

**Endocytic Trafficking is Required for Neuronal Cell Death through
TGF-beta Signaling in *Drosophila melanogaster***

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Dedication

To my parents, Yan Cui and Ryan Wang

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Abstract

Programmed cell death (PCD) is an essential feature during the development of the central nervous system in *Drosophila* as well as in mammals. During metamorphosis, a group of peptidergic neurons (vCrz) are eliminated from the larval central nervous system (CNS) via PCD within 6-7 h after puparium formation. To better understand this process, we first characterized the development of the vCrz neurons including their lineages and birth windows using the MARCM (Mosaic Analysis with a Repressible Cell Marker) assay. Further genetic and MARCM analyses showed that not only Myoglianin (Myo) and its type I receptor Baboon is required for neuron cell death, but also this death signal is extensively regulated by endocytic trafficking in *Drosophila melanogaster*. We found that clathrin-mediated membrane receptor internalization and subsequent endocytic events involved in Rab5-dependent early endosome and Rab11-dependent recycling endosome differentially participate in TGF- β [beta] signaling. Two early endosome-enriched proteins, SARA and Hrs, are found to act as a cytosolic retention factor of Smad2, indicating that endocytosis mediates TGF- β [beta] signaling through regulating the dissociation of Smad2 and its cytosolic retention factor.

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List of abbreviations

AEL: After Egg Laying

AP2: Adaptor Protein 2

APF: After Puparium Formation

APR: Accessory Planta Reactor

Babo: Baboon

BDNF: Brain-Derived Neurotrophic Factor

BMP: Bone Morphogenetic Proteins (BMPs),

BR-C: Broad Complex

CAP: Crz-Associated Peptide

C. elegans: *Caenorhabditis elegans*

CCV: Clathrin-Coated Vesicle

CHC: Clathrin Heavy Chain

CNS: Central Nervous System

Crz: Corazonin

Dcp-1: *Drosophila* caspase-1

DIAP: *Drosophila* Inhibitor of Apoptosis

DL: Dorsal Lateral

DLP: Dorsal-Lateral-Posterior

DM: Dorsal Medial

EcR: Ecdysone Receptor

EEA1: Early Endosome Antigen 1

FGF: Fibroblast Growth Factor

FLP: Flippase

FRT: Flippase Recombination Target

FYVE: Fab1, YOTB, Vac1 and EEA1

Gbb: Glass bottom boat

GDF: Growth and Differentiation Factor

GFP: Green Fluorescent Protein

GMC: Ganglion Mother Cell

Hid: Head Involution Defective

Hrs: Hepatocyte growth-factor-regulated tyrosine-kinase substrate

IAP: Inhibitor of Apoptosis

IHC: Immunohistochemistry

IR: Immunoreactive

IRy: Immunoreactivity

JH: Juvenile Hormone

MARCM: Mosaic Analysis with a Repressible Cell Marker

Mav: Maverick

MB: Mushroom Body

M. sexta: Manduca sexta

Myo: Myoglianin

NB: Neuroblasts

NGF: Nerve Growth Factor

NT: Neurotrophin

PCD: Programmed Cell Death

PDGF: Platelet-derived Growth Factor

PI-3K: Phosphoinositide 3-kinase

PRD: Proline-rich Domain

SARA: Smad Anchor for Receptor Activation

SG: Salivary Gland

Shh: Sonic hedgehog

RHG: Rpr, Hid, Grim

TGF: Transforming Growth Factor

TNF: Tumor Necrosis Factor

Ubx: Ultrabithorax

UIM: Ubiquitin Interacting Motif

USP: Ultrspiracle

vCrz: Crz in the ventral nerve cord

VNC: Ventral Nerve Cord

Wit: Wishful thinking

Chapter One

Background and Significance

Extensive evidence showed that neuronal cell death occurs in almost all regions and cell types in the nervous system from the early embryonic proliferating stages until the late adult stage. It includes neural stem cells, proliferating precursors, and differentiated neurons, such as motoneurons and sensory neurons (Oppenheim, 1989). Even long project neurons and local circuit neurons in the brain and spinal cord are found to display restricted periods of cell death (Oppenheim, 1991). Although the magnitude and duration of the cell death may vary through different stages and different populations, it is estimated that at least half of the original cell population undergoes cell death and is eliminated in the developing nervous system (Yeo and Gautier, 2004). In extreme cases, most or all cells of some units die since those neuronal structures degenerate during insect and amphibian metamorphosis. Thus, cell death in the nervous system occurs on a very large scale, indicating that it plays an important role in normal development.

I. Neuronal cell death in embryonic stage

In the developing nervous system, programmed cell death (PCD) occurs as early as neural tube formation and carries on throughout terminal differentiation of the neural network in adult stages (Naruse and Keino, 1995). Early PCD is characterized as an integral part of CNS morphogenesis during embryonic stages. It takes place early during embryogenesis within populations of proliferating neural precursors and newly postmitotic neuroblasts. For

example, during *Drosophila* embryogenesis, the first PCD occurring within the neural population is observed in the head region at stage 11, spatially and temporally overlapping with some embryonic neuroblasts or neuronal precursors (Abrams et al., 1993; Urbach et al., 2003). The functional basis of early neural cell death is not well known. Some possible roles are proposed to be involved in cell number adjustment, removal of damaged or misspecified cells (Boya and de la Rosa, 2005). Study from developmental PCD in *C.elegans* and *Drosophila* found various signaling pathways integrating this process, including bone morphogenetic proteins (BMPs), Wnts, fibroblast growth factors (FGFs), and Sonic Hedgehog (Shh) (Yeo and Gautier, 2004). Since those signals are also associated with multiple cellular and developmental processes such as proliferation and differentiation of neuroepithelial cells, it indicates that early neuronal cell death is an integral part of neurogenesis.

The most distinct period of neuronal cell death in embryonic stage is observed at stage 16 when the first twitching movements occur in the neuromuscular system (Abrams et al., 1993). At least 4% of the neural population in the ventral nerve cord is eliminated through PCD during this period. Their loss is thought to reflect a competition for trophic signals produced by their innervating target cells; thus it is also called neurotrophic cell death (Boya and de la Rosa, 2005). The neurotrophic theory postulates that excessive neurons are generated in the beginning and they compete for the limited amount of survival factor. Only the neurons that receive enough neurotrophic factors survive, while the others undergo PCD (Davies, 2003). Therefore, the regulation of neuronal cell number is postulated as a major role of this process,

comparing with other function such as refinement of connection and carving of the sophisticated cytoarchitecture of the nervous system.

Neurotrophic factors include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin (NT) and they are small, basic, secretory proteins that are essential for survival of postmitotic differentiated neurons both in vivo and in vitro (Binder and Scharfman, 2004). Deprivation of growth factor source or early removal of the innervated target leads to neuron PCD during development. A similar function of the neurotrophic factors is also observed in the adult brain. In a variety of neurological diseases, neurotrophic factors are known to be capable of protecting against excitotoxic neuronal death (Mattson et al., 1993). Aspects of the potential relevance of these findings to the neurodegenerative disease need to be revealed.

II. Neuronal cell death associated with postembryonic stage and vCrz cell death

The occurrence of massive neuron cell death after embryogenesis is a phenomenon characteristic of animals that undergo dramatic life-history changes. For these cells, tissues, and organs that may have had a transient function in early stage but that becomes useless at a later stage will be eliminated during stage transition. This type of degeneration can be termed metamorphic cell death and has been well documented in insects, especially in tobacco hornworm *Manduca sexta* and *Drosophila melanogaster* (Truman et al., 1992).

In the central nervous system (CNS) of *M.sexta*, the highly organized clusters of neurons and their processes form a series of ganglia joined by paired longitudinal nerve cords. During metamorphosis, two pair of excitatory accessory planta reactor (APR) motoneurons in A5

and A6 segment (Weeks and Truman, 1984), which innervate their larval muscle targets, die during the prepupal stage. Surprisingly however, similar motoneurons in segments A3 and A4 die later at the end of pupal stage. Furthermore, approximately half of the nerve cells in the abdominal region degenerates within three days after the adult emerges (Weeks and Truman, 1984). Most neurons that die during this stage are motor neurons that have lost their innervated muscle or had been involved in the regulation of behavior displayed by one life stage and not the other.

During metamorphosis of *D. melanogaster*, neuronal death is found to be prominent mostly in the thoracic and abdominal neuromeres of the CNS (Kimura and Truman, 1990; Robinow et al., 1993; Lee et al., 2011). Unlike in *M. Sexta*, the smaller size of the nerve cells and the fused nature of the ventral neural mass in *Drosophila* make it more challenging to identify individual neuron lineages that are doomed to die. Lack of appropriate neuron lineages result in a large restriction of the studies in fly and thus researchers still largely depend on microscopic cell counts to identify degenerating neurons. Recently, a subgroup of Corazonin producing neurons (called vCrz) in *Drosophila* CNS were characterized and found to undergo cell death during metamorphosis (Choi et al., 2006). These peptidergic neurons display large cell bodies (somata) with high level of steady-state secretory activity and extend long and complex neuronal processes, which allow us to visualize them easily by anti-Crz antibody or Crz gene reporters (Fig. 1-1).

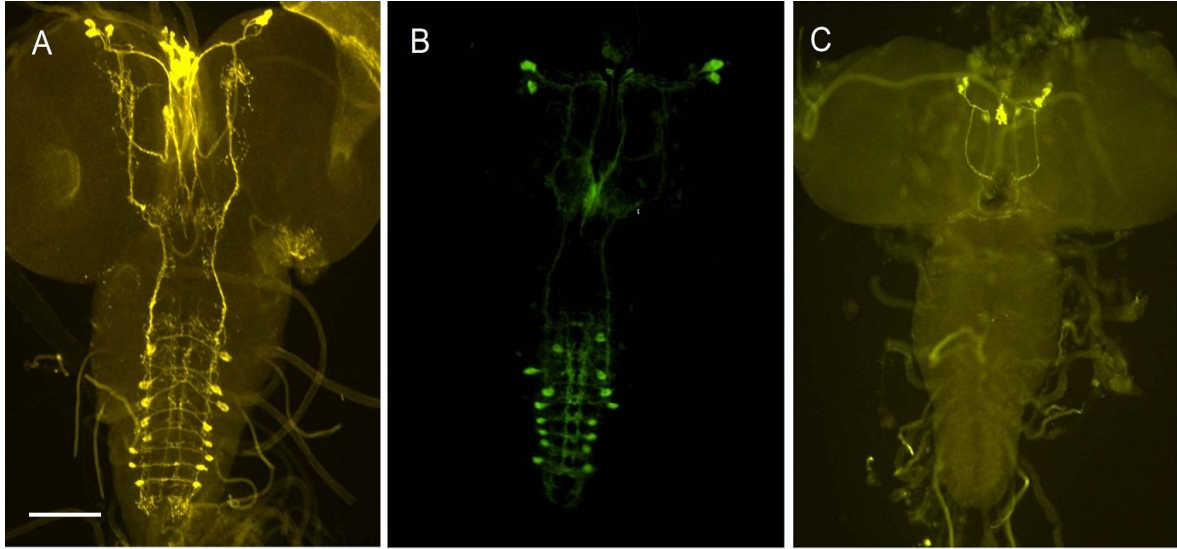


Figure 1- 1. Crz neurons in WL3 and prepupal stage.

(A) Crz-immunoreactive neuron at WL3. (B) Crz-gal4 induced GFP expression at WL3. (C) Crz-immunoreactive neuron at 6 hours APF (after puparium formation). Scale bar = 100 μ m.

III. Ecdysone-dependent neuronal cell death

Ecdysteroids are the steroidal hormones, regulating molting, metamorphosis, reproduction and diapause of insects (Dinan and Lafont, 2006). During metamorphosis, ecdysteroid is critical for regulation of neuronal death in the CNS. Truman and colleagues demonstrated that a decline in ecdysone titers triggered neuronal death after adult emergence and manipulating its levels altered the timing of neuronal death (Truman and Schwartz 1984). On the other hand, the same steroid also regulates neuronal death in the ventral ganglia during early stage of metamorphosis. A peak of ecdysone signals death, not life at the larval-pupal transition (Truman and Schwartz, 1984; Truman et al., 1992). In *M.sexta*, APR neurons die as a result of exposure to the rising levels of ecdysone at the end of the caterpillar stage (Weeks 1987). These neurons also appear to express nuclear receptors for ecdysteroids at this time. According to its dual nature at different stages, ecdysone is more likely acting as a timing signal, rather than directly triggering cell death.

One possible explanation for this multiple- effects with only one hormone is that more than one receptor exists and they may execute different functions. Cloning and sequencing of the nuclear receptor for ecdysone (EcR) demonstrated that fruit fly has three different isoforms of receptor: EcR-A, EcR-B1, and EcR-B2. These isoforms share C-terminal DNA- and ligand binding domains, but are variable in their N-termini (Talbot et al., 1993). Interestingly, they have different expression profiles and distinct biological functions in the CNS (Truman et al., 1994). It has been shown that each of these EcR isoform forms a heterodimer with Ultraspiracle (USP), another receptor (Yao et al., 1993). The interaction

between them significantly increases the binding affinity for ecdysone and in turn stabilizes the interaction of complex with their target DNA. Once bound by ecdysone, EcR induces the transcription of a set of transcription factors (White et al., 1997).

EcR-induced transcriptional hierarchy has been extensively documented in cell death of salivary glands and midgut during metamorphosis. It includes sequential activation of primary-response genes, which in turn regulates the expression of secondary-response genes (Thummel, 1996). The primary-response genes include: *Broad Complex (BR-C)*, which encodes several isoforms of BTB-zinc finger transcription factors; *E74*, which encodes an ETS-domain transcription factor; *E75*, which encodes the nuclear receptor; and *E93* which encodes a novel DNA binding protein. These primary response genes play essential roles in developmental responses to ecdysone. For example, *E93* mutant results in early stage arrest of salivary gland degeneration (Lee et al., 2000a). A similar scenario occurs in *BR-C* and *E74* mutants (Lee and Baehrecke, 2001). The secondary ecdysone-response genes are core cell death genes, including *rpr*, *hid*, *grim*, *dark*, and caspase gene, *dronc* (Fletcher and Thummel, 1995). As proapoptotic proteins, Rpr, Hid and Grim control caspase activation by multiple mechanisms, including activation of caspase through Dark, or through antagonizing caspase inhibitors such as DIAP1 (Yoo et al., 2002). Taken together, ecdysone orchestrates a large variety of molecular events temporally and spatially for the destruction of obsolete larval tissues via upregulation of death activators, caspase activation, and repression of apoptosis inhibiting proteins.

IV. TGF- β dependent cell growth arrest and cell death

Transforming growth factor β is a member of a large family of multifunctional secreted polypeptides involved in cell proliferation, cell differentiation, cell death and various morphogenetic processes during development (Derynck and Akhurst, 2007; Kingsley, 1994).

The diversity of TGF- β responses in different cell types and different developmental processes is generated by controlling transcription of various target genes (Siegel and Massague, 2003). Furthermore, many cofactors modulate TGF- β to activate and repress gene transcription according to its developmental as well as environmental cues (ten Dijke and Hill, 2004). It has been reported that TGF- β is a negative regulator of the maintenance of the T-cell homeostasis, and loss of TGF- β signaling in T cells results in abnormal T-cell differentiation and autoimmune disease (Gorelik and Flavell, 2000; Hahm et al., 2000). TGF- β also possesses a strong inhibitory effect on the proliferation of epithelial cells. Such anti-mitotic effect is associated with the cancer development in the absence of TGF- β signaling (Yue and Mulder, 2001).

A growing body of evidence indicates that TGF- β induces cell death in many different cell types, including normal B cells and a lymphoma B-cell line (Chaouchi et al., 1995). Extensive lymphocytic hyperproliferation and systemic lupus erythematosus-like autoantibodies are seen in TGF- β 1-deficient mice (Dang et al., 1995; Shull et al., 1992). Conversely, ectopic expression of TGF- β 1 in the liver results in hepatic fibrosis and cell death (Sanderson et al., 1995).

On the other hand, TGF- β is also a stimulator of cell proliferation and differentiation and

acts as an antiapoptotic factor. The presence of exogenous TGF- β 1 prevents cell death in serum-deprived macrophages and human lung carcinoma cells (Chin et al., 1999; Huang et al., 2000). Therefore, studying the signaling mechanism is very important for better understanding of this diverse or antagonistic effect in different scenarios.

V. TGF- β signal transduction

The TGF- β superfamily of growth factors comprises 7 members in *Drosophila* and more than 30 members in mammals. The mammalian members include three TGF- β isoforms, four activin β -chains, the protein nodal, ten bone morphogenetic proteins (BMP) and eleven growth and differentiation factors (GDFs) (Schmierer and Hill, 2007). All ligands are first synthesized as inactive precursor forms, a dimeric pre-proprotein, which contains an N-terminal prodomain and a C-terminal mature domain (Derynck et al., 1985). After the cleavage of the prodomain, the mature dimeric forms are secreted. A characterized structural feature is found in the mature protein, the so-called ‘cysteine knot’, a structure motif that is formed by three intramolecular disulfide bonds between six highly conserved Cys residues (Shi and Massague, 2003). The cysteine knot facilitates the protein to dimerize, which avoiding exposure of hydrophobic residues to the aqueous surrounding. This conformation is not uniquely possessed by TGF- β proteins and is also found in other cytokine families, such as NGF, PDGF, glycoprotein hormone (GPH) and IL-17, which together constitute a large superfamily of cysteine knot proteins (Sun and Davies, 1995).

To initiate its intracellular signaling cascade, the ligand binds and brings together pairs of receptors, named type I and type II receptors (Fig. 1-2). Both of them are a single-pass

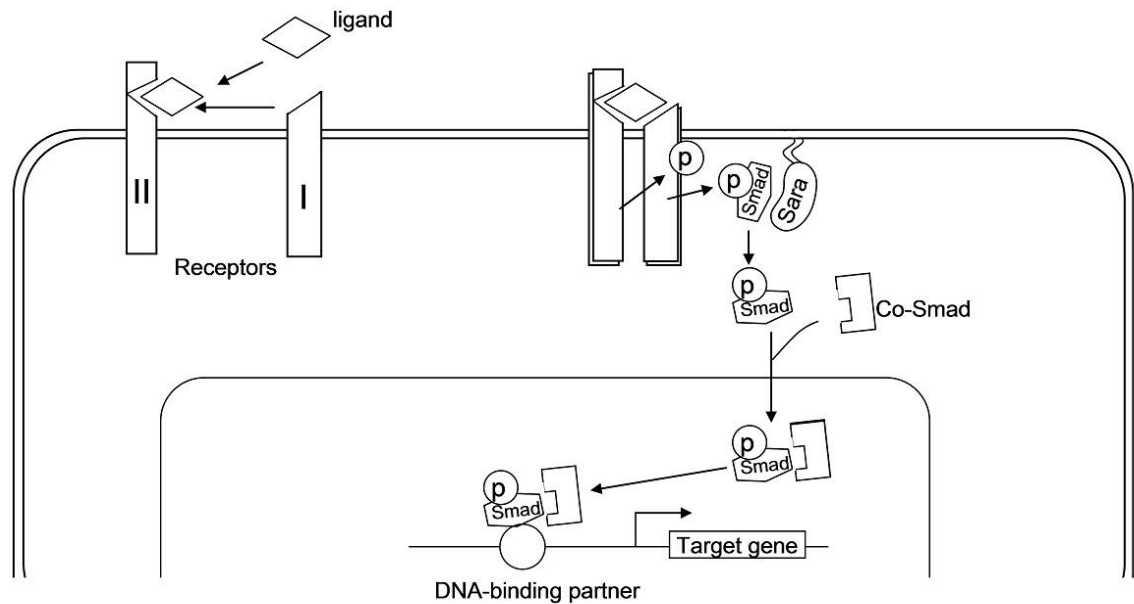


Figure 1- 2. Conventional TGF- β signal pathway.

TGF- β signals via two receptor Ser/Thr kinases, type I and type II receptor. In the presence of ligand, type II phosphorylates specific Ser residues in type I, mediating its activation. In turn, R-Smads are recruited to the receptor complex through the membrane-associated molecule SARA. The subsequent phosphorylation in the SXS motif releases Smads from retention in the cytoplasm and allows them to translocate into the nucleus. Smad4 serves as a common partner of activated Smads and helps to execute their function together. In the nucleus, activated Smad proteins interact with DNA and transcription factors, generating hundreds of early gene responses.

transmembrane protein with a large intracellular Ser-Thr kinase domain and an extracellular domain (Shi and Massague, 2003). Generally, the ligand first binds the pre-formed dimers of its high affinity receptor, the type II receptor, but for BMP-2, its high affinity receptor is the type I receptor. After binding of the high-affinity receptor, the lower-affinity receptor is then brought to the complex (Massague, 1998). In most cases, type II and type I receptors are not in direct contact with each other, but are held close together by the bound ligand (Allendorph et al., 2006). Following binding of ligands to the receptors, the type I receptor is phosphorylated by the constitutively active type II receptor kinase. The phosphorylation event specifically occurs at several Ser and Thr residues in Gly/Ser-enriched domain (GS domain), which is conserved in all type I receptors. Upon phosphorylation in the GS domain, the type I receptor activates, by phosphorylation, a subset of Smad transcription factors, called receptor-regulated Smads (R-Smads). In addition to the R-Smads, two other functional classes of Smad proteins are found, the co-mediator Smads (co-Smads) and the inhibitory Smads (I-Smads). The phosphorylated R-Smads are able to form the heteromeric complex with the co-Smad and then translocate into the nucleus. In the basal state, the R-Smads are retained in the cytoplasm, possibly through the interaction with Smad anchor for receptor activation (SARA). SARA is highly concentrated in EEA1-positive early endosomes through the interaction of the FYVE domain with the membrane lipid PtdIns(3)P. Following receptor-mediated phosphorylation, SARA is disassociated from Smads by decreasing their affinity between each other. However its affinity to the co-Smad is concomitantly increased (Xu et al., 2000). Hrs, another FYVE domain protein involved in

endosomal trafficking, may play a role similar to that of SARA (Miura et al., 2000).

