Pim-1 as an additional regulator of DNA damage induced neuronal death. We focused on (i) whether Pim-1 activity was changed (ii) whether Pim-1 participates functionally in death and (iii) how Pim-1 is regulated and the mechanism(s) by which it signals death.

Pim-1 is up-regulated after exposure to DNA damage and a pro-death regulator

We first examined whether Pim-1 levels change in embryonic cortical neurons after DNA damage. Cortical neurons derived from E15 mouse brain were exposed camptothecin (10 µM) for various time periods and were analyzed for Pim-1 mRNA and protein by RT-PCR and western immunoblotting, respectively. Pim-1 transcripts were detectably up-regulated as early as 2 h of camptothecin exposure and peaked by 4-h treatment (Fig. 1a). As shown in Fig. 1(b), Pim-1 signals were quantified by densitometry and normalized to the corresponding S12 control for each sample. This quantitation showed that camptothecin treatment increased Pim-1 mRNA levels by 3.5-, 5.3- and 3-fold at the 2-h, 4-h and 8-h time points, respectively. To determine whether camptothecin-induced increase in Pim-1 mRNA level was translated into an increase in Pim-1 protein level, we performed immunoblot analyses using anti-Pim-1 antibody. The murine Pim-1 is expressed as two isoforms: a 33-kDa protein (p33 Pim-1) and a 44-kDa protein (p44 Pim-1) (Saris *et al.* 1991). 44-kDa Pim-1 is the product of the same gene from which an upstream alternative translation initiation site was used during translation (Saris *et al.* 1991) and has comparable kinase activity as 33-kDa protein (Lilly *et al.* 1999). The Pim-1 antibody utilized recognizes both isoforms by western blot analyses which are not detected in Pim-1 deficient neurons (Fig. 1e). Figure 1(c) and (d) show that camptothecin treatment induced a significant increase in the level of both Pim-1 isoforms in cortical neurons. Pim-1 p44 isoform is more readily detectable than the p33 form under basal conditions. Induction of Pim-1 protein was detected as early as 1 h (data not shown). Pim-1 protein

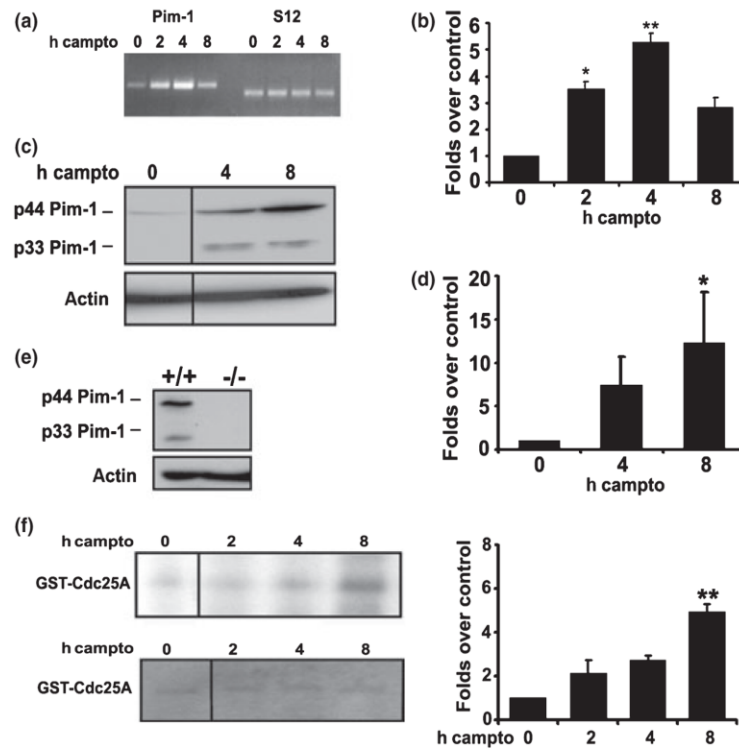


Fig. 1 Pim-1 activation in response to DNA damage. (a) Pim-1 mRNA increases early in response to camptothecin treatment. Total RNA was extracted from cortical neurons at the indicated times after camptothecin treatment (10 μM) and analyzed for Pim-1 mRNA expression using RT-PCR. S12 levels are shown as a control for equal input. (b) Pim-1 and S12 bands from three independent experiments were analyzed by densitometry using NIH ImageJ. Data for Pim-1 expression were normalized to S12 and expressed relative to the zero time point. Each point represents the mean \pm SEM. Significance comparisons with untreated controls. * $p < 0.05$, ** $p < 0.01$. (c) Pim-1 protein level increases with camptothecin treatment. Western blot showing relative Pim-1 levels from the whole cell lysates of cortical neurons following camptothecin (10 μM) treatment for the indicated times. An antibody that recognizes both the 44- and 33-kDa forms of Pim-1 was used. Actin is provided as loading control. (d) Densitometric analysis

of western immunoblots. Data are the mean \pm SEM and are expressed relative to the amount of Pim-1 at time 0. Significance comparisons with untreated controls. * $p < 0.05$. (e) The specificity of the Pim-1 antibody is demonstrated by using cortical cultures obtained from Pim-1-deficient and wild-type littermate controls. (f) Pim-1 kinase activity is rapidly increased in response to DNA damage. Pim-1 activity was calculated by measuring the incorporation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ onto GST-Cdc25A substrate and subtracting the control background value. Left panel, an example of autoradiogram showing the incorporation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ onto Cdc25A. Right panel, the kinase activity was quantified by densitometry using NIH ImageJ. Data represent the mean \pm SEM from three independent experiments. Significance comparisons with untreated controls. * $p < 0.05$. Bottom panel, SDS-polyacrylamide gel electrophoresis gel showing the substrate GST-Cdc25A.

levels were highest at the 8-h time point examined. This is in contrast to the peak of Pim-1 message observed at 4 h. This indicates that the stability of message and protein may differ or that there may be additional modes of Pim-1 regulation. Taken together, these data indicate that the early induction of Pim-1 RNA levels correlates well with increases in protein expression. To determine whether the elevation in Pim-1 levels is accompanied by an increase in kinase activity, we performed an *in vitro* kinase assay of Pim-1 immunoprecipitated from cortical neurons treated with camptothecin for various time periods utilizing Cdc25A as substrate. As shown in Fig. 1(f), activity of Pim-1 was increased throughout the time course of

camptothecin treatment up to 8 h when compared to basal untreated controls.