The I-Smads act as negative regulators of TGF- β signaling by either binding to the activated type I receptor and thus interfering with the phosphorylation of R-Smads (Kavsak et al., 2000; Suzuki et al., 2002) or by direct binding to the phosphorylated R-Smads thereby competing with the co-Smad (Hata et al., 1998). More indirect mechanisms by which I-Smads inactivate receptors have been documented. The I-Smads interact constitutively with ubiquitin ligase SMURFs (Smad ubiquitylation regulatory factors) (Zhu et al., 1999) and therefore target them to the activated receptors, which causes the degradation of the receptors in the proteosome (Ebisawa et al., 2001; Tajima et al., 2003). I-Smads themselves also undergo ubiquitination and degradation in this process.

The TGF- β superfamily can be further divided into two major branches: BMP or Activin/TGF- β , according to their intracellular effectors (Feng and Derynck, 2005). The BMP subfamily signals through R-Smads1/5/8 whereas the Activin/TGF- β utilizes R-Smads2/3. In *Drosophila*, seven members of the TGF- β family have been identified. Decapentaplegic (Dpp), Screw (Scw), and Glass bottom boat (Gbb) belong to BMP family and have been shown to specifically signal through type-I receptors Thickveins (Tkv) and Saxophone (Sax), whereas dActivin (dAct), Dawdle (Daw), Myoglianin (Myo), and Maverick (Mav) are members of Activin/TGF- β branch and propagate signal through only one type-I receptor, Baboon (Babo) (Lo and Frasch, 1999; Nguyen et al., 2000; Serpe and O'Connor, 2006; Zhu et al., 2008). Both subfamilies share the type II receptors: Punt (Put) or Wishful thinking (Wit). As the only type-I receptor in Activin pathway, Babo has been

known to regulate several developmental processes including mushroom body remodeling during metamorphosis (Zheng et al., 2003), morphogenesis of ellipsoid body neurons in the adult (Zheng et al., 2006), motor axon guidance in the embryo (Serpe and O'Connor, 2006) and neuron proliferation in the larval brain (Brummel et al., 1999). The contrast between the wide spectrum of cellular and developmental responses elicited by TGF- β signal and the fact that they utilize a relatively simple signal transduction mechanism with a few Smads or receptors imply that there is an extensive regulation controlling the different biological responses on different cellular levels. Several lines of evidence showed that transcriptional activation by Smads in the nucleus play an important role for the specificity and versatility of signaling (Feng and Derynck, 2005). A remarkable diversity of DNA sequence-binding transcription factor were identified to be able to directly interact with Smads (Feng and Derynck, 2005). In addition to transcriptional regulation in nucleus, the endocytic regulation of TGF- β signal transduction has been intensely investigated on the mechanism of receptor trafficking and dynamic maintenance of nuclear Smad accumulation.

VI. Endocytosis and molecular sorting

VI-1. Membrane internalization

Endocytosis begins with membrane internalization, by which extracellular materials are enclosed and moved into a vesicle within the cell. The content of the vesicle is then delivered to an endosomal compartment where it is sorted to multiple locations such as recycling back to the cell surface or trafficking to lysosomes for degradation (Fig. 1-3) (Seto

et al., 2002).

In higher eukaryotic cells, endocytosis occurs by one of two general mechanisms: clathrin-dependent and clathrin-independent pathway (Le Roy and Wrana, 2005).

Clathrin-dependent endocytosis involves the formation of pit using a clathrin coat. It is induced through the interaction of cytosolic proteins with components of the invaginated membrane. Clathrin-coated pit, as an assembled structure, is specialized to concentrate surface proteins for internalization. It possesses a striking lattice morphology that results from the polymerization of clathrin from the cytosol to the membrane (Brodsky et al., 2001). Clathrin-independent endocytosis occurs at sites of lipid rafts, where cholesterol is highly enriched in the plasma membrane (Le Roy and Wrana, 2005). It was known that some of the clathrin-independent events are mediated by calveolin, accompanied by forming membrane invaginations called caveolae. The mechanisms underlying clathrin-independent pathway are not well understood.

Clathrin-coated vesicle biogenesis is extensively studied in nerve terminals since a large number of synaptic vesicle formation occur during neuronal information flows along nerve system. It is known to be essentially required for recycling of synaptic-vesicle components after release of neurotransmitter in response to action potentials. Later, non-neuronal cells also are found to have a similar process. According to their intermediate morphology during the formation of clathrin-coated vesicle, three mechanistically sequential stages are recognized: (1) assembly of clathrin into a polygonal lattice at the site of endocytosis, (2) invagination of the membrane to form coated pits, and (3) pinching-off of the coated pit,

forming clathrin-coated vesicles (Mousavi et al., 2004).

Clathrin-mediated internalization is initiated by the reallocation of membrane proteins into clathrin-coated pits (Brodsky et al., 2001). During formation of the pits, AP2, a clathrin-binding adaptor complex, binds directly or indirectly to transmembrane receptors (Kirchhausen et al., 1997), allowing clathrin to cluster into a lattice morphology that pulls membrane inwardly. The clathrin-coated pit is a multi-component endocytic unit; besides clathrin and AP-2, several accessory proteins are also implicated in the formation of clathrin-coated pits.

Clathrin

Clathrin is assembled as a triskelion shape composed of three heavy chains and three light chains. The three heavy chains form the backbone of the clathrin lattice, and the three light chains regulate assembling of a clathrin lattice (Ybe et al., 1998). The heavy chain is remarkably invariant among species and contains multiple subdomain: the globular N-terminal domain, a relatively curved region called ankle, distal leg, the knee, proximal leg, and the C-terminal end (Greene et al., 2000; Schmid, 1997). Based on the study of crystal structure, the N-terminal domain forms a β -propeller morphology and contains a binding site, which allows interacting with a number of endocytic proteins, including AP-2 (Mousavi et al., 2004). Two isoforms of the light chain exist named LCa and LCb. They are known to be associated randomly with clathrin heavy chains in the triskelion.

Dynamin

Dynamin is a large GTPase implicated in the regulation of budding and scission of the coated

pit during endocytosis. The GTPase domain, localized in the N terminal, is known to have low affinities for GTP and high intrinsic GTPase activity. The PH (pleckstrin homology) domain in the central region is involved in binding to PtdIns(4,5)P₂. The GED (GTPase effector domain) domain existed as a coiled-coil structure in the C-terminal region regulates dynamin oligomerization and its assembly. It is known that self-assembly is important for dynamin's GTPase activity *in vivo*. Finally, PRD (proline-rich domain) domain interacts with other proteins, such as SH3 (Src homology 3) domain containing protein and actin binding protein (Hinshaw, 1999; Kessels et al., 2001; McNiven et al., 2000; Muhlberg et al., 1997; Schmid et al., 1998). A temperature sensitive mutant of dynamin was found in *Drosophila* (*shibire*, *shi* for short). At non-permissive temperatures, the mutants display paralysis and a presynaptic failure of evoked synaptic transmission because of synaptic vesicle recycling defect (Kitamoto, 2002).

VI-2. Early endosome fusion and sorting

Clathrin-coated pits are pinched off from the membrane, followed by the formation of clathrin-coated vesicles (CCVs). This process is concerted with disassembly of coat components, which is required for fusion of the primary endocytic vesicle. These small, primary endocytic vesicles then fuse with the early endosome, and early endosomes fuse with one another. The mechanism of the early endosome fusion has been well characterized and several components are found to be crucial during this process.

Rab proteins are small GTPases that are implicated principally in the control of vesicle

docking and fusion (Gonzalez and Scheller, 1999; Mohrmann and van der Sluijs, 1999).

Like other small GTPase, Rab proteins possess a capacity of conformational transition upon binding to either GDP or GTP. Upon external stimulation, GDP/GTP exchange factors (GEFs) interact with Rab, and catalyze the conformation switch from GDP-bound inactive form to GTP-bound active form. This transition can be reverted by GTPase activating protein (GAP). In the GTP-bound active form, Rab protein, along their cognate partners (effectors), facilitates targeting of endocytic vesicles to different acceptor membranes (Molendijk et al., 2004; Pfeffer and Aivazian, 2004). Below are several protein components essential for such vesicular trafficking.

Rab5

Rab5 is specifically associated with early endosomes. It regulates the fusion between early endosomes with endocytic vesicles, as well as the homotypic fusion between each other (Olkkonen and Stenmark, 1997). The expression of a Rab5 constitutively active, GTPase-deficient form (Q79L) mutant results in the formation of giant endosomes; conversely, the expression of the mutant that preferentially binds GDP (S34N) inhibits endocytosis, resulting in the formation of very small endosomes (Stenmark et al., 1994). The active Rab5 is equipped to fulfill their various roles in membrane trafficking through binding to their specific effectors. Among their potential effectors, Rabaptin-5 (Stenmark et al., 1995) and EEA1 (Simonsen et al., 1998) are well known.

EEA1 (Early endosome Antigen 1)

As one of the most important effectors of Rab5, EEA1 is a membrane bound endosome

fusion promoting protein. Homotypic fusion study showed that EEA-1 is essential of early endosome fusion, and at high levels, it is the only cytosolic factor required for conferring minimal fusion activity (Christoforidis et al., 1999). EEA1 is a large coiled-coil protein that can potentially interact with other proteins. It contains phosphatidyl inositol-3-phosphate [PI(3)P]-binding FYVE domain in its N-terminal and two Rab5 binding domains, among which, one is adjacent to the FYVE-finger, and the other is a C2H2-type zinc finger at the N terminus (Kutateladze and Overduin, 2001; Mu et al., 1995). The affinity of EEA1 for PI3P and Rab5-GTP is relative low, allowing for the specific enrichment in membranes that contain both of these components under certain physiological conditions (Lawe et al., 2000; Simonsen et al., 1998). This is presumably underlying reason that EEA1 is exclusive targeted to early endosome (Simonsen et al., 1998).

Hrs (hepatocyte growth-factor-regulated tyrosine-kinase substrate)

Another FYVE finger protein that has been implicated in membrane fusion and trafficking is HRS, a hepatocyte growth-factor-regulated tyrosine-kinase substrate (Komada and Kitamura, 1995). Like EEA1, it is also present in early endosomal membranes and is required for sorting of endocytosed proteins. Hrs contains an ubiquitin interacting motif (UIM) and a C-terminal clathrin interacting domain, allowing it to bind ubiquitinated proteins and sorts them into clathrin-coated microdomains of the early endosomes. By using the chimeric combination of the ubiquitin and human transferrin receptor (TfR), Raiborg and its colleague showed that overexpression of Hrs strongly inhibits recycling of ubiquitinated receptors by concentrating them in the early endosome. The ubiquitinated proteins are subsequently

sorted to the lysosome for degradation (Raiborg et al., 2002).

VI-3. Recycling endosome and late endosome

After sorting in early endosome, the endocytic cargo is either transported to the plasma membrane for recycling or to the lysosome for degradation (Gu and Gruenberg, 1999). The lysosome is the last compartment of the endocytic pathway, and most of the materials delivered to this degradative compartment must pass through the early and late endosomes (Bright et al., 1997). For recycling route, recycling endosome is essential compartment and it is believed to bud off from early endosome membranes and then fuse directly or indirectly with the plasma membrane (Mellman, 1996). Therefore, early endosomes act as a sorting station, directing the cargo to the different destinations with precision. However, the mechanism of endocytic cargo sorting and recycling is still not well understood. A small G-protein Rab7 is known to be associated with late endosomes. Expression of a dominant negative Rab7 blocks the regular trafficking of certain cargo molecules from early endosomes to late endosomes (Feng et al., 1995), indicating that Rab7 is required for regulating early to late endosome transport.

In contrast, another Rab protein, Rab11 is enriched in the pericentriolar recycling endosome, where it controls the traffic route through the recycling endosome (Green et al., 1997; Ren et al., 1998; Ullrich et al., 1996). Overexpression of Rab11 mutants results in changes of the morphology of recycling endosome and the corresponding endocytic trafficking. It indicates that Rab11 is essential of structural and functional properties of the

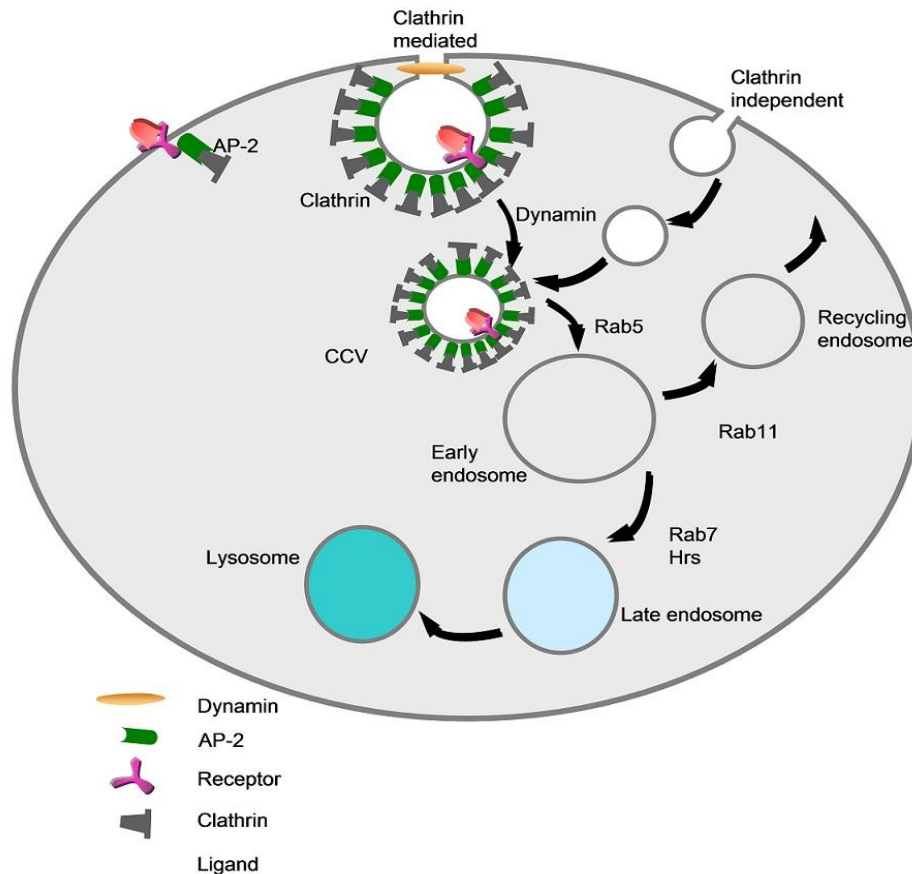


Figure 1- 3. Endocytosis pathway and players.

Transmembrane receptors and their ligands are mainly internalized through Clathrin-mediated endocytosis. Receptors are sequestered into the pits through direct interaction with clathrin coat adaptor protein complex AP2 or other adaptor proteins. Clathrin undergoes polymerization and drives the invagination of the pit. During this process, GTPase dynamin serves as a clipper to pinch off the vesicle. Following their internalization into early Rab5-containing endosomes, receptors can traffic to the recycling compartment that contains Rab11 or undergo a Rab7- and Hrs-dependent, degradative route through late endosomes and then lysosomes. Several clathrin independent pathways of endocytosis also exist, even though the exact mechanisms of these pathways are not fully understood.

recycling endosome. Overall, Rab proteins are functionally specialized for the trafficking of endocytic vesicles, determining the fate of the cargos included in the vesicles.

VII. Endocytic regulation of TGF- β signaling

Receptor internalization can play either a positive or a negative role in signal transduction.

The classical view is that endocytosis act as a negative and passive regulatory mode by inducing receptor degradation, thus terminates prolonged signaling. However, a wealth of evidence recently indicates that endocytic organelles not only function as a “sink” of signaling complex, they can also play a more active role in signal propagation and amplification (Miaczynska et al., 2004).

TGF- β controls the proliferation, differentiation, cell death and various morphogenetic processes during development. Like other cell surface receptors, TGF- β receptors are internalized into the cell and this process now is emerging as an important regulatory framework in TGF- β signaling.

TGF- β receptor internalization proceeds through two major pathways, clathrin-mediated endocytosis and non-clathrin-mediated endocytosis (caveolar/lipid-raft mediated pathway). It is known that these two pathways fulfill separate functions in TGF- β signal transduction. The former is important for promoting signaling whereas the latter mediates receptor degradation (Chen, 2009; Le Roy and Wrana, 2005).

For the clathrin-dependent endocytosis of the TGF- β receptors, a short specific sequence in the cytoplasmic domain of the receptor is found to play an important role during this process. For example, type II receptor contains a di-leucine-based motif, which lends itself

the capacity of binding the $\beta 2$ subunit of AP2 in clathrin-coated pits (Bonifacino and Lippincott-Schwartz, 2003; Bonifacino and Traub, 2003). After internalization into clathrin-coated vesicles, TGF- β receptor is retained for extended periods in EEA1-positive early endosome and is also found in Rab11 positive recycling endosome (Hayes et al., 2002; Panopoulou et al., 2002). Furthermore, SARA, which contains a FYVE domain, is highly concentrated in PtdIns3p-containing early endosomes. Interfering with SARA localization by depleting the endosomal pool of PtdIns3P, or overexpression of SARA mutant lacking the FYVE domain blocks TGF- β induced Smad2 activation and signaling (Itoh et al., 2002). Therefore, the clathrin-mediated endocytic pathway might promote TGF- β signaling via mediating interaction of receptor/Smad2 complex with other proteins in early endosome. By using the chimeric combination of the granulocyte-macrophage colony-stimulating factor (GM-CSF) and TGF- β receptor, Penheiter and its colleagues found that R-smads activation and downstream signaling depend on endocytic vesicle formation (Penheiter et al., 2002). Treatments blocking receptor endocytosis, such as low temperatures, potassium depletion, and expression of dominant-negative K44A dynamin mutant, inhibit Smad2 phosphorylation and Smad3 nuclear translocation. However, these treatments do not impair the early events in TGF- β signaling such as type I receptor phosphorylation and association of SARA and Smad2 with the TGF- β complex. It suggests that endocytic regulation on TGF- β signaling is much more sophisticated than previously recognized. The relationship between receptor endocytosis and the subsequent signaling event requires more investigation (Ceresa and Schmid, 2000; Di Fiore and Gill, 1999). In the TGF- β system, it is still unclear how

clathrin-mediated endocytosis initiates Smad2/3 phosphorylation and activation in the receptor-SARA-Smad complex, whose assembling event takes place presumably at the plasma membrane. Runyan et al. (2005) proposed a different mechanism that inhibition of clathrin-mediated endocytosis only slightly affects Smad2 phosphorylation and Smad2-Smad4 association, even though the nuclear accumulation of Smad2 and its downstream transcriptional responses were significantly impaired. Potassium depletion treatment prolonged the SARA-Smad2 complex formation, indicating endocytosis is possibly required to release Smad2 from the SARA complex.

VIII. Study of neural cell death using genetic mosaic technique in *Drosophila*

A genetic mosaic technique is a method used to generate a group of somatic cells containing different genotypes (usually homozygous mutations) in otherwise wild-type background. It has been widely used to analyze gene functions in many biological processes. Particularly, the techniques provide a means of examining the function of genes whose mutations cause pleiotropic developmental defects. For example, a number of loss-of-function mutants are homozygous lethal in embryonic or early larval stages of development, disallowing us to study the roles of these genes during the later stages of development. This problem could be overcome by using *temperature-sensitive* (*ts*) mutant alleles; for instance, genetic analysis of the cell cycle regulators had been successfully done with *ts* mutations in yeasts (Hartwell et al., 1974). However, it is not very easy to generate the *ts* alleles of a desired gene. Moreover, the *ts* alleles do not provide clear information as to whether the mutational effect is cell-autonomous. Because mosaic techniques produce only in a small number of cells

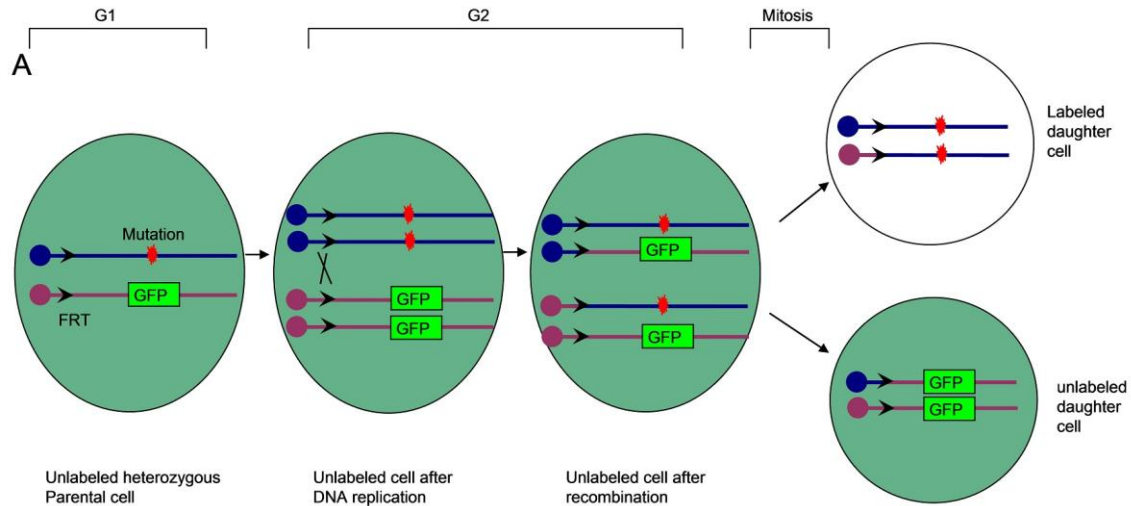


Figure 1- 4. Schematic representation of clonal analysis.

It requires (i) two FRT sites on homologous chromosomes, (ii) UAS-mCD8GFP located distal to one of the FRT sites, (iii) FLP recombinase in the genome, (iv) GAL4 in the genome, (v) a mutation distal to FRT. FRT (black arrowheads) site-specific mitotic recombination gives birth to two daughter cells. The cell which loses UAS-mCD8GFP is homozygous mutant without labeling.

carrying the homozygous mutation in a whole animal, early lethality can often be avoided.

In *Drosophila*, with the introduction of FLP/FRT systems (Golic and Lindquist, 1989), a highly efficient and effective genetic mosaic technique has been established (Fig. 1-4) (Xu and Rubin, 1993). It allowed for both functional analysis of candidate genes and screening new mutants in late stage. This approach has been widely utilized to help understanding the development of late-developing adult tissues, such as compound eyes, and oocyte.

In the traditional clonal system, the marker transgene locates distal to the FRT (Flippase Recognition Target) site and is in *trans* to the mutant allele. Following site-specific mitotic recombination, homozygous mutant cells are the only cells that are not labeled. In many cases, this negative labeling system can trigger a problem, in particular in nervous system. Since there are millions of neurons are densely packed in the CNS, it would be not very easy for us to track the small number of unlabeled homozygous mutant cells from overwhelmingly labeled background. Therefore, it is desirable to have a positive labeling system in which only the homozygous mutant cells are marked, thus distinguishable from their wild type siblings.

A new mosaic technique, MARCM (for Mosaic Analysis with a Repressible Cell Marker) system was developed (Lee and Luo, 1999; Lee and Luo, 2001) to fulfill this goal. The principle of the MARCM system is schematically described in Figure I-5. Compared to the traditional mosaic technique, the introduction of a dominant repressor of a cell marker in *trans* to the mutation allows it to positively mark the mutant cells. This system requires at least 6 transgenes: two FRTs, one FLP recombinase, one UAS-marker, one GAL4 driver, and

one tubp-GAL80. In heterozygous cells, the presence of GAL80 inhibits the activation of UAS-GAL4 system, whereas in homozygous mutant cells the marker is expressed due to the loss of GAL80 (Lee and Luo, 1999).

This new mosaic method in this thesis works greatly in the analysis of neural lineage and the following homozygous mutant clone labeling in CNS. The results largely elucidate the complexity of the underlying cellular and molecular mechanisms in neuron cell death.

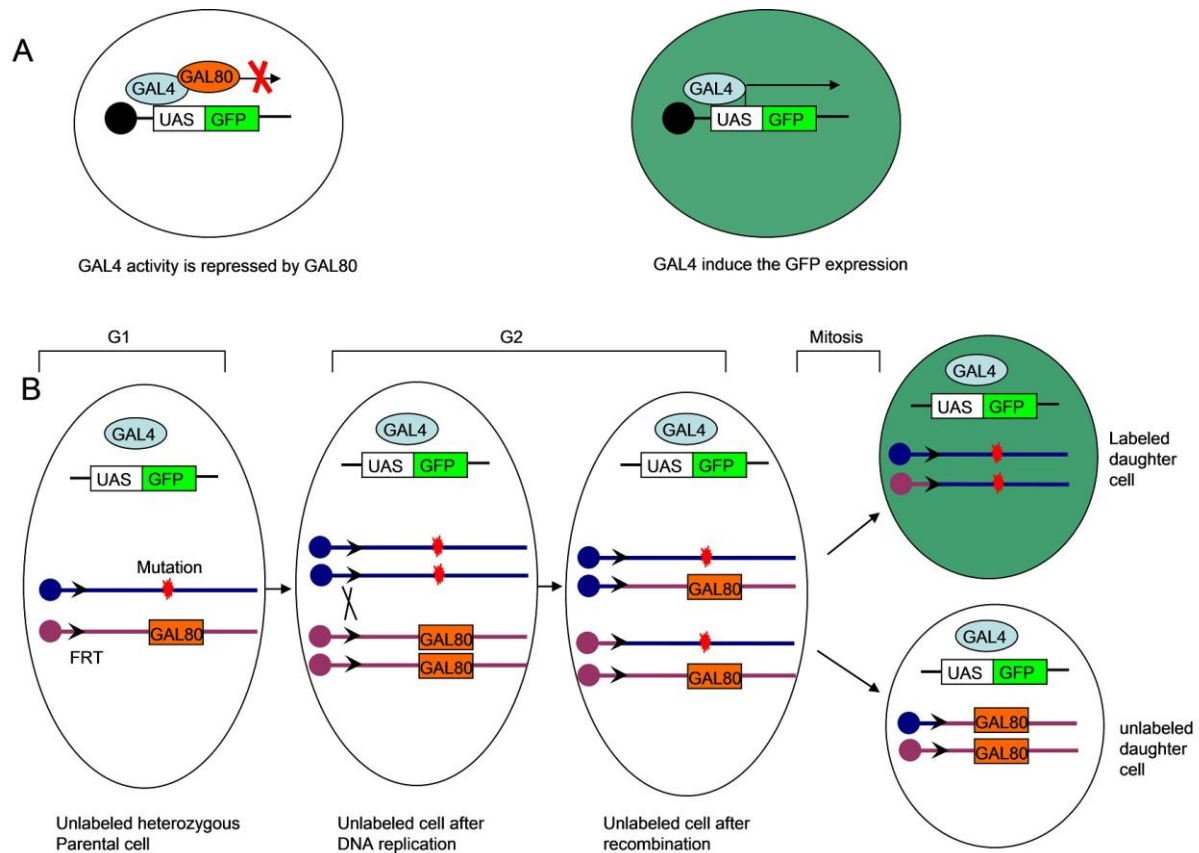


Figure 1- 5. Schematic representation of the MARCM genetic system.

(A) GAL80 protein suppresses the GAL4-dependent expression of UAS-gene. Therefore, in contrast to the cell with UAS-gene and GAL4, the cell containing GAL80 protein is not labeled. (B) MARCM includes (i) two duplicated FRT located on homologous chromosomes, (ii) GAL80 located distal to the FRT site on each chromosome, (iii) FLP recombinase, (iv) GAL4 and UAS-marker, (v) a mutation distal to FRT. After site-specific mitotic recombination at FRT sites (black arrowheads), two daughter cells are generated. Because of loss of GAL80, the homozygous mutant cells are specifically labeled by the marker. Modified from (Wu and Luo, 2006).

Chapter Two

Developmental analysis of Corazonin neurons in *Drosophila*, using genetic mosaic techniques

I. Abstract:

Our various studies indicated that eight pairs of ventrally located Crz neurons (vCrz) are heterogenous with respect to their neuroanatomy. Since conventional labeling system using antibody or reporter gene expression that we routinely employed cannot characterize individual vCrz neurons, we could not clarify such heterogeneity. Using MARCM, we were able to label and trace individual vCrz axonal projections and we found that there are three distinct subgroups of vCrz neurons according to their projection patterns and two subgroups in terms of birth order. Furthermore, we showed that MARCM is a very useful tool to study the roles of genes associated with vCrz apoptotic death. As an example, we induced mitotic recombination of a *usp* lethal allele in the vCrz neurons. The results show that the *usp* gene is absolutely required for cell death of vCrz neurons. We also compared traditional genetic mosaic methods with MARCM, while dissecting the function of homozygous mutant alleles in vCrz cell death.

II. Introduction

Genetic mosaic analysis can generate specific homozygous mutant cell or tissue with background of a phenotypically wild-type heterozygous organism. It allows us to study gene functions at later stages of development. Traditional genetic mosaic system labels

negatively the mutant homozygous cells. In particular, this poses a severe problem in the CNS, since a single unmarked neuron or a small subset of these neurons are not easily tractable in the background of numerous surrounding cells. The MARCM (mosaic analysis with a repressible cell marker) system was developed as a new generation of clonal analysis, a particularly effective method in the CNS. It has been widely used to dissect various gene functions in neuron remodeling in *Drosophila* (Lee and Luo, 1999; Wu and Luo, 2006).

MARCM is also a useful and versatile tool to study cell lineage and morphology in the CNS. Neurogenesis starts the sequential generation of distinct neurons from common progenitors, called neuroblasts (Nbs) (Ito and Hotta, 1992; Truman and Bate, 1988; Urbach et al., 2003). Each Nb undergoes a series of asymmetric cell divisions to produce multiple neuron types in an invariant sequence. In *Drosophila*, a stereotypic pattern of these neuron types derived from an Nb includes neuroblast, ganglion mother cells (GMCs), neurons and/or glia, and they form a specific cell lineage during development. For example, the best-characterized Nb lineage in *Drosophila* CNS is Nb7-3 (Fig. 2-1A). First asymmetric division of the Nb7-3 yields a regenerating Nb and GMC-1. Following a mitotic division, GMC-1 gives rise to two daughter cells; one (EW1) develops into a serotonergic interneuron and the other a GW motoneurons. Subsequently born GMC-2 divides once, producing one daughter cell (EW3) that becomes another serotonergic interneuron. The other daughter cell is removed via programmed cell death. The third progeny of the Nb7-3 is GMC-3, which gives rise to an EW3 interneuron, which later produces Crz neuropeptide (Karcavich and Doe, 2005; Lundell et al., 2003; Novotny et al., 2002). All those information including the

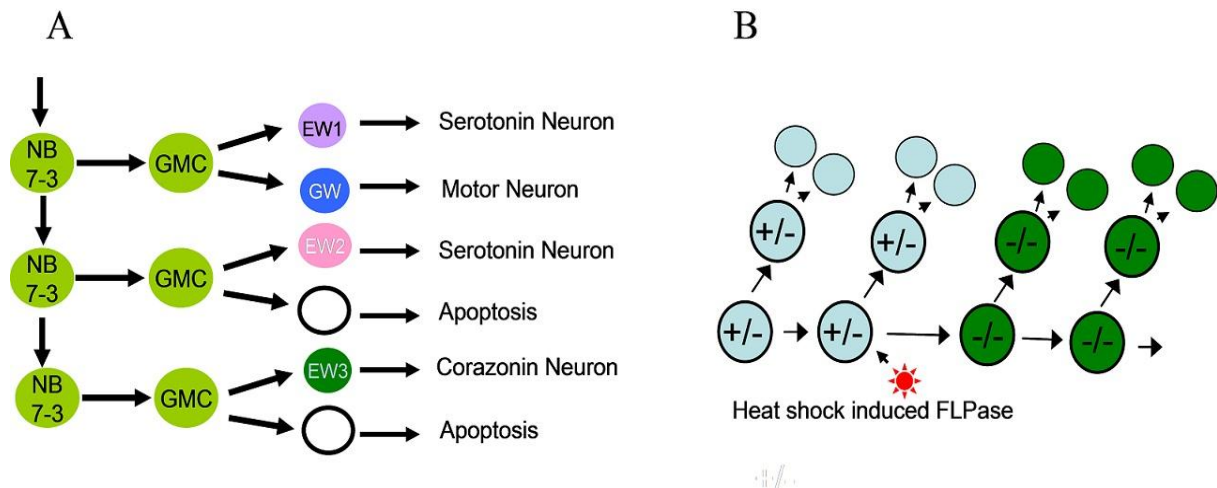


Figure 2- 1. NB7-3 lineage development and MARCM labeling.

(A) Three contralaterally projecting interneurons (EW1, EW2, and EW3) and one motoneuron (GW) are derived from NB7-3. GW motoneuron is the most posterior cell in NB7-3 derived cell cluster. EW1 and EW2, expressing neurotransmitter Serotonin (5-HT) are located medially. Additionally, two sister cells are eliminated through apoptosis during development. The NB7-3 and all of its progenies express transcription factor eagle (eg), which is widely used as a genetic marker of NB7-3 lineage. (B) In the MARCM system, following Flipase-mediated mitotic recombination, a Nb losses GAL80 and subsequently, all neurons derived from this GAL80 negative Nb are specifically labeled. It can be used in lineage study.

precursor and its descendants, the cell compositions, the birth timing and birth order of the progeny and the development of the cells is described as a cell lineage (Anderson, 1992).

The versatility and power of MARCM assay in the cell lineage study has been exemplified vastly in olfactory system in *Drosophila* (Komiyama et al., 2003).

The MARCM system emerges as a useful tool to study cell lineage because of the following properties. First, one can express FLP expression by heat shock treatment at a specific stage. Thus only precursors that divide actively at that particular stage might possibly prone to mitotic recombination. Therefore, by controlling the timing of mitotic recombination during development, the timing of cell birth and the birth order of different cells within a given lineage can be easily tracked (Fig. 2-1B). Second, the neuron set in a multicellular Nb clone is generated from a common progenitor, so study of these neurons and their respective projection patterns reveals the role of cell lineage in the construction of neural circuits. Third, single-cell marking can differentiate morphological features from their corresponding neuron set (Jefferis et al., 2001; Lee and Luo, 2001). With lineage analysis of the development of mushroom bodies (MB) with MARCM, it is illustrated how adult MBs acquire their mature morphologies after production of postmitotic neurons along larval and pupal stages. Three distinct axon projection patterns in MB are further revealed by examining single-cell clones systematically generated at different stages (Lee et al., 1999). Similar strategies can be utilized to analyze the development of other neural structures in the *Drosophila* CNS.

Corazonin (Crz) is a neuropeptide and has been shown to have diverse functions in

insects. In *D. melanogaster* larva, Crz expression is found in four pairs of neurons per cerebral lobe and in eight pairs of bilateral neurons in the ventral nerve cord (vCrz). vCrz are programmed to die during early metamorphosis. Characterization of the development of individual Crz neurons will be important for elucidating their functions.

Here a conventional mosaic analysis and MARCM assay were used to study the development of Crz neurons. We showed that the 8 pairs of Crz neurons can be classified into three groups according to their projection patterns, and two groups based on their birth order. Further, we demonstrated the effectiveness of mosaic analysis and MARCM assay in studying program cell death. Two types of candidate gene, the cell death promoting gene and cell death blocking gene were exemplified to show how to label homozygous mutant clone during cell death.

III. Materials and methods

***Drosophila* strains and species**

Generation of clones in lineage analysis (1) *y w, hs-FLP; FRTG13, UAS-mCD8GFP*; (2) *FRTG13, tubP-GAL80; Crz-gal4*. *usp* mutant analysis (Lee et al., 2000b) (3) *FRT19A, tubP-GAL80, hs-FLP; Crz-gal4, UAS-mCD8GFP*; (4) *FRT19A, usp³*; (5) *FRT19A, usp⁴; FRTG13*, (6) *FRT19A, usp⁵*, (7) *UAS-usp-miRNA* (Lin et al., 2009). Clonal analysis (7) *y w, hs-FLP; FRTG13*. (8) *FRTG13, UAS-mCD8GFP; Crz-gal4*. *Fkh* mutant clone (9) *fkh⁶* (Bloomington Stock Center); (10) *FRT82B*; (11) *y w, hs-FLP, UAS-mCD8GFP; FRT82B, Crz-gal4*

Recombination of a mutant allele with FRT

The mutant line was crossed with the corresponding P[ry, hsneo, FRT] line. The F1 virgin was collected and then crossed with the corresponding balancer to undergo G418 selection in G418-containing medium. Flies carrying the corresponding P[ry, hs-neo, FRT] element can be selected by their resistance to G418 (Geneticin, GIBCO laboratories). Homozygous lethality was used to screen the flies carrying the mutant allele. G418-containing medium was made as follows: a fresh vial was taken and few holes were made in food surface with toothpicks, and 100 ul of 180 ug/ml freshly made G418 solution was added per 13 ml of food and the vials were allowed to air-dry for overnight.

Generation of clones

To produce clones in the Crz neurons, eggs from the appropriate crosses were collected at specific time before Crz neurons are born (8.5 hours after egg laying at 25°C), and then were incubated in a 37°C water bath for 60 minutes to induce mitotic recombination. After the heat shock treatment, the animals were kept at 25°C until the CNSs were dissected for immunohistochemical analyses at the desired stage.

Immunohistochemistry

Whole-mount Crz immunohistochemistry was performed, as described previously (Choi et al., 2005). Briefly, dissected CNSs were fixed in 4% paraformaldehyde in PBS for 30 min followed by rinsing with TNT buffer (0.1 M Tris, 0.3 M NaCl, 0.5 % Triton X-100, pH 7.4) three times for 15 min each. After that, the CNS samples were applied with anti-Crz antibody and incubated overnight at 4°C. After rinsing 6 times with TNT for 10 minutes each at room temperature, the TRITC-conjugated secondary antibodies was applied to the

samples with dilution of 1:200 (Jackson ImmunoResearch) for 1-h incubation. The samples were then cleared in 60% glycerol and mounted in 10% glycerol. Fluorescent signals were acquired by Olympus BX61 connected with CC-12 camera.

IV. Results

Characterization of vCrz neurons for genetic mosaic analysis

As previously reported, vCrz are generated from NB7-3 lineage on each neuromere. These NBs undergo 4 rounds of division and produce 8 pair of vCrz before 8.5h After Egg Lay (AEL) during embryogenesis. According to this, embryos (*hs-FLP/Y or X; FRTG13, tubP-gal80/ FRTG13, UAS-mCD8GFP; Crz-gal4/+*) were heat-shocked at 37°C for 1 hr during early embryogenesis (0-8 h AEL) to induce FLP/FRT-mediated mitotic recombination. After these animals reach wandering third-instar larval stage, CNSs were dissected for Crz IHC. As a result, we were able to identify larval CNSs bearing 1-3 GFP labeling vCrz neurons (Fig.2-1). These studies indicate that MARCM is useful for clonal analysis of neurons that are produced from embryonic NBs.