We next asked whether the observed induction of Pim-1 was general to all inducers of apoptosis. To test this, we used the general apoptotic initiator and kinase inhibitor, staurosporine. Staurosporine can induce neuronal apoptosis at relatively low concentration (1 μM) (Koh *et al.* 1995). As shown in Fig. 2(a–c), neither Pim-1 message, protein level nor kinase activity changed upon staurosporine treatment. This indicates that Pim-1 involvement is not general to all apoptotic contexts.

The above evidence indicates that Pim-1 activity and levels is increased following camptothecin exposure. We

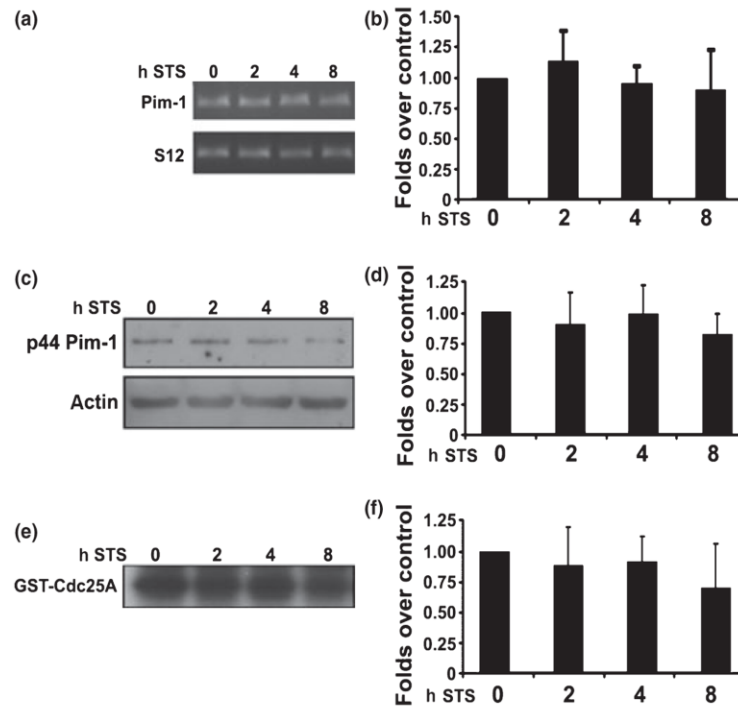


Fig. 2 Pim-1 is not activated in response to staurosporine. (a) Pim-1 mRNA does not increase in response to staurosporine (STS) treatment. Total RNA was extracted from cortical neurons at the indicated times after staurosporine treatment (1 μ M) and analyzed for Pim-1 mRNA expression using RT-PCR. S12 levels are shown as a control for equal input. (b) Pim-1 and S12 bands from three independent experiments were analyzed by densitometry using ImageJ. Data for Pim-1 expression were normalized to S12 and expressed relative to the zero time point. Each point represents the mean \pm SEM. (c) Pim-1 protein level does not change with staurosporine treatment. Western blot showing relative Pim-1 levels from the whole cell lysates of cortical

neurons following staurosporine (1 μ M) treatment for the indicated times. An antibody that recognizes both the 44- and 33-kDa forms of Pim-1 was used. Actin is provided as loading control. (d) Densitometric analysis of western immunoblots. Data are the mean \pm SEM and are expressed relative to the amount of Pim-1 at time 0. (e) Pim-1 kinase activity does not change significantly in response to staurosporine treatment. Pim-1 activity was calculated by measuring the incorporation of [γ - 32 P]ATP onto GST-Cdc25A substrate and subtracting the control background value. (f) The kinase activity was quantified by densitometry using NIH ImageJ. Data represent the mean \pm SEM from three independent experiments.

next examined the function of Pim-1 as it relates to neuronal death in this system. To do this, neurons were cultured from Pim-1 deficient embryos or heterozygous or wild-type littermate controls. As shown in Fig. 3(a), Pim-1-deficient neurons were significantly resistant to camptothecin-induced death when compared with wild-type controls (83% survival in knockout vs. 57% in wild-type controls at 8 h and 34% survival in wild-type vs. 58% knockout at 12 h). This finding supports a pro-apoptotic role for acute Pim-1 activation in response to DNA damage. We also performed Pim-1 over-expression experiment to test whether exogenous Pim-1 expression induces neuronal death. As shown in Fig. 3(b), Pim-1 expression alone is insufficient to induce death. It is also important to note that protection afforded by Pim-1 deficiency is not complete and transient. This suggests the participation of other death signals which act independently of Pim-1 (see Discussion below).

Cdc25A as downstream effector of Pim-1 in camptothecin-induced neuronal death

The above results suggest that Pim-1 is a pro-death factor in our paradigm of neuronal death. We next examined the mechanism(s) by which Pim-1 modulates death. Pim-1 has been shown to act on a number of substrates involved in cell cycle regulation such as Cdc25A, Cdc25C, p21 and C-TAK1 (Mochizuki *et al.* 1999; Wang *et al.* 2002; Bachmann *et al.* 2004, 2006). Relevant to the present work, however, is the report that Pim-1 can potentially regulate cell cycle through direct phosphorylation and resultant activation of Cdc25A phosphatase in proliferating cells (Mochizuki *et al.* 1999). To examine this potential mechanism, we first tested whether there is a physical interaction between Pim-1 and Cdc25A in post-mitotic neurons. We carried out immunoprecipitation (IP)-western blot assay using mouse brain extracts. Cdc25A was immunoprecipitated using an anti-Cdc25A antibody and co-immunoprecipitated proteins were examined for the