After analyzing a number of CNSs bearing a few GFP-marked vCrz neurons at different positions, we found segment-specific anatomical variations among vCrz neurons. In the T2-A3 neuromeres, the neurons project contralaterally into the opposite hemimere, and then turn anteriorly to the medial protocerebrum. In contrast, neurons in the A5-A6 neuromeres send projections contra-laterally, and then posteriorly, terminating at the caudal region of the abdominal ganglion. Unlike the two groups just described, A4 neuronal projection bifurcates into two directions after passing midline, one is going anteriorly until the T2

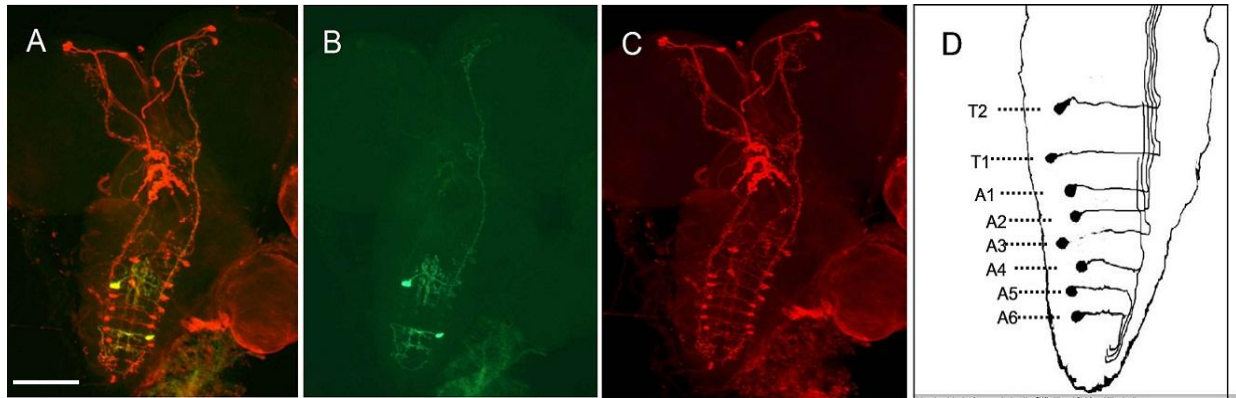


Figure 2- 2. Lineage analysis of Crz using MARCM

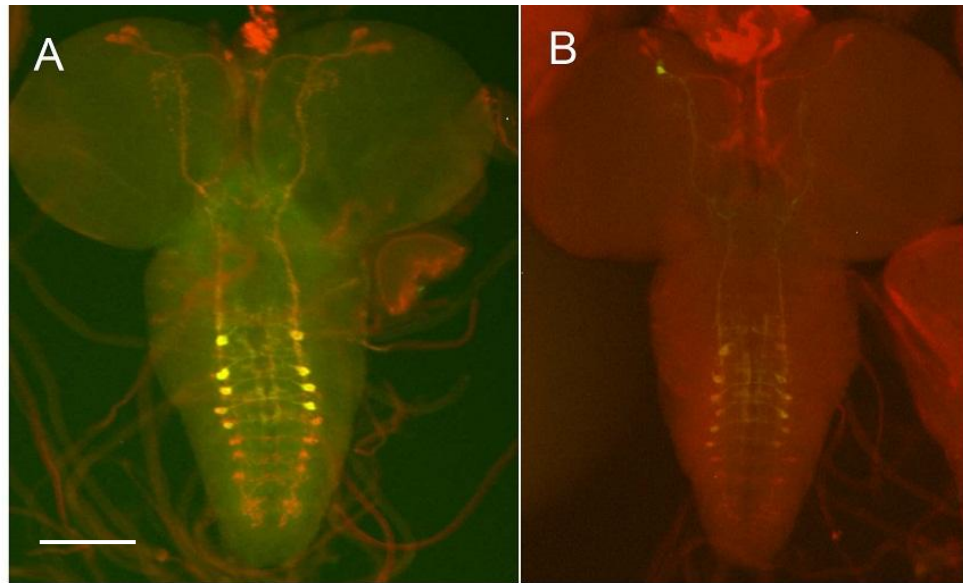
(A, B, C) MARCM with a heat shock during 0-6 h AEL. In this sample, A1 and A5 neurons show distinct neuroanatomical features in their arborization patterns. (genotype: *hs-FLP/Y; FRT^{G13}, tubP-gal80/FRT^{G13}, UAS-mCD8GFP; Crz-gal4/+*). (D) Schematic diagram of each segmental vCrz neuron. Only one side is drawn here and detailed arborizations in the midline are not shown for the sake of simplicity. Scale bars = 100 μ m.

neuron, and the other one travel posteriorly and converges with the projection of A5 and A6. It indicates that there exist inherent interdependence between the projection pattern of each vCrz and their specific spatial properties, such as segment information.

Segment specific generation of vCrz neuron

VNC neurons originally arise from a sheet of neuroectodermal cells located in the ventral-lateral region of the embryo. In early stage of embryogenesis, the sheet is subdivided into several neural equivalence groups along anterior-posterior and dorsal-ventral axes. Interactions among the cells in each group ultimately select one cell to acquire the CNS stem cell or neuroblast fate. The selected cell enlarges and segregates to the interior of the embryo. As a result from 5 sequential waves of NB segregation, 30 NBs are patterned stereotypically in each hemisegment (Skeath and Thor, 2003). One of them, NB7-3 is vCrz's ancestor.

Previously, BrdU labeling in NB7-3 derived neuron showed vCrz neurons are born around stage 15 at 6.5h AEL and until 8.5h AEL (Novotny et al., 2002). Using MARCM, we determined whether there is heterogeneity of birth timing among vCrz neurons. To do this, we manipulated heat-shock timing, thus inducing mitotic recombination at various embryonic stages, and then dissected around 20 larval CNSs bearing differential vCrz neurons labeled. When heat-shock was given randomly during 0-8 h AEL, we observed that most of samples acquired one or two vCrz neurons each. However, if mitotic recombination was induced in embryos after 8.5 h AEL, none of the CNS samples showed vCrz neurons labeled. These results indicate that all vCrz neurons are born before 8.5 h AEL, which is



C Summary of the Crz neuron development



Figure 2- 3. The birth order analysis with MARCM in Crz.

(A) posterior-most pairs (A4-A6) are not labeled with GFP. (B) A5-A6 pairs are not labeled, note that DL neuron is labeled and the A4 pair has a very faint GFP expression. (red, anti-Crz; green, GFP; yellow, overlapping signals of red and green). (C) Summary of the Crz development. One vCrz is derived from one NB7-3 in T2-A6 segment. Within 7h AEL, all Crz clone can be captured by MARCM labeling. However after 7h AEL, the most posterior segment A5-A6 can not be labeled. It indicates that A5-A6 pairs are born before 7h AEL, whereas T2-A4 and DL neuron are born late (genotype: $hs-FLP/Y$; FRT^{G13} , $tubP-gal80/FRT^{G13}$, $UAS-mCD8GFP$; $Crz-gal4/+$). Scale bars = 100 μm .

consistent with previous BrdU labeling study. Interestingly, we observed that 8 pairs of vCrz neurons randomly were labeled in samples with 0h AEL-6h AEL; however, with heat shock treatment after 6h AEL, the abdominal 2 pairs of vCrz (A5, A6) were unlabeled in any sample (Fig. 2-3A, B) (n=50). It means that these 2 pairs are generated before 6h AEL, whereas anterior 6 pair vCrz are generated until 8h AEL. Taken together, there is a different birth order of vCrz along anterior posterior axes (Fig. 2-3C).

MARCM labeling homozygous mutant clone

In the CNSs taken from prepupa aged 6 h APF, *Crz* expression is undetectable in the VNC, and we have shown that such disappearance is due to the programmed death (Choi et al., 2006). Since many of the mutations that completely remove the function of a given gene are homozygous lethal in embryonic or early larval stages of development, it would be impossible to undertake directly mutant dissection for neuronal PCD.

Previously, we have shown that vCrz PCD requires ecdysone receptor (EcR) activity. Since EcR's functional heterodimeric partner is Ultraspiracle (Usp), we expected that vCrz PCD would be impaired in a loss-of-*usp* function mutant. Since all known *usp*-null mutants are embryonic lethal, we took advantage of MARCM to generate *usp* homozygous mutant vCrz neurons.

As shown in Fig. 2-4B, a few GFP-labeled neurons bearing homozygous *usp*⁵ allele are clearly viable and have a normal cell body and projection during larval stage, suggesting that normal morphogenesis of the vCrz neurons does not require Usp activity. During metamorphosis, the *usp*⁵ mutant vCrz neurons and its projection are intact at 6 h APF,

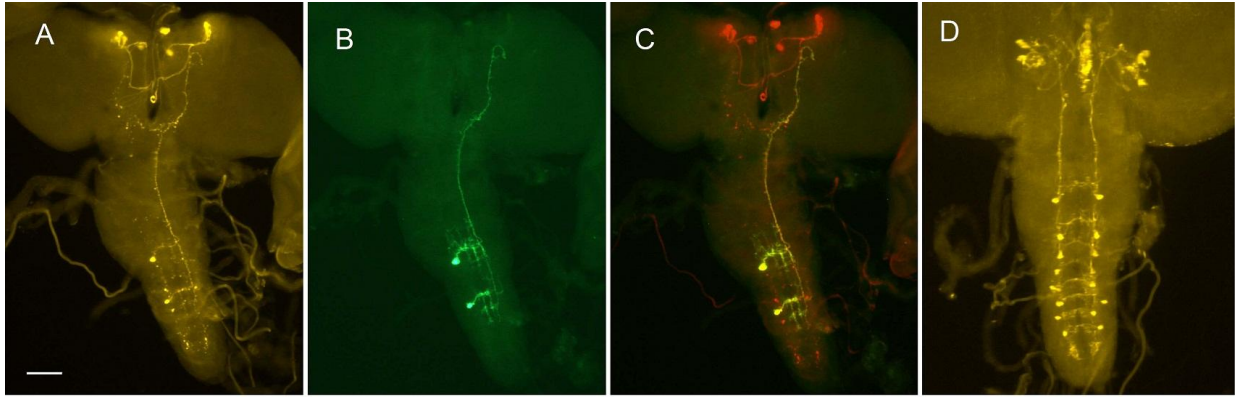


Figure 2- 4. Lack of cell death in *usp* mutant Crz neurons.

(A) At 6h APF, two vCrz neurons are detectable with intact cell body and projection. (B) As marked by GFP, two survival neurons are homozygous mutant of *usp*⁵. (C) Overlapping of A and B (genotype: $FRT^{19A},usp^5/FRT^{19A},hs-FLP,tubp-GAL80; ;UAS-mCD8GFP,Crz-gal4/+$). (D) RNAi-mediated knockdown of the *usp* results in complete cell death defect (n=6). Scale bars = 100 μm .

supporting that vCrz PCD requires Usp as a partner of EcR (Fig. 2-4, A-C). Consistent with clonal analysis, RNAi-mediated knockdown of the *usp* resulted in a complete block of vCrz PCD at 6 h APF (Fig. 2-4D).

Mosaic analysis of *fkh* mutant

Following neurogenesis, neurons have multiple fates to choose: to survive, differentiate and connect with its targets, or to undergo cell death. Two groups of genes are found to regulate this important decision-making process, pro-survival genes and pro-apoptotic genes. In vertebrates, the known pro-survival genes in regulation of program cell death include Bcl-2 family, neurotrophic factors, and other survival related genes (Schmidt et al., 2004). Also there is clear evidence for similar regulation of PCD in *Drosophila*. Since MARCM assay can only be used to label homozygous mutant cells, it is not appropriate to study the pro-survival gene function, because homozygous mutant cells will die prematurely due to the lack of survival factors. Compared to positive labeling with MARCM assay, traditional genetic mosaic technique marks the clone in opposite way, as it labels only the heterozygous precursor cell and wild-type cell. Thus it gives an opportunity to study the gene function, whose genetic disruption causes early cell death.

As described in MARCM assay, 1-h heat treatment at 37°C was applied to embryos during early embryogenesis (0-8 h AEL). After these animals reach wandering third-instar larval stage, CNSs were dissected for Crz IHC. As a result, we found some sample CNSs bearing 1-3 GFP-negative vCrz neurons (Fig. 2-5A,B,C). These GFP unlabeled neurons are the result of mitotic recombination between homologous chromosomes, *FRTG13 UAS-mGFP*

and *FRTG13*. These results indicate that traditional genetic mosaic technique can be used to reversely label the homozygous mutant clone. Here we exemplified how to use candidate gene approach to test whether mutations in known pro-survival genes would affect vCrz cell death.

Forkhead genes are a family of transcription factors that play important roles in cell growth, proliferation, differentiation, and longevity (Kaufmann and Knochel, 1996). In *Drosophila*, *fkh* is required for early embryogenesis, the formation of several organs and tissues, such as the gut, the malpighian tubules and the larval salivary glands (Myat and Andrew, 2000). Expression of the *fkh* in larval and pupal stage depends on ecdysone signal. Ectopic expression of *fkh* in late prepupae blocks the cell death of salivary glands (Liu and Lehmann, 2008; Wang et al., 2004). However its function has not been well characterized in the *Drosophila* CNS. It was reported that *fkh* regulate apoptosis in dMP2 neuron during embryogenesis, as 95% of anterior dMP2 neurons fail to undergo cell death in *fkh* mutants (Miguel-Aliaga et al., 2008). Here we examined its function in vCrz PCD by applying genetic mosaic analysis.

In order to use the FRT/FLP recombination system to produce clones homozygous for *fkh*⁶ mutation, the mutant allele was recombined with FRT^{82B}. Since *fkh*⁶ mutation is embryonic homozygous lethal, we screened for both *neoR* and homozygous lethality, but balanced heterozygous survival mutation. Mosaic animals were generated after crossing this recombinant to a strain that carries a chromosome arm with the same P[ry⁺, hs-*neo*, FRT] element, FRT^{82B} combined Crz-gal4 and a cell marker as well as the hsFLP gene on a

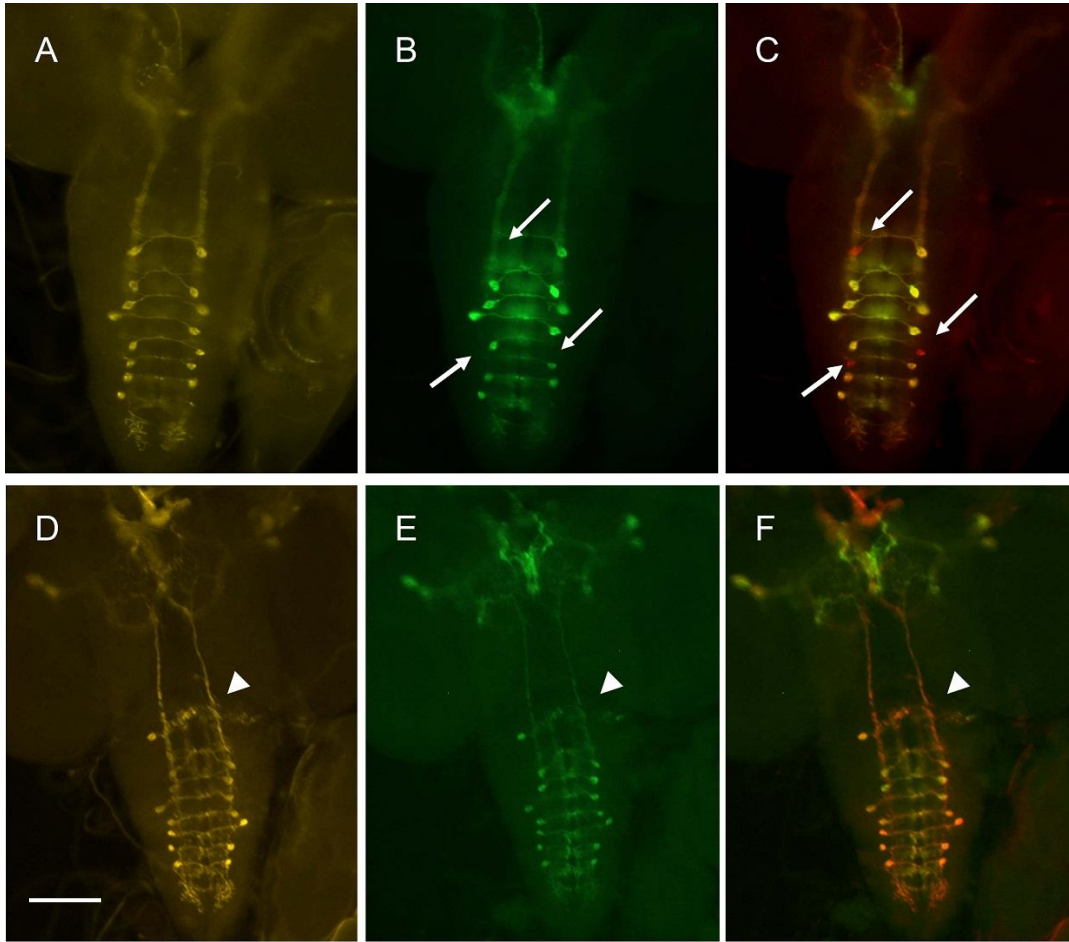


Figure 2- 5. *fkh* is required for vCrz survival during development

(A, B, C) Marking vCrz clone. Three vCrz clones (arrow) are not labeled by GFP because of mitotic recombination (genotype: *hs-FLP/Y; FRT^{G13}, UAS-mCD8GFP/FRT^{G13}; Crz-gal4/+*).

(D, E, F) Clonal analysis of *fkh⁶* mutant. 15 GFP labeled vCrz which carry wildtype *fkh* gene are survival at WL3. One missing neuron (arrow head) is possibly caused by lack of Fkh

function (genotype: *hs-FLP, UAS-mCD8GFP/Y; FRT^{82B}, fkh⁶/FRT^{82B}, Crz-gal4*). Scale bars

= 100 μ m.

separate chromosome. The FLP recombinase was then induced in the heterozygous progeny by a brief heat-shock treatment at 0-8h AEL.

After these animals reach wandering third-instar larval stage, CNSs were dissected for Crz IHC. As a result, we found that more than half of the samples (n=14) carried 8 pairs of GFP-marked vCrz and rest of them lacked one of vCrz neurons in T2 segment (Fig. 2-5D-F). Since mitotic recombination results in GFP-negative homozygous mutant cells, we speculate that the missing vCrz neuron is *fkh*⁶ mutant cell. Furthermore, such cell was not labeled by Crz-immunohistochemistry (Fig. 2-5D), most likely reflecting cell death. Considering the Fkh as a pro-survival factor, it is reasonably assumed that *fkh* mutant vCrz died prematurely.

V. Discussion:

MARCM based cell labeling

Genetic mosaic analysis can be used to generate homozygous mutant cells from heterozygous precursors, and is a powerful tool to study gene function in many systems (Lee and Luo, 1999; Xu and Rubin, 1993; Zong et al., 2005). MARCM is an advanced genetic mosaic technique. Through mitotic recombination, MARCM positively labels a clone of cells that are generated from a common GAL80-minus precursor cell (Lee and Luo, 1999, 2001). This feature gives it a great advantage of gene function study and lineage analysis in CNS. In MARCM, the labeling method depends on the GAL4 driver used in the system. Mostly, they are designed to selectively label the cell of interest, called cell-subtype-specific GAL4 driver (Brand and Perrimon, 1993; Manseau et al., 1997). For example, Crz neuron specific GAL4 driver is controlled by genomic regulatory sequence of Crz gene that specifically labels Crz in CNS

(Choi et al., 2005). However, using subtype-specific GAL4 to label MARCM clones has potential problems. Since clones are most likely to be generated in a non-tissue-specific manner, MARCM cannot give full profile of the GAL80-minus cell lineage without using a ubiquitous GAL4 driver. In vCrz cell death, Crz-gal4 only labels Crz neuron in all GAL80-minus progeny of NB7-3. Therefore, it is difficult to tell whether the cell death defect occurred due to cell-autonomous or non-autonomous phenotypes by examining subsets of mosaic patterns. It is also the same scenario in cell lineage study. Since usually all the progeny derived from a single GAL80-minus precursor can not be labeled in MARCM, it become insufficient for a comprehensive lineage analysis.

MARCM analysis in Crz PCD

vCrz neurons are born in late embryonic stage (Lee et al., 2008). Since vCrz neurons are terminally differentiated cells, they have no chance to undergo mitotic recombination. Therefore, an early embryonic stage mitotic recombination was conducted to generate chimeras and then only label those homozygous mutant clones among Crz using *Crz-Gal4* and UAS-mCD8GFP. It allows us to examine homozygous mutant phenotype in vCrz during late stage. However, since the recombination is mediated by heat shock-induced Flpase, homozygous mutant cell clones are randomly generated not only in the vCrz neurons but also in the entire animal. Often this could cause severe detrimental effect on normal animal development, which makes it very hard to obtain later stages of animals with recombination. This could explain the difficulty of generating mutant cells with some mutant alleles (for example, *dSmad2* mutant analysis in our study). To overcome this

problem, it is necessary to restrict *Flpase* expression to the target progenitor cells. For instance, the *ey-Flp* transgene has been used to conduct mutant clonal analysis only in the photoreceptor cells. Similarly, neuron lineage specific expression of the Flp recombinase might be useful to limit mitotic recombination to the dividing NBs or GMCs.

Segmental diversity and roles of *Hox* genes

The underlying molecular and cellular mechanisms to control the generation of cell diversity have been studied in many systems. Two sources of information, spatial and temporal factors, have been shown to be required for the generation of neural diversity during development (Pearson and Doe, 2003). In *Drosophila*, the pattern of segregating neural stem cells, the neuroblasts (NBs) is identical in the ventral nerve cord and all of NBs are derived from the ventral neurogenic region of the ectoderm (Bate, 1976). However, significant differences occur to these progenitor cells during development. Each NB gives rise to lineages of specific size and cell composition along anterioposteior axis (Bossing and Technau, 1994; Murance and Gould, 2005; Schmidt et al., 1997; Udolph et al., 1993).

NB 7-3, one of the last neuroblasts delaminated from the neuroepithelium in CNS has been well studied in lineage analysis. It generates three contralaterally projecting interneurons (EW1, EW2, and EW3) and one motoneuron (Higashijima et al., 1996). EW3 is known as Crz neuron as it synthesizes the neuropeptide Corazonin. However, Corazonin antibody staining only marked 8 pairs in VNC and the subesophageal segment, the first thoracic, T1, and the abdominal segment A7, A8, and A9 misses its expression. It indicates differently sized lineages exist along anteroposterior axis.

Previous studies have shown that Hox homeotic genes play a key role, acting in several different ways to regulate segment-specific behaviors of NB lineages (Carpenter, 2002; Dalla Torre di Sanguinetto et al., 2008; Rogulja-Ortmann and Technau, 2008). Recent studies have shed new light on these events. For instance, Hox genes and their cofactors, Pbx and Meis act in tight interplay with temporal genes to prevent formation of abdominal-specific neurons through blocking lineage progression within an early temporal window in NB5-6 lineage. Another known mechanism of controlling lineage size in NB 7-3 is related to cell death. Detailed studies showed that lineage size increased from 4 up to 10 cells when cell death is genetically blocked (Lundell et al., 2003; Novotny et al., 2002). In *H99* deficient line, supernumerary cells are detectable within the NB7-3 lineage. Ectopic expression of p35 in NB7-3 and its progeny during embryogenesis produce ectopic Crz neuron in some segments (Novotny et al., 2002). Numb/Notch signaling was found to be necessary for regulating cell death. When Numb inhibits Notch signaling, cells undergo neuronal differentiation, whereas cells that maintain Notch signaling initiate cell death (Lundell et al., 2003). However, our understanding of the segment-specific neuronal subtype specification is still rudimentary, in particular with respect to how the spatial factor and temporal cues are integrated with lineage progression. We find that Crz neurons exclusively appear in thoracic segments, T2 and T3 and abdominal segment, A1-A6. How does the identifiable neural lineage generate diverse cell numbers along anteroposterior axis? Here, several combined elements present us with an opportunity for understanding the underlying molecular and cellular mechanism to control the generation of neural diversity, including; 1) Genetically

blocking cell death does not recover any Crz neuron in T1 and posterior abdominal segment A7, A8, and A9. 2) Birth window study of vCrz along anterior posterior axis showed the abdominal segments, A5 and A6, have early temporal birth window than other segments. Therefore this set of studies demonstrates that early lineage progression termination caused by NB cell cycle exit could be a key factor of preventing formation of vCrz neurons in the T1 and A7-9 segments.

Previous studies have shown that Hox input plays a key role in controlling segment-specific behaviors of NB lineages. We find that manipulation of the *Hox* gene expression in NB7-3 and its progeny can change the pattern of vCrz neurons along anteroposterior axis. Over-expression of Hox genes was achieved using a UAS-Hox transgene driven by an *eagle-GAL4* transgene. At larval stage, overexpression of *AbdA* and *Ubx*, respectively, leads to extra vCrz neurons at subesophageal and thoracic segment (Fig. 2-6A, D), whereas *AbdB* and *Antp* overexpression cause less number of neuron at VNC (Fig. 2-6B, C). These preliminary data provide the first insight into the mechanisms by which positional cues conferred by differential *Hox* gene expression and temporal cues are integrated within a defined lineage to control unique neuronal identities only in specific segments.

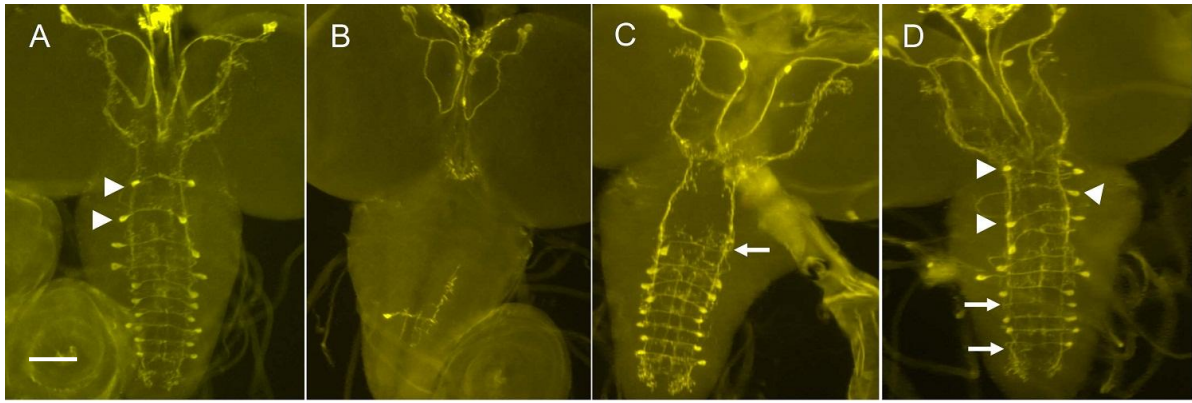


Figure 2- 6. Hox genes regulate segment dependent vCrz development

(A) Overexpression of AbdA leads to the generation extra Crz at T1 and S3 segment (arrow head). (Genotype, *eagle-GAL4/UAS-AbdA*). (B) Overexpression of AbdB inhibits development of most of vCrz neuron (genotype, *eagle-GAL4/UAS-AbdB*). (C) T2 segment misses one Crz neuron (arrow) when Antp is overexpressed in NB7-3 lineage (arrow). (Genotype, *eagle-GAL4/UAS-Antp*). (D) In contrast to the generation of extra neuron at thoracic and subesophageal segment (arrow head), There is losing of Crz in some abdominal and thoracic segments when Ubx is overexpressed (arrow). (Genotype, *eagle-GAL4/UAS-Ubx*). Scale bars = 100 μ m.

Chapter Three

**Signaling involving Baboon receptor and its ligand Myoglianin is required for the
programmed death of peptidergic neurons during metamorphosis**

This study has been submitted for publication

I. Abstract

Program cell death (PCD) is an essential feature during the development of the central nervous system in *Drosophila* as well as in mammals. During metamorphosis, a group of peptidergic neurons (vCrz) are eliminated from the larval central nervous system (CNS) via PCD within 6-7 h after puparium formation. To better understand this process, we first characterized the development of the vCrz neuron including its lineage and its birth window using MARCM assay. Further genetic and MARCM analyses showed that Myoglianin (Myo), a ligand TGF- β , and its type I receptor Baboon mediate TGF- β signaling in vCrz PCD. Genetic analyses of the components downstream of the receptors suggest that Myo does not signal exclusively through Babo, indicating complicated signaling pathway of Myo. Interestingly, an adaptor protein, Sara is also required in vCrz PCD and function as a concentration-dependent biphasic modulator of TGF- β signaling.

II. Introduction

Establishment of a functional nervous system is achieved through precise controlling of the staggering neuronal cell number and assembling their highly complex neuronal circuits. Programmed cell death (PCD) plays a key role in regulating the number of neurons in the nervous system throughout animal development. During embryogenesis, prominent cell death occurs in populations of proliferating neuroblasts, early postmitotic cells, and neurons that fail to make functional connections with their targets (Davies 2003). A well established idea is that the fate of neurons during this period is largely regulated by a variety of secreted

proteins that either promote survival or bring about cell death after binding to receptors expressed on the neurons (Davies, 2003). For example, neurotrophic factors secreted from the innervated targets and associated glial cells can promote the survival of these developing neurons (Levi-Montalcini, 1987). As a result of competition for limiting neurotrophic factors in their target organs, removal of a subpopulation of initially equivalent neurons is random and called stochastic PCD (Miguel-Aliaga and Thor, 2009). In contrast, a predetermined PCD by its neuronal lineage is called stereotyped PCD. It evolved a conserved mechanism that removes specific cells with precision and reproducibility and has been observed in *Drosophila*, *C. elegans* and vertebrate nervous systems (Miguel-Aliaga and Thor, 2009). For example, in *Drosophila*, lineage-specific PCD of differentiated neurons occurs in a segment manner. The dMP2 and MP1 pioneer/visceral neurons and the GW and anterior NB2-4t motor neuron are eliminated during embryonic development (Miguel-Aliaga and Thor, 2004; Rogulja-Ortmann et al., 2008). Despite these studies, however, the mechanism of how these stereotyped PCD is initiated is still unknown.

Holometabolous insects, which undergo complete metamorphosis, offer a model system for studying developmentally-regulated neuronal cell death, because the formation of the adult CNS during metamorphosis involves reorganization of larval neural circuits (Robinow et al., 1993; Truman, 1990). A number of unwanted larval neurons are programmed to die, while other neurons are persistent into adulthood but usually remodeled through pruning of axon and dendritic processes (Robinow et al., 1993). Previously, we found that a group of peptidergic neurons producing Crz neuropeptide are eliminated from the larval CNS via PCD

within 6-7 h APF in *Drosophila* (Choi et al., 2006), which provides us with a model to study how this subgroup of neurons are eliminated in CNS.

Members of the TGF- β superfamily have been shown to control many aspects of cellular behavior, including cell differentiation, cell growth and death, and various morphogenetic processes during development (Derynck and Akhurst, 2007; Kingsley, 1994). A growing body of evidence has shown that TGF- β induces cell death in many different cell types, including normal B cells and a lymphoma B-cell line (Chaouchi et al., 1995). Extensive lymphocytic hyperproliferation and systemic lupus erythematosus-like autoantibodies was seen in TGF- β 1-deficient mice (Dang et al., 1995; Shull et al., 1992). Likewise, ectopic expression of TGF- β 1 in the liver results in hepatic fibrosis and cell death (Sanderson et al., 1995). However its function in the CNS is still elusive.

According to the conventional model, binding of TGF- β ligands induces the formation of a serine/threonine kinase complex that is composed of type-II receptor and type I receptor. The type II receptor kinase phosphorylates the GS domain, a regulatory region within type-I receptor and activates its kinase activity. The active type-I receptor kinase phosphorylates receptor-regulated Smad (R-Smad) proteins (Shi and Massague, 2003). In the basal state, R-Smads are retained in the cytosol because of its interaction with some adaptor proteins, such as SARA (Xu et al., 2000) and HRS (Miura et al., 2000). It is known that SARA provides an anchor where R-smads binds the activated TGF- β receptor complex and undergoes phosphorylation (Tsukazaki et al., 1998). However, the phosphorylated R-Smad has lower affinity with SARA, releasing R-Smad from the complex and allowing it to form a

complex with co-Smad, Smad4. The resulting R-Smad:co-Smad complex translocates to the nucleus and regulates gene expression of diverse target genes (Chen et al., 1997; Labbe et al., 1998; Yanagisawa et al., 1999).

In *Drosophila*, seven members of the TGF- β family are identified. Decapentaplegic (Dpp), Screw (Scw), or Glass bottom boat (Gbb) belong to BMP family and have been shown to specifically signal through type-I receptors, Thickveins (Tkv) and Saxophone (Sax), and type-II receptors, Punt and Wit (Bangi and Wharton, 2006; Shimmi and O'Connor, 2003). dActivin (dAct), Dawdle (Daw), Myoglianin (Myo), and Maverick (Mav) are members of Activin/TGF- β branch and propagate signal through only one type-I receptor, Baboon (Babo) and they share the type II receptor with BMP pathway (Lo and Frasch, 1999; Nguyen et al., 2000; Serpe and O'Connor, 2006; Zhu et al., 2008). Therefore pathway specificity is only provided by the type-I receptors. As an only type-I receptor in Activin pathway, Babo has been known to regulate several developmental processes including mushroom body remodeling during metamorphosis (Zheng et al., 2003), morphogenesis of ellipsoid body neuron in the adult (Zheng et al., 2006), motor axon guidance in the embryo (Serpe and O'Connor, 2006) and neuron proliferation in the larval brain (Brummel et al., 1999). It indicates that Babo may respond to different ligands in different developmental context.

Here we demonstrate that Babo/dSmad2 mediates TGF- β signaling through another ligand Myo, and plays a central role in the PCD of vCrz neurons during metamorphosis. Interestingly, we further observed that endosome enriched protein SARA is also involved in vCrz PCD, adding a complexity of TGF- β signaling.

III. Materials and methods

Fly strains and genetic crossing

The recombinant fly lines were generated and employed for MARCM of Crz neurons. The Crz-gal4 lines were from our previous studies (Choi et al., 2008). For *babo* mutation: (1) hs-FLP, UAS-mCD8GFP; FRT^{G13}, tubP-Gal80/CyO, y⁺. (2) FRT^{G13}, *babo*⁵²/CyO, y⁺; Crz-gal4. (3) FRT^{G13}, *babo*^{Fd4}/CyO, y⁺. (4) FRT^{G13}, *babo*^{Fd4}; Crz-gal4, UAS-babo. For *dSmad2*¹ (Zheng et al., 2003): (5) FRT^{19A}, tubP-Gal80, hs-FLP; Crz-gal4 and (6) FRT^{19A}, *dSmad2*¹, UAS-mCD8GFP/FM7C. For *sara* mutation: (7) FRT^{G13}, *sara*¹²/CyO, y⁺; Crz-gal4 (Bokel et al., 2006). For a wild-type clone: (8) FRT^{G13}, UAS-mCD8GFP; Crz-gal4.

The following mutants for the type-II receptors were also used: *wit*^{G15}/TM6B (Marques et al., 2002), *punt*⁶² and *punt*¹³⁵ (Simin et al., 1998). In an experiment, *wit*^{G15} was recombined with the *punt* mutants. To induce transgenic RNAi-mediated knockdown, the following UAS-lines (gift from M. O'Connor) were crossed to a Crz-gal4: UAS-*punt*^{RNAi}, UAS-*wit*^{RNAi}, and UAS-*dSmad2*^{RNAi} (Vienna Drosophila RNAi Center). The *myo*^{D1} mutant, *myo*-gal4 and UAS-babo lines were also obtained from M. O'Connor (Awasaki et al., 2011; Zhu et al., 2008b), and UAS-Akt and UAS-ras^{v12} lines from E. Baehrecke.

Generation of MARCM clones

Embryos (0-8 hours old) were collected and placed on standard fly food. A 60-min heat shock at 37°C was applied to induce the expression of the hs-FLP transgene, and hence mitotic recombination. After the heat pulse, the animals were kept at 25°C until the CNSs

were dissected for immunocytochemistry.

Detection of MARCM clones by immunohistochemistry

Whole-mount Crz immunohistochemistry was performed, as described previously (Choi et al., 2005). The primary antibodies were detected by FITC- or TRITC-conjugated secondary antibodies (Jackson ImmunoResearch) at 1:200 dilution. The specimens were then cleared in 60% glycerol and mounted in 10% glycerol. Fluorescent signals were acquired by Olympus BX61 connected with CC-12 camera.

IV. Results

Babo and Smad2 are required for vCrz PCD

As most homozygous mutations of PCD-associated genes cause embryonic or early larval lethality, it is difficult to assess these genes' functions in late developmental events. As previously described, clonal analysis of vCrz neurons based MARCM assay poses a good reason that we can use it to study homozygous gene function in vCrz PCD (Lee and Luo, 1999). Therefore, we conducted a MARCM-based screen for mutations that disrupt normal cell death in vCrz. Since several lines of evidence indicate that TGF- β has a role in cell death, thus a collection of known mutations of the TGF- β receptor was examined

Two independent *babo*-null alleles, *babo*⁵² and *babo*^{Fd4} were further characterized (Brummel et al., 1999). We found around 85% specimens contain surviving vCrz neurons at 6 h APF (n=50); all of these vCrz are GFP labeled and therefore bear *babo*^{Fd4} mutant alleles (Fig. 3-1A-C), whereas only 35% specimens retain surviving vCrz in *babo*⁵² MARCM analysis (n=50) (Fig. 3-1D-F). This is consistent with the previous study and supports the

notion that *babo*^{Fd4} may function as an antimorphic allele since it is still capable of binding ligands, but not signaling (Zheng et al 2003). Furthermore, *babo* hemizygous mutant CNS showed significant rescue of vCrz PCD (Fig. 3-1G). Together, the data strongly suggest that autonomous TGF- β signaling mediated by Babo is crucial for the PCD of vCrz neurons. The *babo* locus codes for three isoforms, Babo-a, Babo-b, and Babo-c, varying the fourth exon through differential splicing and each isoform seems to perform functions specific to the type of ligand (Jensen et al., 2009). Four cysteine residues interspersed within this domain are conserved and involved in the formation of disulfide bonds upon ligand binding (Brummel et al., 1999; Wrana et al., 1994). To determine the isoform that is responsible for the vCrz PCD, each isoform was expressed in *babo*^{Fd4} mutant background. Transgenic expression of the *babo-a* complemented *babo*^{Fd4}, as vCrz PCD took place normally, leaving no detectable vCrz neurons in the tissue (n=50, Fig. 3-1H). In contrast, expression of the *babo-b* or *babo-c* did not rescue *babo*^{Fd4} mutation, as ~40% sample of *babo-b* expression (n=32, Fig. 3-1I) and 30% of *babo-c* expression (n=50, data not shown) showed defective PCD phenotype, which is comparable to that observed with *babo*^{Fd4} mutant alone. The result is also consistent with the fact that *babo-a* is the predominant form expressed in the CNS (Jensen et al., 2009).

Since dSmad2 is a likely candidate as an R-Smad for the Babo-mediated signal transduction, we investigated the role of dSmad2 as a proapoptotic factor. Genetic analyses of *dSmad2*¹ mutant as well as RNAi-mediated knockdown resulted in the defective PCD of vCrz neurons, as the prepupal CNS at 6 h APF showed 13-14 vCrz neurons alive (Fig.

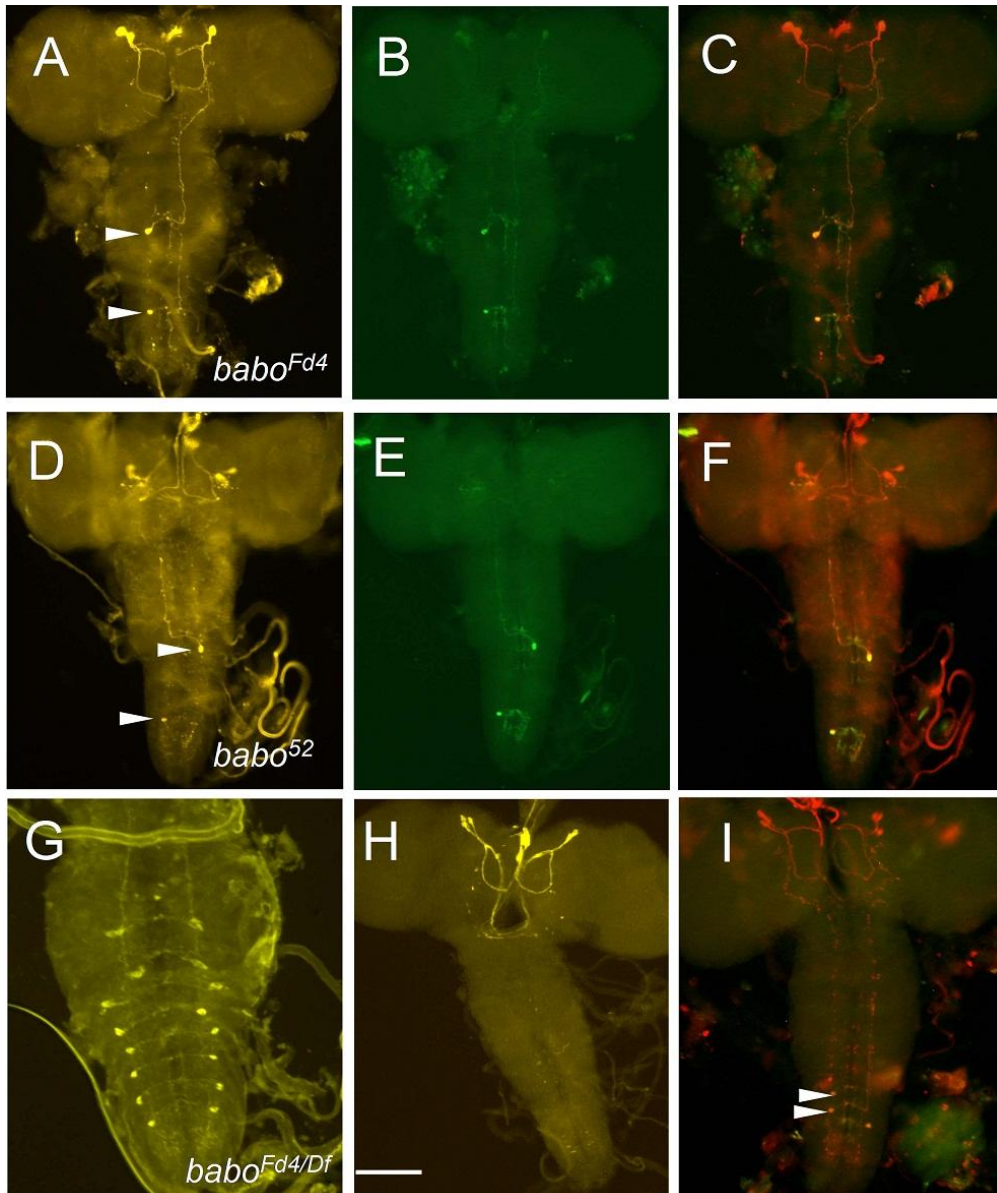


Figure 3- 1. PCD of vCrz neurons is prevented in homozygous *babo* mutant clones.

Mitotic recombination was induced by a heat-shock of embryos at 0-8 h AEL and then vCrz neurons were examined at 6-7 h APF. (A-C) *babo*^{Fd4} mutant clones (arrowheads). Note that only GFP-labeled vCrz neurons bearing *babo*^{Fd4} homozygous mutation survive, thus become Crz-immunoreactive. (D-F) Same as A-C, except for *babo*⁵² allele used (G) Hemizygous *babo* mutant VNC (*babo*^{Fd4/Df}) at 20 h APF. Most vCrz neurons do not undergo PCD. (H) Expression of UAS-*babo*-a complemented *babo*^{Fd4} with respect to vCrz PCD, as

no GFP-labeled mutant vCrz neurons are detectable. (I) Defective PCD in *babo*^{Fd4} mutant CNS is not rescued by the expression of UAS-babo-b (or UAS-babo-c, data not shown), suggesting that these two isoforms do not complement *babo*^{Fd4} mutation. Scale bars = 100 μ m.