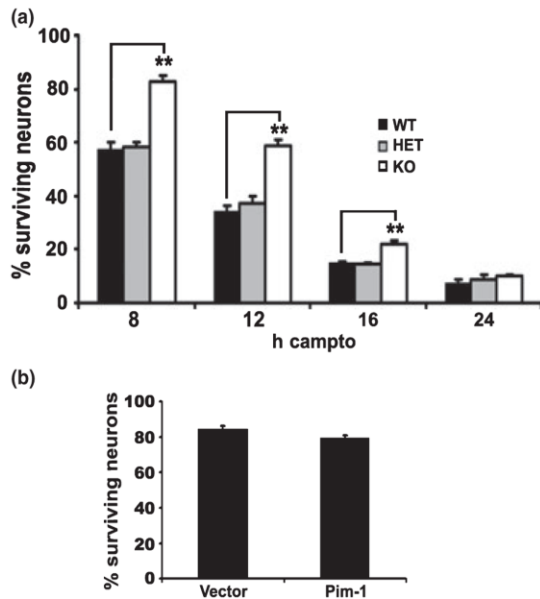


Fig. 3 (a) Pim-1 deficiency is protective against camptothecin-induced neuronal death. Cortical neurons were obtained from E15 embryos derived from a double heterozygote breeding of Pim-1 mice. Cultures were exposed to camptothecin (10 μ M) for 8, 12, 16 and 24 h and cell viability was analyzed by nuclear counts. ** $p < 0.01$ compared with control. Each data point is the mean \pm SEM from at least three independent embryos. (b) Pim-1 over-expression does not induce significant cell death. Cultured cortical neurons were co-transfected with pEGFP and the indicated constructs. 24 h after transfection, cells were fixed and stained with Hoechst. GFP-positive neurons were scored for survival by evaluating nuclear integrity.

presence of Pim-1 by western blot analyses. In Fig. 4(a), we show that IP with Cdc25A antibody but not an IgG control antibody pulls down Pim-1. The reciprocal experiment was also performed where IP was performed using Pim-1 antibody followed by probing for Cdc25A. Consistent with the above results, an interaction between Pim-1 and Cdc25A was also observed (Fig. 4b). This indicates that these two proteins can interact endogenously in brain. To further support the interaction of Pim-1 and Cdc25A, we performed co-immunoprecipitation assay using cultured neurons. As shown in Fig. 4(c), endogenous Cdc25A was detected by western blot analyses when Pim-1 was immunoprecipitated utilizing Pim-1 antibody. Finally, we examined whether Cdc25A and Pim-1 would colabel upon immunofluorescence analyses in cortical neurons (Fig. 4d). Upon camptothecin treatment there was a noticeable increase in Pim-1 staining as predicted by our previous biochemical experiments. There was also significant overlap between the Pim-1 and Cdc25A signals supporting the observed physical interaction between the two proteins as detected by IP analyses.

Next, to test the hypothesis that Pim-1 modulates neuronal death by enhancing Cdc25A activity in this paradigm, we

determined whether/how Pim-1 deficiency would affect Cdc25A activation previously observed following DNA damage (Zhang *et al.* 2006). To do this, Cdc25A activity was measured from Pim-1 deficient or littermate wild-type neuronal cultures treated with camptothecin utilizing 4-nitrophenol phosphate as a substrate. As shown in Fig. 4(e), Cdc25A phosphatase activity was increased in wild-type neuronal cultures upon camptothecin exposure, consistent with our previously reported results. However, this increase was blunted in Pim-1 deficient cultures. Pim-1 expression alone was not sufficient to Cdc25a activation (Fig. S1). Cyclin D1-associated kinase activity is also increased in wild-type neurons following DNA damage in a Cdc25A dependent fashion. If Pim-1 truly affects Cdc25A activity, we would expect that Cyclin-D1 associated kinase activity would also decrease. As shown in Fig. 4(f), the induction of Cyclin D1 activity was also abolished in Pim-1 deficient neurons. These findings indicate that Pim-1 mediates neuronal death upstream of Cdc25A and Cdk/Rb/E2F.

NF- κ B as activator for Pim-1 up-regulation at transcriptional level

Thus far we have shown that Pim-1 functions to regulate Cdc25A in this DNA damage paradigm. Next, we investigated how DNA damage up-regulates Pim-1 expression. It has been demonstrated that Pim-1 is induced by transcription factor NF- κ B in CD40 signaling in B lymphocytes (Zhu *et al.* 2002). This is consistent with the observation that the Pim-1 promoter (both human and mouse) contains several putative NF- κ B binding sites situated closely together (Fig. 5a). Accordingly, we first examined whether NF- κ B members can potentially bind these sites. We designed primer sequences (using DNASTAR software) close to the Nfkb binding region and performed chromatin immunoprecipitation (ChIP) analyses for p65 and c-Rel (Fig. 5b and c). Our results demonstrated that both mouse and human Pim-1 promoter contains p65. The human Pim-1 promoter also binds c-Rel which was not detected with the mouse promoter (data not shown). These experiments indicate that NF- κ B subunits can bind to the Pim-1 promoter. Supporting the functional relevance of this binding, we also tested whether association of p65 to Pim-1 promoter is augmented in response to DNA damage. As shown in Fig. 5(d), promoter binding ability of p65 increases with camptothecin treatment. Next we examined for the effects of p65 expression on a luciferase reporter construct driven by the Pim-1 promoter containing the NF- κ B sites. As shown in Fig. 5(e), p65 expression significantly increases the Pim-1-promoter driven luciferase signal. The above evidence combined with our previous report that NF- κ B modulates DNA damage response as a pro-death factor in this death model (Aleyasin *et al.* 2004) prompted us to address the possibility that camptothecin-induced Pim-1 expression might be directly controlled by NF- κ B. To this end, we employed two different