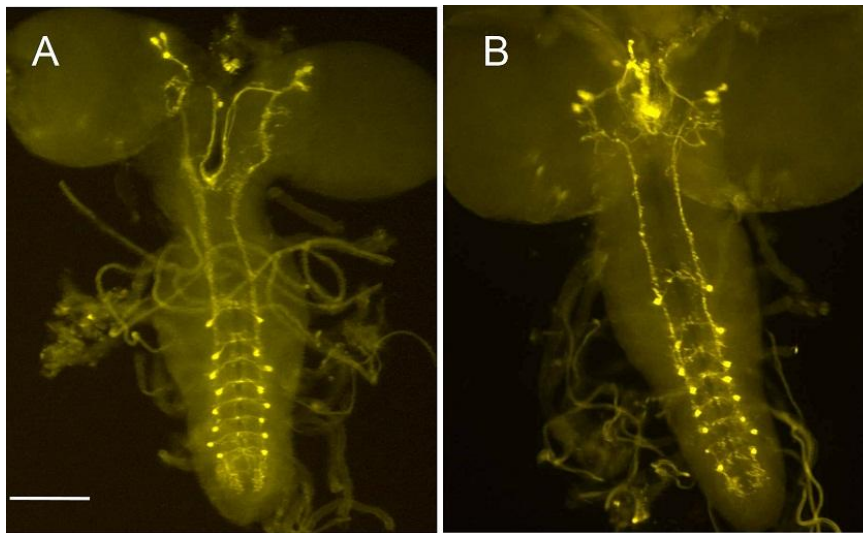


Figure 3- 2. Roles of Smad2 for vCrz PCD.

(A) Most vCrz neurons survive in *dSmad2*¹ homozygous mutant prepupal CNS 6 h APF (16 ± 0 , n=5). (B) Knockdown of *dSmad2* blocks the PCD significantly (13.5 ± 1.0 , n=5). (Genotype: Crz-gal4/+; Crz-gal4/UAS-dSmad2^{RNAi}). Scale bar = 100 μ m.

3-2A,B). Taken together, the results suggest that Babo and dSmad2 are essential signaling components for the neuronal PCD during metamorphosis.

Punt and Wit mutant exhibit different phenotype expressivity as babo

According to the study in mushroom pruning, *punt* and *wit* could function redundantly.

Therefore we combined *punt* and *wit* mutant together, *punt*⁶², *wit*^{G15} and *punt*¹³⁵, *wit*^{G15}. To our surprise, these double transhetero mutant at 25°C also does not significantly block cell death (Figure 3-3C). Result of using double RNAi for Punt and Wit showed similar results with mutant analysis (Figure 3-3D). Since these mutants are not null alleles, we can not conclude whether *punt* and *wit* are required for vCrz PCD. However, considering type I receptor *babo* mutant has strong phenotypic effect in cell death, it is speculated that type II receptor may have different phenotype expressivity.

Myo is required for PCD

The *Drosophila* genome contains four potential ligands for Babo: Activin, Myoglianin (Myo), Maverick (Mav) and Daw (reviewed by (Parker et al., 2006). Both Activin and Myo are known to bind Babo in biochemical assay (Lee-Hoeflich et al., 2005), but only Activin function is well characterized in mushroom body remodeling, neuron proliferation and motor axon guidance during development (Serpe and O'Connor, 2006; Zheng et al., 2003; Zhu et al., 2008). To functionally explore their roles in vCrz PCD, three ligands with available mutant, Activin, Myo and Daw have been examined during metamorphosis. We observed no blocking effect of PCD in *activin* or *dawdle* homozygous mutants but complete blocking in *myo* mutant at 6 h APF (Fig. 3-4A). Even after 20 h APF, 8 pair neurons are also retained

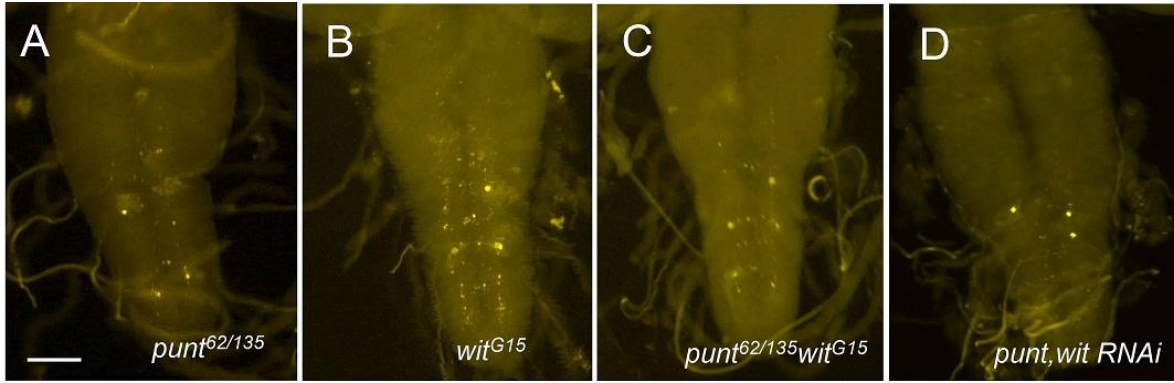


Figure 3- 3. *punt* and *wit* mutant analysis in vCrz death.

(A-C) Anti-Crz staining for the indicated genotypes at 7 h APF. Only a few vCrz cell debris without any projections remain detectable in these mutants. (D) Double RNAi-mediated knockdown of the *punt* and *wit*. The result is similar to that in the double *punt*, *wit* mutant. (Genotype: Crz-gal4/UAS-*punt*^{RNAi}; Crz-gal4/UAS-*wit*^{RNAi}). Scale bar = 50 μ m.

with intact neuronal morphology (Fig. 3-4B). To further study *myo* function, *myo*'s expression pattern was examined using GAL4-*myo* and it shows that *myo* is mainly expressed in glial cells and not Crz neurons (Fig. 3-4C). However there are always a few number of *myo*-expressing glial cell around vCrz at white pupal stage. This discovery is consistent with the notion that Myo is a secreted ligand and not a cell-autonomous factor for vCrz PCD.

Myo is not exclusively transduced into Babo

In vCrz PCD, Myo and its binding receptor, Babo and downstream Smad2 are required for cell death. However their blocking intensity is quite different. Myo has the strongest blocking effect among them. In MB remodeling and vCrz PCD, EcR is a critical factor to synchronize these degeneration process during metamorphosis (Zheng et al., 2003). To further study its mechanism, we examined the EcR gene expression and found out it completely depletes EcR expression in *myo* mutant (Fig. 3-4D). Whereas the expression level of EcR-B1, was not altered in *babo* mutant according to anti-EcR-B1 staining (Fig. 3-5D,E). Furthermore, forced expression of EcR-B1 did not rescue *babo* mutant clonal defects (Fig. 3-5A-C). Combining these lines of evidence, we postulate that Myo is critical factor for EcR expression and therefore it is important for vCrz PCD, however its death signal is not exclusively transduced through Babo and Smad2. Such a model could explain the observed blocking effect difference between *myo* and *babo*. As inferred by myostatin function study in mouse (McCroskery et al., 2003; Yang et al., 2007), it could be that other downstream components bypass and transduce death signal to EcR-B1. Therefore, the

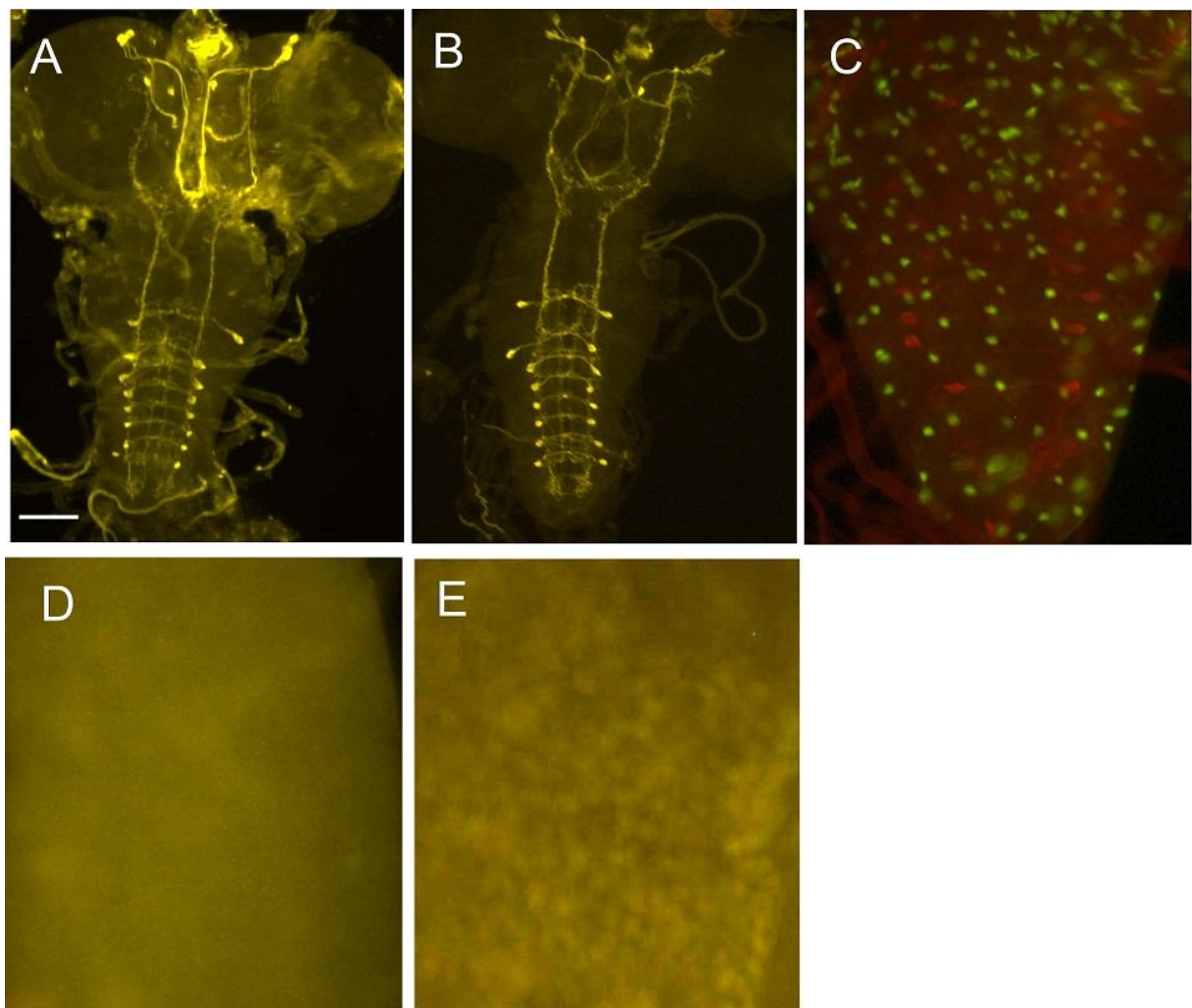


Figure 3- 4. Myoglianin (*myo*) is required for the PCD of vCrz neurons.

(A, B) Anti-Crz immunohistochemistry in homozygous *myo*-null mutant at 7 h APF and 20 h APF, respectively. Most vCrz neurons remain intact even at 20 h APF. (C) Double labeling of the white prepupal CNS for the expression of *myo* (GAL4-*myo*/UAS-nuclear GFP) and Crz (red, immunohistochemistry). Expression of the *myo* does not overlap with Crz. (D) EcR-B1 expression is not detectable in *myo* mutant VNC of the white prepupa. (E) Faint but numerous EcR-B1 immunoreactivity in the wild-type control. Scale bar = 50 μ m.

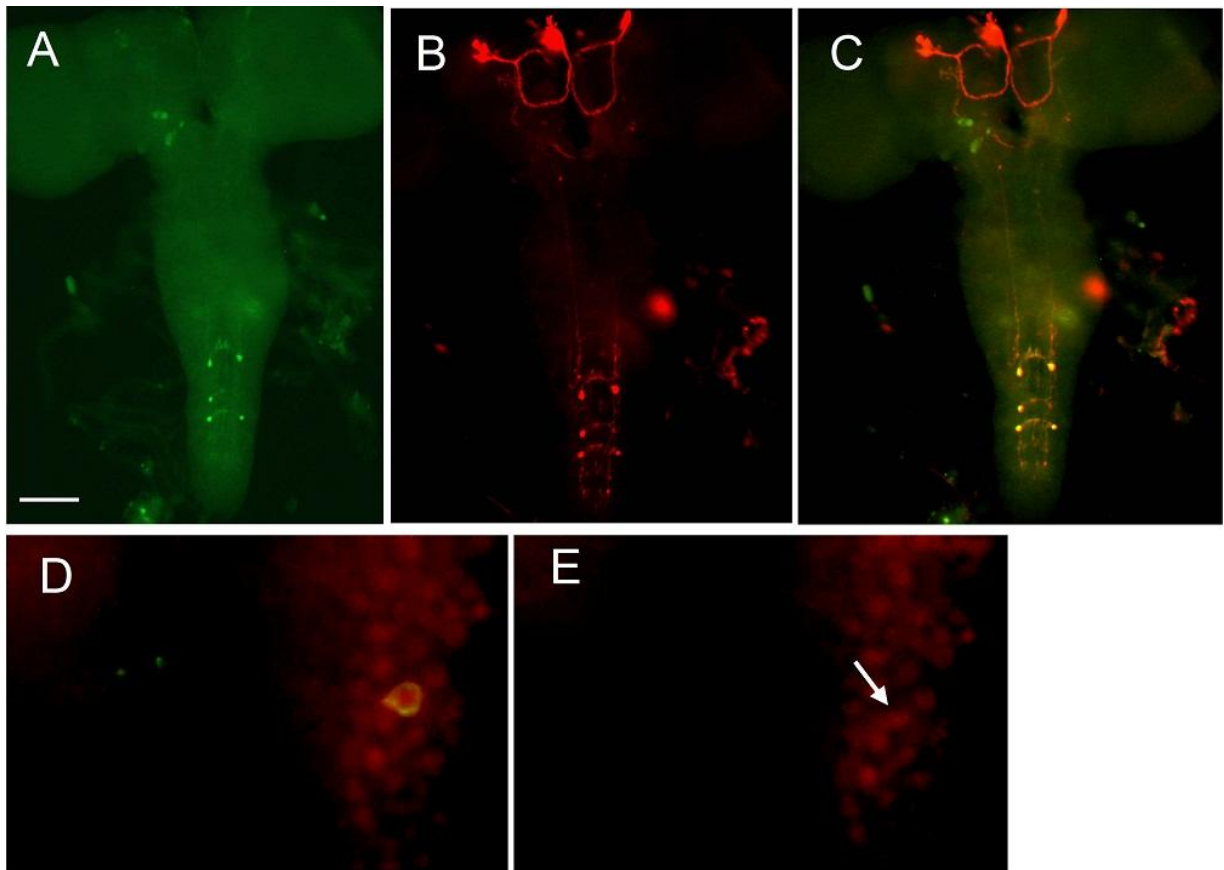


Figure 3- 5. EcR-B1 is not downstream of Babo.

(A-C) Live vCrz neurons carrying *babo*^{Fd4} mutation at 6 h APF after ectopic expression of EcR-B1 (Genotype: hs-FLP, UAS-mCD8GFP/X; FRT^{G13}, *babo*^{Fd4}/FRT^{G13}, tubP-gal80; Crz-gal4, UAS-EcR-B1/+). (D, E) Anti-EcR-B1 staining (red) in a *babo*^{Fd4} mutant clone (green) in white pupal stage is indicated by an arrow. Scale bar = 10 μ m.

relevant transcriptional targets of Babo/Smad2 and other downstream of Myo in vCrz PCD need to be identified.

Sara is a concentration-dependent biphasic modulator of TGF- β signaling

As we know, TGF- β receptor transmits signal to nucleus through phosphorylation of Smad2. Our results showed type I receptor Babo and dSmad2 are essential for activating the TGF- β signaling pathway in vCrz PCD. However, these seemingly simple subcellular translocation of Smad2 is still highly regulated by a number of additional factors. Smad protein can undergo nucleocytoplasmic shuttling freely in the basal state (Inman et al., 2002; Nicolas et al., 2004). However, several cytosolic retention factors can keep smad2 in the cytoplasm through physical interaction between them. Once phosphorylation of Smad2 by type I receptor occurs, affinity between those cytosolic retention factor and smad2 declines and smad2 will accumulate in the nucleus. Therefore, the intra-cellular localization results from the battling between phosphorylation of Smad2 and interaction of retention factors (Derynck and Zhang, 2003; ten Dijke and Hill, 2004). SARA, an adaptor protein that presents an anchor where the activated TGF- β receptor complex interacts and phosphorylates R-Smad, is also the best characterized cytosolic retention factor for Smad2 (Tsukazaki et al., 1998; Xu et al., 2000). Despite some studies on SARA function of Smad nuclear import, the physiological consequences of disruption of proper Smad amount within the nucleus when SARA amount is manipulated in TGF- β signaling have rarely been examined.

sara has two mutant alleles *sara*¹² and *sara*¹. *sara*¹ is a point mutation in FYVE domain, resulting in mistargeting of SARA out of the apical endosomes into the cytosol.

*sara*¹² removes the entire coding region and represents a null mutation. Both mutants fail to target signaling cargo to endosomes associated with the spindle machinery during mitosis (Bokel et al., 2006). We tested flies bearing those mutant alleles in pupal stage. About 50% of vCrz neurons are observed in *sara*¹² tissue (Figure 3-6A), similar to those animals with transallelic mutant of *sara*¹ (Figure 3-6B). To further confirm it, clonal analysis is also applied to those mutants. Around 20% specimens have live vCrz neurons in *sara*¹² MARCM analysis (n=30) (Figure 3-6C-E). It indicates that SARA, as an adaptor factor, is indispensable for phosphorylation of Smad2. Disruption of SARA function will lead to decreased nuclear accumulation of Smad2.

To further study the role of SARA during TGF- β signaling, SARA protein level was manipulated in vCrz using a genetic approach. Over-expression of *sara* in Crz blocks vCrz PCD during metamorphosis (Figure 3-6F). This result validates that SARA may function as a cytosolic retention factor and high expression levels of SARA can interfere with nuclear translocation of Smad2, thereby reducing its transcriptional activator functions. Therefore, the relative amount of SARA and Smad2 is precisely regulated in the cell and disruption their ratio could change the Smad2 subcellular distribution. To demonstrate this, we elevated Smad2 and SARA expression level together. We observed that ectopic co-expression of dSmad2 and SARA completely revert the blocking effect of SARA (Fig. 3-6G). It indicates that high Smad2 levels over saturate SARA and yield free distribution for Smad2. Thus SARA is a concentration-dependent biphasic modulator of TGF- β signaling. Depletion of SARA protein can deteriorate the ability of phosphorylation of Smad2 by the type I receptor,

and then change its subcellular distribution. However elevating SARA levels could retain cytosolic localization of Smad2 and block smad2 nucleus trafficking.

V. Discussion

The TGF- β superfamily including TGF- β , Activins and bone morphogenetic proteins (BMPs) play critical roles in regulating the development of many organisms. TGF- β functions in a context-dependent manner. Upon the cell type, the differentiation stage, it may act differently (Sanchez-Capelo, 2005; Schmierer et al., 2008; Schuster and Krieglstein, 2002). Whereas Activins have been shown to be important apoptotic players in different systems, e.g. in the immune system, in tumorigenesis and in the nervous system, especially in oligodendroglial progenitors (Deli et al., 2008; Schulz et al., 2008; Wang et al., 1996), they are also involved in proliferation and function as survival factor (Ageta et al., 2008; Bilezikjian et al., 2006). Our studies provide the first genetic evidence that TGF- β can function as a death signal and may help understanding the implemented mechanism in a different context.

An intertwined roles of TGF- β in cell death and other signal pathway

As a member of TGF- β superfamily, Myo have several features including a core of hydrophobic amino acids near N-terminal regions as a secretory protein signal and cysteine cluster in the C-terminal which facilitates the forming of a homodimer, and a proteolytic processing site (McPherron et al., 1997). Its mammalian homolog, Myostatin has been known to be expressed specifically in developing and adult skeletal muscle and functions as a negative regulator of skeletal muscle cell proliferation and differentiation. In contrast to

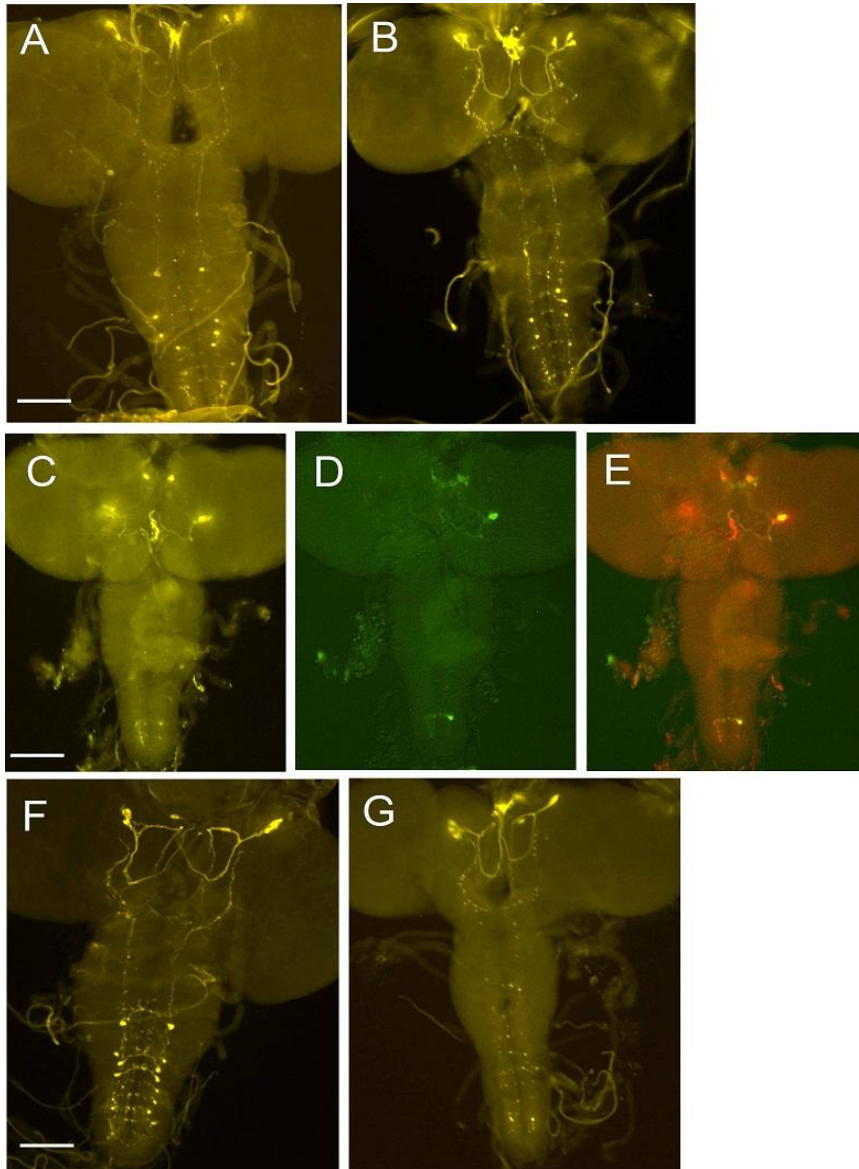


Figure 3- 6. Sara has dual roles for vCrz PCD.

(A, B) *sara* mutant alleles, as indicated, block vCrz death partially. Numbers of vCrz neurons detected are 5.6 ± 0.5 (n=5) for *sara*¹² and 5 ± 0.6 (n=5) for *sara*¹. (C-E) MARCM analysis of *sara*¹² allele. (Genotype: hs-FLP, UAS-mCD8GFP/X; FRT^{G13}, *sara*¹²/FRT^{G13}, tubP-gal80; Crz-gal4/+). (F) Overexpression of wild-type *sara* can block the PCD (11.2 ± 0.9 , n=6). (Genotype: Crz-gal4/UAS-*sara*). (G) Co-expression of *sara* and *dSmad2* reverts blocking effect of *sara*. (Genotype: Crz-gal4, UAS-*dSmad2*/UAS-*sara*). Scale bar = 100 μ m.

Myostatin's muscle-specific expression through development, Myo is mainly expressed in glial and muscle cell during embryogenesis, and later during third larval stages, it is only observable in the CNS. Both myoglianin and myostatin are shown to bind type I and type II receptor complex to activate TGF- β /Activin-like signaling pathways (Lee-Hoeflich et al., 2005; Rebbapragada et al., 2003). Additionally, non-Smad-dependent pathway has also been implicated to signal through inhibiting AKT phosphorylation (McCroskery et al., 2003; Yang et al., 2007). Therefore, consideration of how Myo/Myostatin activates downstream effectors will be paramount in understanding the functional flexibility and molecular specificity in TGF- β signal transduction.

Since loss of EcR expression was observed in *myo* mutant but not in the *babo* mutant, it raises the hypothesis that Myo does not bind exclusively to Babo and transduced to Babo/Smad2 signal pathway in vCrz PCD. Previous structure studies have revealed a similarity between members of the TGF- β superfamily and members of the NGF and platelet-derived growth factor (PDGF) families (McDonald and Hendrickson, 1993). All of these ligands contain the cysteine knot motif. In particular, crystallography studies showed the cores of the monomers are nearly superimposable. NGF and PDGF belong to neurotrophic factor and originally are identified as neuronal survival factors. They exert diverse biological effects by binding a specific receptor. NGF binds TrkA and PDGF binds PDGF receptor and they can activate two pathways including phosphoinositide 3-kinase (PI-3k)/Akt and Ras/MAPK (Kaplan and Miller, 2000). Over the last two decades, extensive research has been carried out to study their function in stochastic neuron PCD

during embryonic stage. However their roles in PCD during later stages are still largely unknown. To elucidate their functions in vCrz PCD, several downstream factors including Ras, PIP3K and Akt have been examined in vCrz PCD. Overexpression of Ras or Akt caused cell death defect as Myo (Fig. 3-7A,B). All these results indicate that neurotrophic factors play an essential role in vCrz PCD. Considering structure similarity between TGF- β ligand and NGF, PDGF, it raises the probability that Myo may regulate survival factor signal through competition to bind their receptors or decreasing their transcription level indirectly.

Babo-a is a type I receptor for Myo

Four potential Activin-like ligands are found in *Drosophila*, Dawdle, Activin, Myo, and Mav. They are known to regulate many cellular functions including cell growth, cell-fate determination and differentiation, and apoptosis (Schmierer and Hill, 2007). The diversity of those signal effects for different ligands relies on large repository of receptor combination. One type-I receptor and two type-II receptors are identified in *Drosophila* and have capacity to form a functional heterocomplex. Recently three type-I receptor Babo isoforms are also discovered to contribute to the specificity of the signal (Jensen et al., 2009). During development, specific combination of these receptors is expressed in different larval tissues. As such, they regulate various activin-type signals. Dawdle was found to preferentially signal through one isoform of type-I receptor, Babo-c (Jensen et al., 2009) and Activin is specifically binding to another isoform Babo-b based on cell-line binding assay (personal communication with M. O'Connor). Of interest, our data indicate that Babo-a is a major signaling component for vCrz cell death. Preferential expression of *babo-a* in the larval

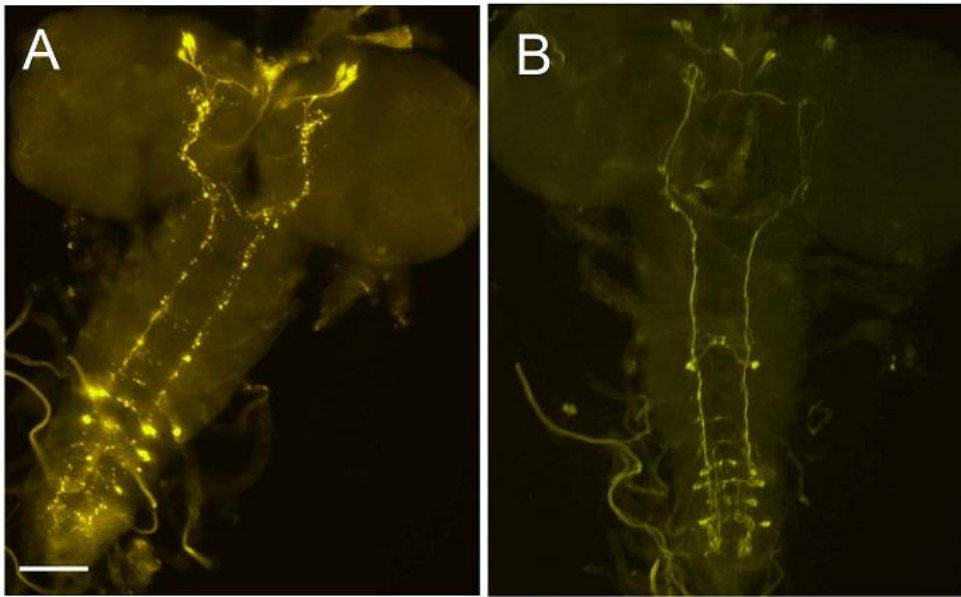


Figure 3- 7. Effect of survival factors on the vCrz PCD.

(A) Overexpression of a UAS-Akt shows cell death defect (5.8 ± 0.9 , $n=6$) (genotype: Crz-gal4/+; Crz-gal4/UAS-Akt). (B) Overexpression of a constitutively active form of Ras blocks the cell death (6 ± 0.5 , $n=5$) (genotype: Crz-gal4/UAS-ras^{v12}). Scale bar = 100 μ m.

CNS supports our data (Jensen et al., 2009). Taking together, we gingerly propose that Myo functions preferentially through the Babo-a isoform to induce various events occurring in the post-embryonic CNS.

Type I receptor and type II receptor has different phenotype expressivity

Although type I and type II TGF- β receptors are presumed to function in the same signaling complex, their respective ligand binding affinity varies among different subfamily. The generally established theory in TGF- β signaling assumes that the type II receptor represents the high-affinity receptor and the binding of ligand to the type II receptor firstly is followed by recruitment of the type I receptor. However for BMP-2, the high affinity receptor is the type I receptor and their sequential ligand binding is reversed even though a measureable low affinity of type II BMP receptor for free BMP-2 exists (Kawabata et al., 1995; Liu et al., 1995). Mutations in two respective binding epitopes of high-affinity and low affinity receptor in BMP2 yielded different effect on biological activity in C2C12 cells. Only epitope for low affinity receptor binding behaved as antagonists partially or completely inhibiting BMP-2 activity (Kirsch et al., 2000). It indicates that high affinity and low affinity receptor exert variable influence on assemble of ternary signaling complex and the related cellular signaling process. Generally, the high affinity ligand-receptor binding implies that a relatively low amount of proteins are needed to form ligand-receptor complex, whereas low affinity binding require a relatively high concentration of a ligand or receptor. Binding study of TGF- β 1 with its low affinity receptor T β R-1 revealed that the forming heteromeric complexes of type I and type II receptors was dependent on the presence of low

concentration of high affinity type II receptor (ten Dijke et al., 1994). In our study, *punt* and *wit* double mutant does not produce a comparable blocking phenotype as *babo* mutant. The similar logic can be used to explain this. First, the *punt* and *wit* mutants in the study are not null mutant. For instance, *punt*¹³⁵ is a moderate allele and it cannot be ruled out the possibility that the small amount of Punt exist (Simin et al., 1998). Second, *punt* and *wit* mutant are zygotic loss-of-function mutant. Perdurance of maternal protein results in the presence of low concentration of protein (Marques et al., 2002 ; Simin et al., 1998). Thus, mutant cells may possibly possess small number of type II receptors, which allows formation of functional ternary signaling complex. In contrast, as a low affinity of receptor, *babo* mutant exhibits a severe cell death defect. In some sense, it validates our hypothesis.

Nucleocytoplasmic shuttling is a dynamic process

Smad2 are key intracellular signal transducers for TGF- β signaling and the level of nuclear R-Smads determine the duration and strength of signaling (ten Dijke and Hill, 2004). Normally, Smads possess intrinsic nucleocytoplasmic shuttling capacity and lots of interacting proteins including nuclear transport factors and cytosolic retention proteins can alter their translocation and determine their subcellular distribution (Pierreux et al., 2000; Schmierer et al., 2008; Watanabe et al., 2000). It is generally thought that the phosphorylation status of R-Smads is coupled with their nuclear accumulation. TGF- β activation can retain more phosphorylated Smad2 in the nucleus through inhibition of Smad2 nuclear export rather than change of the nuclear import rate of Smad2 (Schmierer and Hill, 2005). Also, it is reported that Ran-binding protein 3 (RanBP3) can mediate nuclear export

of R-Smad after R-Smad are dephosphorylated by the nuclear phosphatase (Dai et al., 2009; Lin et al., 2006). In our study, TGF- β signaling is shown to have a biphasic response to SARA concentration. An optimal amount of SARA is required for Smad2 nucleus accumulation, whereas low levels and high levels deteriorate the response. It may explain that SARA has dual roles. As a cytosolic retention factor, SARA can prevent the Smad2 nuclear import, whereas as an anchor protein, it can help in phosphorylation and nuclear accumulation of Smad2, thereby enhancing TGF- β signaling.

Chapter Four

Endocytic trafficking is required for neuron cell death through regulating TGF- β signaling

This part is a manuscript in preparation

I. Abstract

Endocytic trafficking is crucial for various aspects of cell homeostasis. Here, we show that endocytic membrane trafficking is coupled with cell signaling during PCD. Endocytosis and its regulatory signal of TGF- β play essential roles in vCrz PCD during early metamorphosis. By using available genetic tools, we revealed the following events: the clathrin mediated membrane receptor internalization and subsequent endocytic events involved in Rab5 dependent early endosome and Rab11 recycling endosome differentially participates in TGF- β signaling. Two early endosome-enriched proteins SARA and HRS are observed to act as a cytosolic retention factor of Smad2, indicating that endocytosis mediates TGF- β signaling via regulation of the dissociation of Smad2 and its cytosolic retention factor, SARA and Hrs.

II. Introduction

No cell lives in an isolated island. In all multicellular organisms, cell fate determination, cell death or survival is largely controlled by an elaborate intercellular communication network. This is particularly the case in the nervous system. Neural functions are strictly dependent on the cellular interactions. Inter-neuronal communication occurs by means of variable signals such as hormones, neurotransmitters, or secreted proteins. These extracellular modulators may promote survival or favor the cell death according to receptors that the cell bears (Davies, 2003; Oppenheim et al., 1999). For instance, developing motoneurons or sensory neurons deprived of neurotrophic factors died through the interaction

of the death ligand and its receptor. The well-studied case of death ligand is a member of the tumor necrosis factor (TNF) family. Activation of Fas in cultured embryonic motoneurons is even sufficient to trigger death of a significant proportion of motoneurons in the presence of neurotrophic factors (Lewin and Barde, 1996; Raoul et al., 1999). However, aside from the neuron cell death induced by TNF family (Frade and Barde, 1998), other possible death receptors have remained undetermined.

Signal transduction by many surface receptors is largely involved in membrane trafficking and endocytosis. For many years, the internalization of receptors by endocytosis is not only known to be associated with signal attenuation by degradation of the complex of receptor and ligand, but also to play an essential role in propagation of signal transduction (Le Borgne et al., 2005). Experiments demonstrate that active receptors initiate signal from the plasma membrane, and stay in their active states in endosomes, suggesting continuation of signaling from this intracellular compartment (Di Guglielmo et al., 2003). Therefore, endocytosis may serve to bring ligand-bound receptors to the specific intracellular compartments where signal-transducing machinery is assembled (Seto et al., 2002).

Endocytosis is the process of cellular ingestion by which plasma membrane is internalized into the cytoplasm together with ingested molecules. According to the kinetic relevance and the diagnostic molecular markers, the endocytic pathway is defined as several functional organelles, including clathrin-coated vesicles (CCVs), early endosomes, recycling endosomes, late endosomes and lysosomes. As one of molecular markers, the Rab protein in rab family of ras-like GTPases are distributed to distinct intracellular compartments and

regulate transport between organelles (Zerial and McBride, 2001).

Endocytosis is typically initiated by the formation of CCVs in the cell membrane (Gruenberg, 2001). Dynamin has been shown to play a key role in this process. Dynamin has been originally linked to endocytosis through the discovery of a temperature-sensitive *Drosophila* mutant *shibire^{ts}* (*shi^{ts}*). In *shi^{ts}*, endocytosis is inhibited at the restrictive temperature because clathrin-coated pits cannot be pinched off from the plasma membrane (PM) (Kosaka and Ikeda, 1983).

The second station along the endocytic pathway is early endosome, where the endocytosed proteins are delivered by the endocytic vesicle. It is a critical organelle to serve as a sorting station along the endo-lysosomal pathway. Whether an endocytosed protein is recycled back to membrane or delivered to the successive degradative compartment is controlled by the sorting process initiated in the early endosome. Rab proteins mediate trafficking at each distinct step of interorganellar transport. Rab5 mediates traffic from the PM to the early endosome (Bucci et al., 1992), Rab7 the step from the early sorting endosome to the degradative compartment (Bucci et al., 2000) and Rab4 and Rab11 trafficking within the recycling pathway (Zerial and McBride, 2001).

we reported here the investigation of endocytosis dependent signal transduction on neuron cell death. A set of dominant negative, constitutively active, temperature sensitive form of endocytosis transporter proteins were examined in vCrz PCD and they showed significant blocking of cell death. The underneath possible transduced messenger is TGF- β signaling.

III. Methods and Materials

Fly Stocks

Inhibition of dynamin and CHC function (1) w; TM3 UAS-shi^{K44A}/TM6B (Molline et al., 1999) (2) UAS-Shi^{ts} (Awasaki and Ito, 2004) (3) UAS-CHC^{DN} (Bloomington Stock Center). Early endosome analysis (4) UAS-Rab5^{SN} (Entchev et al., 2000) (5) UAS-sara (Bokel et al., 2006)(6) UAS-Hrs (Seto and Bellen, 2006)(7) UAS-hrs^{RNAi} (Bloomington Stock Center). Recycling and late endosome analysis (8) UAS-Rab11^{NI} (Satoh et al., 2005) (9)UAS-Rab11^{RNAi} (Satoh et al., 2005) (10)UAS-Rab7^{QL} (Entchev et al., 2000). TGF- β pathway analysis (11) UAS-Smad2 (12) UAS-Smad2^{SVD} (13) UAS-Smad2-2SA (14) UAS-babo-a

Generation of MARCM clones

Embryos of 0-8 hours old were collected and placed on standard fly food. A 60-min heat shock at 37°C was applied to induce the expression of the hs-FLP transgene, and hence mitotic recombination. After the heat pulse, the animals were kept at 25°C until dissection.

Generation of UAS-babo-aWA transgenic line

Mutagenesis was carried out by two rounds of PCR amplification. First, using the PUASt-babo-a (from Dr. O'Connor) as a template, two DNA fragments were generated using the first pair of primer: baboKpn1_forward (ATCGCGTGGGTACCAAGCGC), baboWA_reverse (GATTGGGATACGCGCACTCCTTC) and second pair of primer: baboWA_forward (GAAGGAGTGCGCGTATCCCAATC) and UAS_reverse (TGTCCAATTATGTCACACCAC). The primer baboWA-reverse and baboWA_forward

are complementary to each other and contain point mutation of Tryptophan to Alanine (TGG-GCG). The two amplified PCR products were then mixed together and re-amplified using primer baboKpn1_forward and UAS-reverse to yield a 500bp fragment. The primer baboKpn1_forward was specifically designed to contain a Kpn I digestion site. With KpnI and XbaI digestion, we combined the mutant sequence with the original PUASt-babo-a construct. After sequencing, the construct was injected into the w^{1118} embryos for germ-line transformation.

IV. Results:

Clathrin-mediated internalization is required for cell death

The binding of an extracellular ligand at the cell surface triggers the internalization of molecules into internal membrane compartments. In most animal cell types and under normal conditions, the complex of receptor and ligands are endocytosed mainly through the formation of CCVs (Mellman, 1996).

The GTPase dynamin is a critical mediator of clathrin-dependent endocytosis, and is required for pinching off invaginated coated pit during formation of CCVs (Schmid et al., 1998). To determine if dynamin regulates the internalization of cell death related receptor, the dominant-negative dynamin mutant dynamin K44A line, which is deficient in GTP binding and GTPase activity, was expressed in vCrz. Immunostaining at 6 h APF showed 14-15 vCrz neurons alive (n=6) (Fig. 4-1A). Moreover, ectopic expression of UAS-shi^{ts} at the nonpermissive temperature (29°C) manifests the comparable result with 14 neurons

surviving at 6 h APF (n=5) (Fig. 4-1B).

Dynamin has also been implicated in forming of caveolae and non-clathrin vesicles (Henley et al., 1998; Le Roy and Wrana, 2005) and non-clathrin dependent endocytosis pathway is speculated to be mainly involved in receptor degradation. To further study the endocytosis pathway that dynamin is involved, the function of clathrin needs to be examined. In the clathrin-mediated endocytic pathway, clathrin is composed of a trimer of clathrin heavy chain (CHC). A dominant negative form of the CHC was overexpressed in vCrz, which caused significant cell death defect, with 12.3 ± 0.19 (n=6) neurons alive (Fig. 4-1C). Considering cell death blocking intensity triggered by the defect of dynamin and CHC are similar, it is speculated that the extracellular signal molecules or membrane proteins are internalized through dynamin- and clathrin-dependent pathway during vCrz cell death.