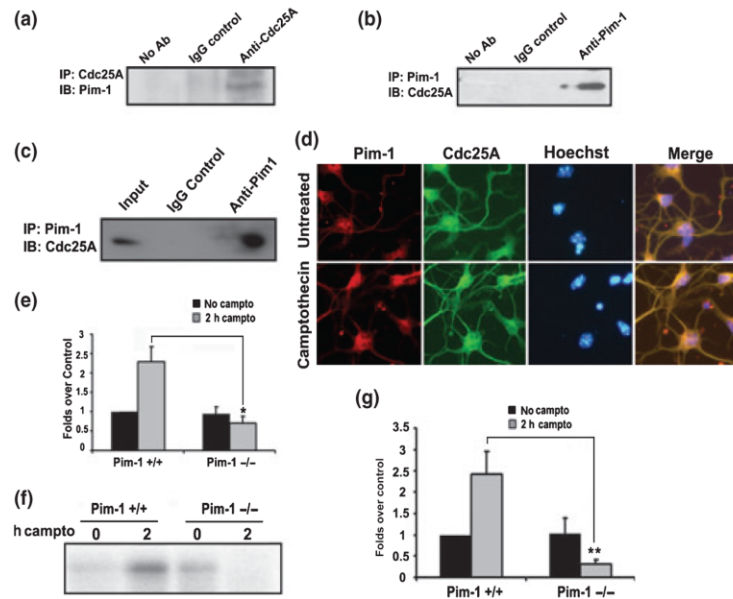


Fig. 4 Pim-1 regulates Cdc25A in response to DNA damage. (a and b) *In vivo* association of Pim-1 and Cdc25A. IP-western blot analysis of mouse brain extracts immunoprecipitated with anti-Cdc25A (or anti-Pim-1) antibody. Immunoprecipitates were run on SDS-polyacrylamide gel electrophoresis followed by western blot analysis with anti-Pim-1 (or anti-Cdc25A) antibody. Parallel IP-western blot analysis using no antibody or non-specific IgG antibody were performed as controls. (c) Association of Pim-1 and Cdc25A in cultured neurons. Cultured cortical neurons were treated with camptothecin for 8 h. Pim-1 protein was immunoprecipitated by an anti-Pim-1 antibody and western blot was performed using an anti-Cdc25A antibody. (d) Pim-1 co-localized with Cdc25A in cortical neurons. Cortical cultures were treated with or without camptothecin for 4 h and subjected to Pim-1 and Cdc25A immunostaining. Nuclei were stained with Hoechst. (e) Pim-1 deficiency attenuates camptothecin-induced Cdc25A phos-

phatase activity. Cortical neurons from Pim-1^{+/+} and Pim-1^{-/-} littermates were treated with or without camptothecin for 2 h. Cdc25A was immunoprecipitated from whole-cell lysate of neurons and subjected to phosphatase assay using pNPP as substrate. Data represent the mean \pm SEM from four independent experiments. * p < 0.05. (f) Pim-1 deficiency attenuates camptothecin-induced Cyclin D1-associated kinase activity in cortical neurons. Cdk4/6 were immunoprecipitated from whole-cell lysate of Pim-1^{+/+} and Pim-1^{-/-} neurons treated with or without camptothecin for 2 h and subjected to kinase assay using pRb as substrate. (g) Densitometric analysis of Cyclin D1-associated kinase activity. The kinase activity was quantified by measuring the incorporation of [γ -³²P]ATP onto pRb substrate and subtracting the wild-type control background value. Data represent the mean \pm SEM from five independent experiments. ** p < 0.01.

approaches to inhibit NF- κ B activation and observe whether camptothecin-evoked up-regulation of Pim-1 is affected. First, we used several different pharmacological inhibitors to block NF- κ B activity via different mechanisms: (i) CAPE inhibits nuclear translocation of NF- κ B possibly via antioxidant effects (Natarajan *et al.* 1996); (ii) Helenalin blocks DNA binding of NF- κ B by specific and irreversible alkylation of the p65 subunit (Lyss *et al.* 1997, 1998); (iii) BAY 11-7082 inhibits I κ B α phosphorylation and degradation thereby results in the inactivation of NF- κ B (Pierce *et al.* 1997). We treated cortical neurons with camptothecin (10 μ M) alone or co-treated with CAPE, BAY 11-7082 and Helenalin for the indicated time periods. Total RNA and protein were harvested for RT-PCR and western blot analysis, respectively. As shown in Fig. 6(a), RT-PCR demonstrated that co-treatment of NF- κ B inhibitors significantly decreases Pim-1 signals at 4 h after camptothecin treatment. Similarly, co-treatment of NF- κ B inhibitors

robustly decreases Pim-1 protein level at 4 h and 8 h after camptothecin treatment (Fig. 6b). Importantly, Helenalin and CAPE also markedly decreased camptothecin induced kinase activity (Fig. 6c).

Because of potential specificity issues related to pharmacological inhibitors, we next examined the functional link of NF- κ B and Pim-1 using siRNA-mediated knockdown approach. In this regard, we transfected neurons with two independent NF- κ B-specific siRNA oligonucleotide reagents and treated with camptothecin for the indicated times. RNA and protein levels of Pim-1 were examined by RT-PCR and western blot analysis respectively. Figures 7a,b and S2a,b show that down-regulation of NF- κ B significantly decreases Pim-1 induction at both RNA and protein levels. Also, siRNA-mediated down-regulation of p65 leads to significantly decreased Pim-1 kinase activity induced by DNA damage (Figs 7c and S2c). These results are consistent with the observations that NF- κ B, as a pro-apoptotic factor, is

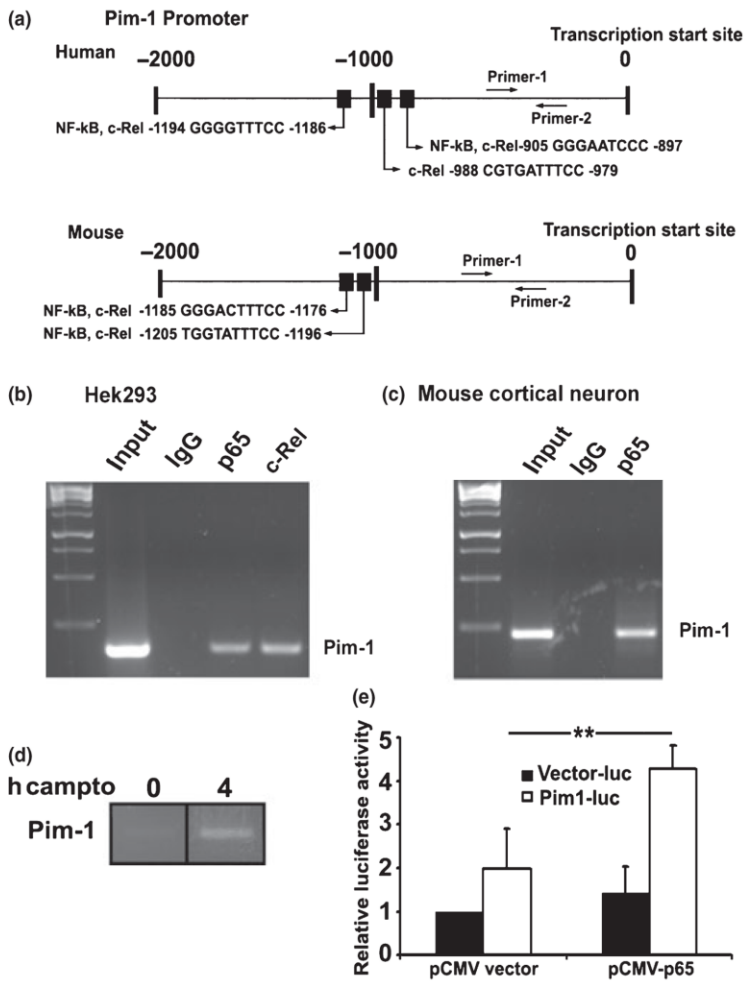


Fig. 5 NF-κB interacts with Pim-1 promoter in both mouse and human cells. (a) NF-κB binding sites in Pim-1 promoter region as identified through the TFSEARCH server (<http://www.cbrc.jp/research/db/TFSEARCH.html>). (b) ChIP assay to show p65 and c-Rel subunits bind to Pim-1 promoter in HEK293 cells. (c) ChIP assay to show p65 subunit binds to Pim-1 promoter in mouse cortical neurons. (d) Promoter binding ability of p65 increases with camptothecin treatment. Cultured neurons were treated with camptothecin for 4 h and ChIP assay was performed as described above. (e) p65/Pim-1 promoter luciferase assay. Luciferase vector (vector-luc) and Pim-1 promoter luciferase construct (Pim-1-luc) were co-transfected with a pCMV control vector or pCMV-p65 plasmid as well as CMV-β-galactosidase plasmid using Lipofectamine in HEK293 cells. 24 h after transfection, cell lysates were processed for both luciferase and β-galactosidase assay. Data are presented as normalized luciferase activity (luciferase/β-galactosidase). ** $p < 0.05$.

activated early (as early as 2 h after camptothecin treatment) in response to DNA damage (Aleyasin *et al.* 2004), and support an important role of NF-κB as upstream activator of cell cycle pathway via Pim-1 in this death model.

p53 is not a downstream effector of Pim-1 in camptothecin-induced neuronal death, and vice versa

As we have previously shown that NF-κB mediates death through induction of p53 following DNA damage (Aleyasin *et al.* 2004), we asked whether NF-κB regulates death and p53 induction via Pim-1 up-regulation in this death paradigm. Previously we have shown that p53 is induced after camptothecin exposure, accompanied by an increase in p53-responsive genes (Morris *et al.* 2001; Cregan *et al.* 2004). The induction of the Bcl-2 family members Noxa and Puma is known to be dependent on p53 (Oda *et al.* 2000; Yu *et al.* 2001). Accordingly, we examined whether Pim-1 acts as an upstream regulator of p53 pathway. As shown in Fig. 8(a), the induction of Noxa and Puma in Pim-1 wild-type neurons

after camptothecin treatment, as determined by RT-PCR, is not reduced in Pim-1-deficient neurons. These results indicate that Pim-1 does not act upstream of p53 in this death model. Next we addressed the question whether p53 regulates Pim-1 up-regulation in this death paradigm. To this end, we examined Pim-1 induction in response to DNA damage in p53 deficient neurons. As shown in Fig. 8(b), p53 deficiency did not affect camptothecin-induced Pim-1 up-regulation.

Discussion

DNA damage triggers a complex series of biochemical and molecular mechanisms which eventually results in rapid neuronal cell death. These intricate processes lead to activation of at least three signaling pathways: (i) a cell cycle pathway which involves Cdc25A phosphatase, Cyclin D1-associated kinases, pRb family members, and E2F transcription factors (Park *et al.* 1997); (ii) a p53 pathway

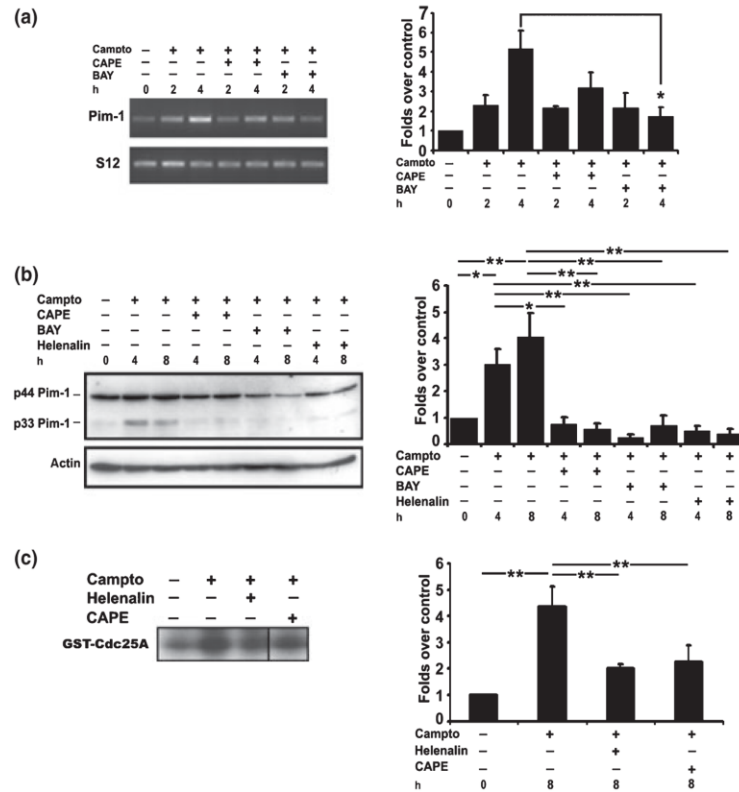


Fig. 6 NF-κB inhibition blocks DNA damage-induced up-regulation of Pim-1. (a) Cortical neurons were treated with camptothecin (10 μM) alone or camptothecin plus CAPE (5 μM) and BAY 11-7082 (5 μM) for 2 and 4 h. Total RNA was extracted and Pim-1 transcripts were analyzed by RT-PCR. S12 was analyzed as a control (left panel). Densitometric analysis of RT-PCR was performed from three independent experiments (right panel). Data represent the mean ± SEM from three independent experiments. **p* < 0.05. (b) Cortical neurons were treated with camptothecin (10 μM) alone or camptothecin plus CAPE (5 μM), BAY 11-7082 (5 μM) and Helenalin (5 μM) for 4 and 8 h. Whole cell extracts were separated by SDS-polyacrylamide gel electrophoresis and then analyzed by immunoblotting with anti-Pim-1

antibody. Actin is provided as loading control (left panel). Densitometric analysis of three independent experiments was performed (right panel). Data represent the mean ± SEM from three independent experiments. **p* < 0.05, ***p* < 0.01. (c) NF-κB inhibition blocks Pim-1 kinase activity induced by DNA damage. Cortical neurons were treated with camptothecin (10 μM) alone or camptothecin plus Helenalin (5 μM) or CAPE (5 μM) for 8 h. Pim-1 were immunoprecipitated and subjected to kinase assay using Cdc25A as substrate (left panel). Densitometric analysis of three independent experiments was performed (right panel). Data represent the mean ± SEM from three independent experiments. **p* < 0.05.

which is regulated by NF-κB and ataxia telangiectasia mutated (ATM) kinase (Aleyasin *et al.* 2004); (Keramiris *et al.* 2003) and (iii) a *c-Jun* N-terminal kinases (JNKs) pathway (Ghahremani *et al.* 2002). The coordination of these and likely other signal transducers is necessary to activate the conserved mitochondrial pathway of death. However, upon DNA damage, the manner by which these cellular events are initiated, regulated and interact to promote neuronal death is not fully understood. The purpose of the present study is to further our understanding of the molecular mechanisms involved in these processes, particularly in regards to cell cycle activation. Presently, the significance of our findings are as follows: (i) we provide critical evidence of how abnormal cell cycle activation is promoted in neurons by Pim-1. This evidence is crucial if we are to understand the

unique phenomenon of cell cycle in neuronal death. (ii) We demonstrate that Pim-1 plays a prodeath role in neurons exposed to DNA damage, contrary to the prosurvival properties attributed to this kinase in dividing cells. (iii) We delineated the mechanism by which Pim-1 is activated (NF-κB) and its downstream target linking it to the cell cycle in neurons (Cdc25A).

Pim-1 and cell cycle regulation in neuronal death

In this study, we report that Pim-1 mediates DNA-damage induced neuronal death through enhancing Cdc25A activity and that this is critical for the inappropriate activation of the cell cycle signals in neurons. The data supporting this model is as follows: (i) Pim-1 levels and activity are induced following DNA damage. (ii) Pim-1 activity is important for

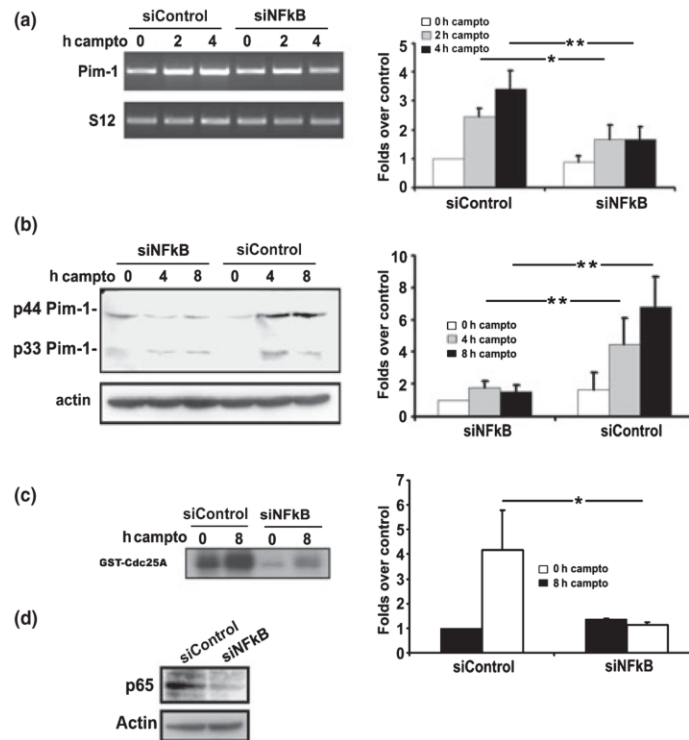


Fig. 7 NF- κ B knockdown blocks DNA damage-induced up-regulation of Pim-1. (a) Cortical neurons were transfected with a control siRNA (siControl) or NF- κ B siRNA oligonucleotides (siNF- κ B) for 24 h and then treated with camptothecin for 2 and 4 h as indicated. Pim-1 transcripts were analyzed by RT-PCR (left panel). Densitometric analysis of three independent experiments are presented as fold increase compared with untreated groups transfected with control siRNA (right panel). Data represent the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$. (b) Cortical neurons were transfected as above mentioned and whole-cell lysates prepared from transfected cortical neurons

were subjected to western blot analysis using anti-Pim-1 antibody. Actin is provided as loading control (left panel). Densitometric analysis of western immunoblots was performed as mention above (right panel). (c) Cortical neurons were transfected as above mentioned and then treated with camptothecin for 8 h as indicated. Pim-1 were immunoprecipitated and subjected to kinase assay using Cdc25A as substrate (left panel). Densitometric analysis was performed as mention above (right panel). (d) Western blot analyses showing down-regulation of NF- κ B p65 subunit in cells treated with NF- κ B siRNA compared with control siRNA. Actin is provided as loading control.

death as its deficiency delays death. (iii) Pim-1 deficiency also blocks Cdc25A activity, an activity that we have previously shown to be important for activation of Cyclin D1-associated kinase activity following DNA damage in neurons and consequent death. (iv) Pim-1 deficiency also blocks Cdk4/6 activity consistent with its importance in regulation of Cdc25A activity. Previously we have also reported that Cdc25A activity is also regulated by the checkpoint kinase Chk1. In this case, loss of basal Chk1 kinase activity contributes to increased Cdc25A activity. Accordingly, taken together with our current data, we suggest a model by which Cdc25A regulation is controlled by at least two different pathways, one involving the loss of Chk1 activity, and the other regulated by an increase in Pim-1 kinase activity. This is consistent with our observation that over-expression of Pim-1 alone does not lead to neuronal death or increased Cdc25A activity (Figs 3b and S1). This

dual control of Cdc25A activation makes sense in light of the need to ensure that activation of the critical Cdk death signal not occur unless warranted. This 'dual key' theme in death regulation is common and likely occurs at multiple levels to prevent accidental death. For example, we have previously shown that activation of the conserved mitochondrial death machinery is also regulated by both p53 and Cdk activation (Morris *et al.* 2001). In the case of Pim-1, this dual regulation is likely even more critical as Pim-1 may have normal neuronal function in limited contexts such as with consolidation of long-term potentiation (Konietzko *et al.* 1999).

While we have provided evidence for a Pim-1/Cdc25A relevant pathway, we do not rule out the possibility that Pim-1 may also act to regulate other factors. Indeed, even in regards to cell cycle regulation, Pim-1 can modulate a number of substrates. This includes the Cdk inhibitors, p21

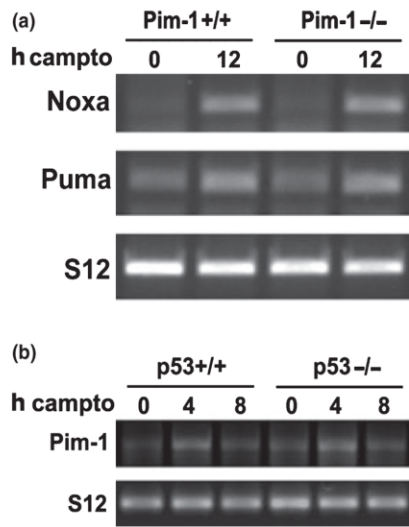


Fig. 8 (a) Pim-1 deficiency does not inhibit the p53-inducible genes Noxa and Puma. Cortical neurons from Pim-1^{+/+} and Pim-1^{-/-} mice were treated with and without camptothecin for 12 h. Noxa and Puma levels were then analyzed by RT-PCR as described in Material and Methods. S12 was also analyzed as a control for equal input. (b) p53 deficiency does not affect Pim-1 induction in response to camptothecin treatment. Cortical neurons from p53^{+/+} and p53^{-/-} mice were treated with and without camptothecin for 4 and 8 h. S12 was also shown as a control for equal input.

involved in G1/S progression and Cdc25C, a Cdc25 family member associated with G2/M progression (Wang *et al.* 2002; Bachmann *et al.* 2006). In regards to the former, Pim-1-mediated phosphorylation of p21 leads to its cytoplasmic sequestration, at least in proliferating cells (Wang *et al.* 2002). However, we did not observe any translocation of p21 in Pim-1 deficient neurons when compared to wild-type controls either basally or in the presence of DNA damage (unpublished data). Cdc25C directs dephosphorylation of Cyclin B-bound Cdc2 and triggers entry into mitosis during G2/M transition (Strausfeld *et al.* 1991). Interestingly, Cdc2 is implicated in multiple neuronal death models including K deprivation and in response to DNA damaging agent (Konishi *et al.* 2002; Konishi and Bonni 2003; Ueno *et al.* 2006). Whether this cell cycle Cdk member as well as a potential corresponding Pim-1/Cdc25C pathway of regulation is important in neuronal death will be interesting to explore more carefully.

Finally, it is important to reiterate that death regulation likely involves numerous pathways acting in concert to execute the life-death decision. In this regard, Pim-1 is only one player participating in this balancing act. This is evidenced by the fact that Pim-1 deficiency only delays neuronal death and does not completely prevent it. It is also possible that activation of the other two Pim family members: Pim-2 and Pim-3, which have similar activity

and overlapping functions as Pim-1 (Bachmann and Moroy 2005; Bullock *et al.* 2005), may compensate for the loss of Pim-1 in this death model. However, it is also equally likely that there are other death signals which act independently of Pim-1 regulated pathways to promote death. This includes p53 which is activated independently of abnormal cell cycle activation (Morris *et al.* 2001).

Regulation of Pim-1 upon DNA damage

What regulates the increase in Pim-1 levels following DNA damage? We had previously shown that NF- κ B plays a dual role in regulating neuronal loss where basal NF- κ B activity is critical for survival while acute NF- κ B activation signals death through induction of p53 message (Aleyasin *et al.* 2004). It is important to point out that NF- κ B, however, is not the only regulator of p53. For example, post-translational modification of p53 is also regulated by upstream kinases such as ATM (Canman *et al.* 1998), which presumably regulates the stability of the p53 protein. p53 is also regulated by calpain activation in a manner which is not clear yet (Sedarous *et al.* 2003). Interestingly, we now show that NF- κ B is also critical for induction of Pim-1 message/protein which in turn is critical for Cdk activation. This evidence suggests that NF- κ B promotes the up-regulation of at least two pathways critical for DNA damage-induced death, one involving p53 and the other Cdk activation. This work is interesting in light of our previous findings that inhibition of Cdks did not affect p53 activation and that conversely, p53 deficiency did not affect DNA damage-induced pRb phosphorylation (Morris *et al.* 2001). We interpreted these observations to mean, at the time, that Cdks and p53 were on parallel pathways. However, our new data suggest that the signaling picture is a bit more complex and that there is some degree of cross-talk upstream of Cdks and p53. Finally, while our evidence suggests that regulation of Pim-1 levels is important, it does not rule out the potential importance of post-translational modification of Pim-1. For example, protein phosphatase 2A (PP2A), a serine/threonine phosphatase, which is reported to associate with Pim-1 *in vivo*, dephosphorylates Pim-1 and decreases its kinase activity (Losman *et al.* 2003; Ma *et al.* 2007). Interestingly, a recent study shows that PP2A appears to be a pro-survival factor during neuronal death and its phosphatase activity is significantly decreased following cerebral hypoxia (Truttmann *et al.* 2004). Whether or not PP2A is critical for Pim-1 regulation in neuronal death will be of interest for further study.

Relevance of this pathway in injury and disease

The potential importance of the NF- κ B/Pim-1/Cdc25A/Cdk pathway in a wider context than that presented here, is potentially intriguing. Just as one example, many of the players described here have also been implicated in ischemic injury. There is now increasing evidence in multiple stroke models that cell cycle Cdks are activated and required for

death (Osuga *et al.* 2000; Katchanov *et al.* 2001; Rashidian *et al.* 2005, 2007). Our previous report has shown that inhibition of Cdk4, and its activator Cyclin D1, plays critical roles in delayed death component of ischemic/hypoxic stress by regulating the pRb protein (Rashidian *et al.* 2005). Although the potential role of the cell cycle regulators in neuronal death has been hypothesized, little has been done concerning how cell-cycle Cdks are activated, particularly in an *in vivo* context. Interestingly, NF- κ B is activated following stroke and appears to have both protective and deleterious roles similar to our DNA damage models *in vitro* (Schneider *et al.* 1999). Finally, Cdc25A phosphatase activity increases in cerebellar granule neurons upon hypoxia/reoxygenation and one of the pharmaceutical inhibitors for Cdc25A, NSC95397 protects these neurons from hypoxia-induced death (unpublished data). Whether or not these signals are linked and regulated by Pim-1 in a similar fashion in injury models such as stroke or other degenerative diseases where Cdks appear to be activated will be an important question for future research.

In conclusion, past and present data present a growing model by which a complex array of signals regulate three major pathways of death cell cycle Cdks, p53, and the JNKs (see Fig. 9). In the Cdk branch of signals, Pim-1 activation and loss of Chk1 activity both contribute to Cdc25A activation and consequent increase in Cdk4/6 activity. Pim-1 activity is regulated, at least in part by an increase in levels

regulated by NF- κ B. NF- κ B also works in conjunction with other regulators such as ATM and calpains to regulate p53 activation. Cdks act on the tumor suppressor pRb family members which in turn regulate downstream effectors such as B-myb and C-myb (Liu *et al.* 2004). Cdks also activate the PP2A inhibitor I2PP2A protein (Qu *et al.* 2007) and the transcription co-activator Cited2 which acts in turn to regulate a peroxisome proliferator-activated receptor- γ mediated pathway of death (65). p53, once activated regulates a series of pro-death molecules such as Puma and Apaf (Fortin *et al.* 2001; Cregan *et al.* 2004; Uo *et al.* 2007). There is also at least a third pathway involving the stress activated JNKs (Ghahremani *et al.* 2002). This pathway appears to be regulated by upstream Cdc42 and plenty of SH3 domains/JNK interacting proteins as well as Cdks (Ghahremani *et al.* 2002; Kukekov *et al.* 2006). These three pathways coordinate ultimately, in ways which are not completely clear, to control the translocation and activation of Bax to the mitochondria, cytochrome *c* release and activation of downstream effector caspases. Accordingly, our present data delineate the regulatory steps of a critical pathway in DNA damage induced death provides insight into the understanding of mechanisms of cell cycle activation.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Pim-1 over-expression does not significantly enhance Cdc25A activity.

Figure S2. NF- κ B down-regulation blocks DNA damage-induced up-regulation of Pim-1.

Appendix S1. Supplementary Materials and Methods.

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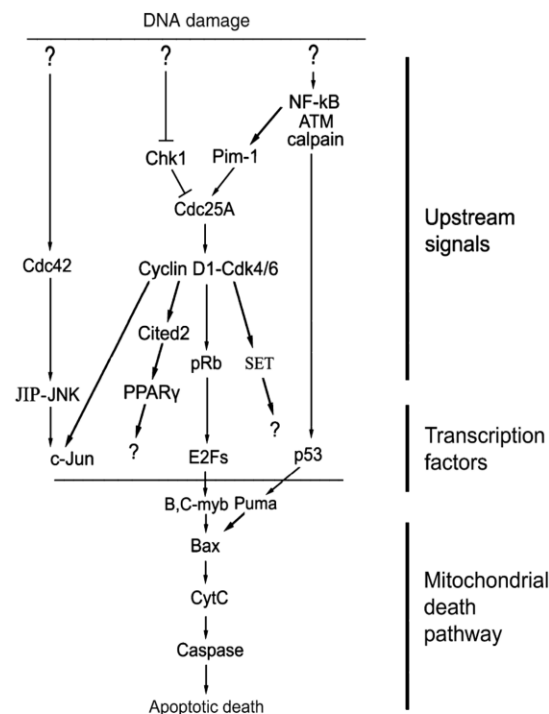


Fig. 9 Schematic model of the signaling pathways involved in DNA damage induced death.

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