Trafficking through early endosome and recycling endosome are necessary for cell death

After dynamin-mediated internalization, endocytic vesicles undergo Rab5 mediated fusion with the endosome. To determine the possible role of early endosome in cell death, we overexpress dominant negative Rab5, a constitutively GDP-bound form that inhibits endosome fusion. Subsequently, the vCrz neuron with expressing Rab5^{SN} has cell death defect and 7 pairs of vCrz neurons still survived at 6 h APF (n=6) (Fig. 4-2A).

Following their trafficking into early Rab5-containing endosomes, receptors can rapidly recycle back to the plasma membrane. Through a Rab11-dependent mechanism, traffic route is steered to the recycling endosome. We next examined whether the recycling

dependent endocytic regulation is required for signal transduction in cell death. We perturbed the endocytic routes by suppressing the function of Rab11. Crz-immunofluorescence experiment with *Crz-Gal4/UAS-Rab11^{DN}* CNSs (Fig. 4-2B, n=5) showed severe deficit in the vCrz PCD, as about 46% of neurons were detectable at 6 h APF. Less significant cell death defect was also observed with the knockdown of Rab11 (*Crz-Gal4/UAS-Rab11^{RNAi}*), which showed ~29% vCrz neurons survived (Fig. 4-2C). Nevertheless, the results suggest that levels of signaling are reduced by impaired recycling endosomal transport.

Endocytic suppression affects TGF- β signaling

We next sought to identify the molecular cargo that is transported by the clathrin and Rab5 dependent pathway. Our previous work showed that TGF- β plays a role in inducing cell death. Signaling involving Baboon receptor and its ligand Myoglianin is required for Smad2 phosphorylation in vCrz PCD during metamorphosis. Once phosphorylated, the Smad2 undergoes subcellular translocation from cytoplasm to nucleus, where Smad2 can regulate the signaling outcome. Considering that TGF- β dependent death signal emanates from the cell surface, endocytosis may be the efficient way to conduct signaling molecules translocation from cell membrane to nucleus.

To test this possibility, we performed genetic rescue experiment by overexpressing Babo-a, dSmad2 with Rab5^{SN}, Shi^{K44A} respectively. It showed that ectopic expression of dSmad2 or Babo-a can significantly rescue the cell death defect triggered by overexpression

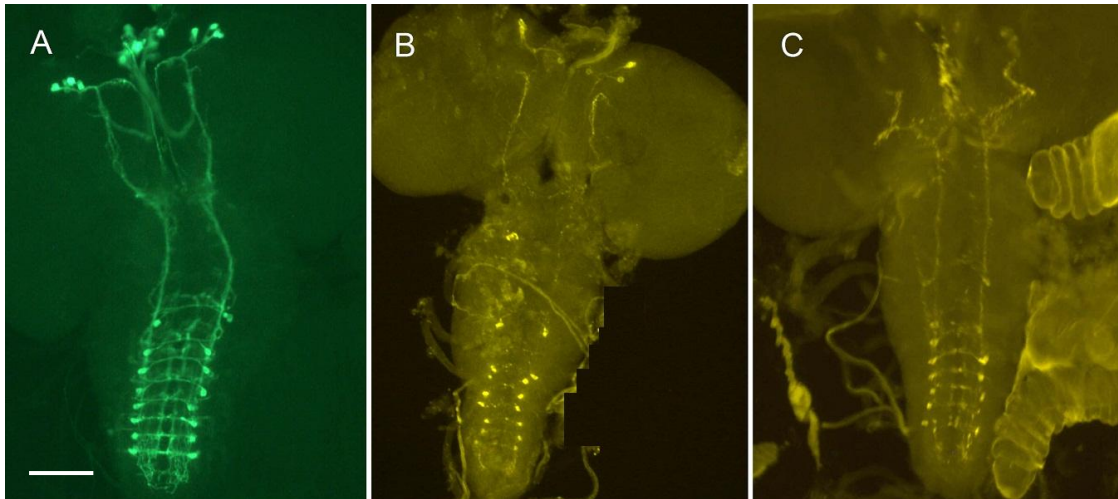


Figure 4- 1. Effect of impaired clathrin dependent internalization.

(A) Overexpression of shibre dominant negative shi^{K44A} inhibits cell death in vCrz (14.5 ± 0.67 , n=6). (Genotype: Crz-gal4/Y;; UAS- shi^{K44A}). (B) Overexpression of temperature

sensitive shi mutant at 29°C cause a comparable cell death defect as A (14.2 ± 0.66 , n=5).

(Genotype: Crz-gal4/+;; Crz-gal4/ shi^{ts}). (C) Overexpression of dominant negative CHC

also significantly blocks the cell death (12.3 ± 0.56 , n=6). (Genotype: Crz-gal4/UAS-CHC^{DN}).

Scale bars = 100 μ m.

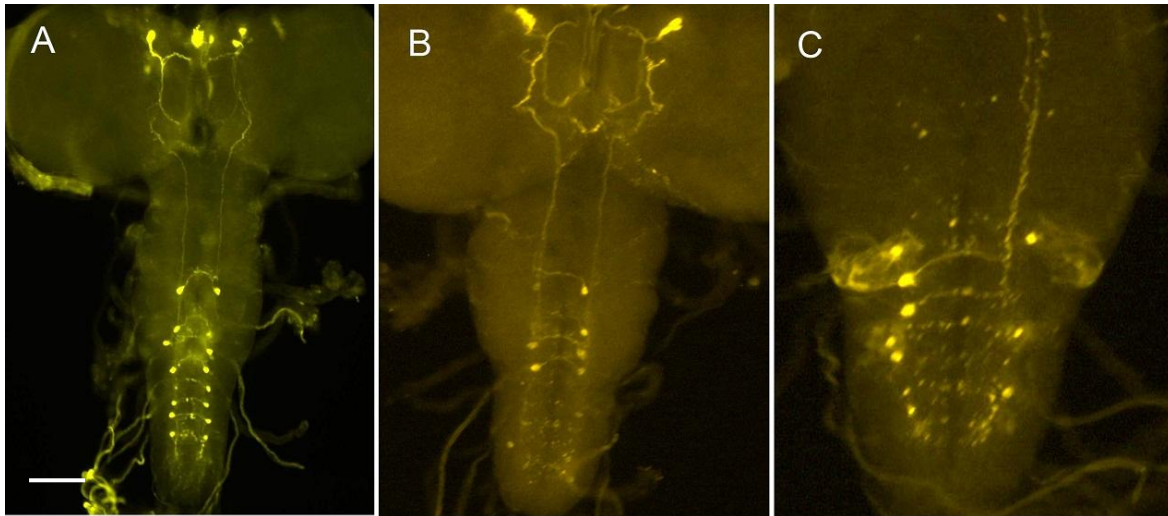


Figure 4- 2. Inhibition of Rab5-dependent, Rab11-dependent endocytic route causes cell death defect.

(A) Overexpression of dominant-negative Rab5 in Crz inhibits vCrz cell death (14.3 ± 0.91 , $n=6$). (Genotype: UAS-Rab5^{SN}/Crz-gal4). (B, C) Overexpression of dominant negative Rab11 (7.4 ± 0.92 , $n=5$) (Genotype: UAS-Rab11^{N124I}) or RNAi mediated knock down also cause cell death defect significantly (4.6 ± 0.42 , $n=6$) (genotype: UAS-Rab11^{RNAi}/Crz-gal4).

Scale bars = 100 μm .

of *shi*^{K44A} (Fig. 4-3B,E), even though the *babo-a* has a stronger restoring effect with 4-5 neurons surviving (n=5). It indicates that dynamin dependent signal transduction is more involved with receptor than the downstream effector dSmad2. However, this is not the case in Rab5 dependent pathway. In contrast to non-significant rescue of *babo-a*, *dSmad2* can completely revert the defect of cell death triggered by overexpression of *rab5*^{SN} (Fig. 4-3C,F). These data indicate that the death signal transduced through endocytosis is TGF-β signaling. As an intracellular effector of TGF-β mediated signaling, subcellular localization of Smad2 is regulated by several factors. In addition to the well known phosphorylation state dependent nucleus accumulation, some cytoplasmic retention factors also regulate the subcellular location of dSmad2. Here, we sought to investigate the role of phosphorylation state dependent signal regulation in vCrz PCD. Ectopic expression of *dSmad2* cannot completely revert the cell death in *babo* mutant clone (Fig. 4-4A,B). Despite of significant reduction of homozygous *babo* mutant clone percentage (85% sample of *babo*^{fd4} bear homozygous clone), there is still 22% of CNS samples bearing GFP labeled clone which can not be rescued by overexpression of *dSmad2*. It indicates that TGF-beta signaling propagates information from receptor to dSmad2 in a non-transcriptional manner. As a signaling effector, dSmad2 encodes information derived from membrane receptor through phosphorylation. In line with this, overexpression of the phospho-mimicking mutation of Smad2, Smad2-DMD leads to accelerated cell death in vCrz. At wandering L3 stage, most of samples carry no neuron except at the posterior abdominal segment A5 and A6 (Fig. 4-4C). Therefore, phosphorylation is a prerequisite for Smad2

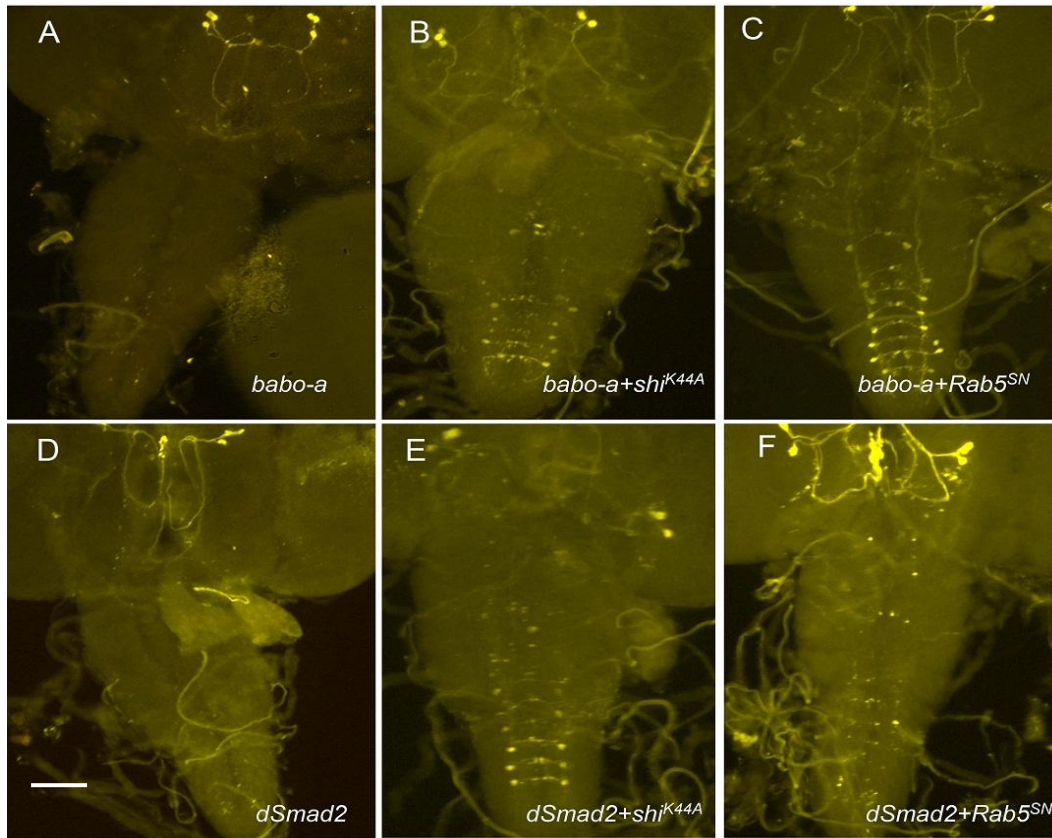


Figure 4- 3. Involvement of TGF- β signaling in endocytosis dependent cell death

(A, D) overexpression of *babo-a* or dSmad2 in Crz does not cause cell death defect. (B, C)

Co-expression of *babo-a* with *shi^{K44A}* (4.6 ± 0.40 , $n=5$) has a stronger restoring effect than its corresponding rescue of *Rab5^{SN}* (11.4 ± 0.81 , $n=5$). (Genotype: UAS- *shi^{K44A}* /Crz-gal4, UAS-*babo-a*; UAS-*Rab5^{SN}* / Crz-gal4, UAS-*babo-a*).

(E, F) In contrast to its mild rescue of *shi^{K44A}* (6.0 ± 0.68 , $n=6$), dSmad2 has completely can completely revert the defect of cell death triggered by overexpression of *Rab5^{SN}*.

Scale bars = 100 μm .

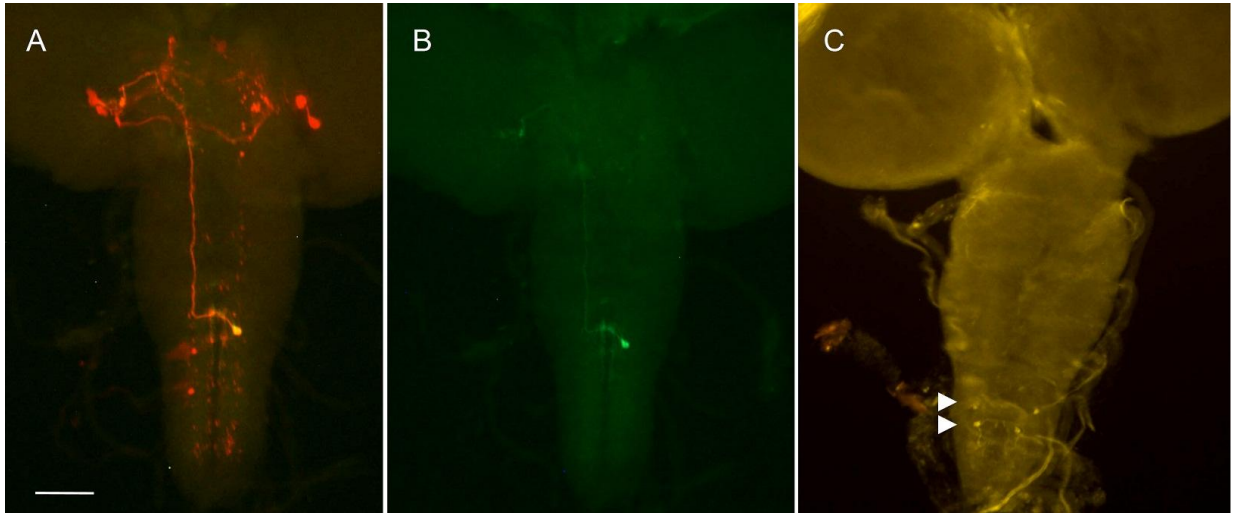


Figure 4- 4. Phosphorylation of Smad2 is required for TGF- β signaling

(A, B) ectopic expression of Smad2 did not restore the defect of cell death in *babo^{Fd}* mutant homologous clone. (Genotype: hs-FLP, UAS-mCD8GFP/Y; FRT^{G13}, tubP-gal80/FRT^{G13}BaboFd4; Crz-gal4,UAS-dSmad2/+). (C) Overexpression of the phospho-mimicking mutation of dSmad2, dSmad2-DMD leads to premature cell death at WL3 stage. Note that abdominal segment A5 and A6 carry surviving neuron. (Genotype: UAS- dSmad2^{DMD} /Crz-gal4). Scale bars = 100 μ m.

nucleus accumulation in TGF- β receptor dependent regulation of signal transduction.

The foregoing study showed that Dynamin dependent signal transduction is more involved in receptor than downstream effector, Smad2, indicating that Dynamin regulate the TGF- β signaling through receptor dependent Smad2 phosphorylation. However, it is not the case in Rab5 dependent signaling. Smad2 but not receptor Babo-a can rescue the cell death defect caused by the inhibition of early endosome formation. It is speculated that early endosome controls cell death via regulating Smad2' cytosolic retention factor.

SARA and HRS functions redundantly as a cytosolic retention factor

SARA acts as a cytosolic retention factor of Smad2 and regulates TGF- β signaling by controlling nuclear translocation of Smad2. By virtue of the FYVE domain binding to PI3P, SARA is localized to EEA1-positive and Rab5-containing early endosome (Hu et al., 2002), providing a potential link between membrane trafficking and TGF- β signaling. As indicated by our previous data, SARA acts as a biphasic regulator of Smad2 nucleus translocation and is required for cell death. To further characterize other cytosolic retention factor in vCrz PCD, we investigated another FYVE finger-membrane protein Hrs. HRS localizes to flat clathrin lattices, a complimentary part of EEA1 locale on early endosomes (Raiborg et al., 2002). Overexpression of *hrs* in Crz neurons significantly triggered defective cell death with 7-8 vCrz neurons alive at 6 h APF (n=6) (Fig. 4-5A). However, co-expression of *hrs* and *Smad2* completely reverted the blocking effect by *hrs* (Fig. 4-5C). Furthermore, RNAi-mediated knockdown of *hrs* also partially inhibited cell death and causes 5 vCrz neurons surviving (n=5) (Fig. 4-5B). Therefore, *hrs* knockdown in vCrz

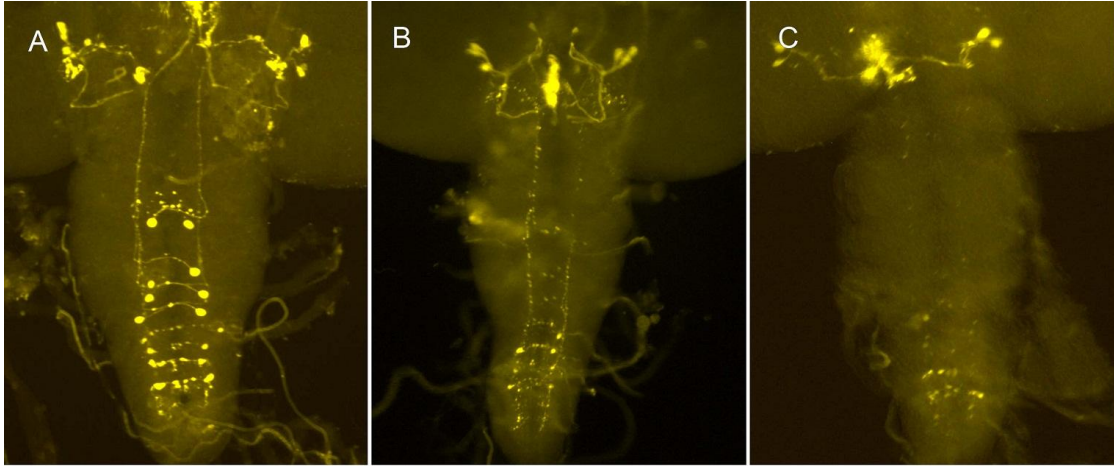


Figure 4- 5. Hrs is required for vCrz PCD.

(A) Overexpression of wild-type Hrs can block the PCD (7.3 ± 0.6 , $n=6$). (Genotype: Crz-gal4/UAS-hrs). (B) Knockdown of Hrs blocks the PCD partially (5.0 ± 0.77 , $n=5$). (Genotype: Crz-gal4/+; Crz-gal4/UAS-hrs^{RNAi}). (C) Co-expression of *hrs* and *dSmad2* completely reverts blocking effect by Hrs ($n=5$). (Genotype: Crz-gal4, UAS-dSmad2/UAS-hrs). Scale bars = 100 μ m.

phenocopies the loss of *sara*. Moreover, the blocking magnitude of their loss gene is less than the effect triggered by their overexpression. These studies indicate that SARA and HRS may act, partially redundantly, to recruit Smad2 to the receptor. Simultaneous depletion of HRS and SARA shows more severe effect than individual one (to be done). Therefore, considering SARA and Hrs possess the direct binding site of Smad2 and their endocytic roles in membrane trafficking, it is reasonably speculated that endocytosis allows the Smad2 nucleus translocation by regulating the interaction of Smad2 and its cytosolic retention factors during vCrz PCD.

Babo-a carrying Trp573 to Ala mutation is defective in the vCrz cell death

The endocytosis of cell surface receptor occurs in response to the ligand-induced receptor activation. The activated signaling receptors are internalized to clathrin-coated vesicle by directly interacting with the clathrin coat adaptor protein. A short, specific signal sequence at signaling receptor is known to be involved in recognition by the endocytic machinery (Bonifacino and Jackson, 2003; Mukherjee et al., 1997). Type II TGF- β receptors contain an internalization signal belonging to the dileucine family (Ehrlich et al., 2001), which is one of motifs recognized by the clathrin-associated adapter complex AP2 (adaptor protein 2) (Traub, 2003). In addition, a NANDOR box (nonactivating-non-down-regulating), localized at the carboxyl terminus, is found to be well conserved in type-I receptors and play a role in receptor endocytosis (Garamszegi et al., 2001). However, later studies identified Trp477 within the kinase domain of the type-I activin receptor Alk4 as a key residue responsible for the ligand-dependent internalization (Zhou et al., 2004). We found that this

residue is also conserved in *Drosophila* Babo. Babo is known to encode three isoforms that differ only in their extracellular portion, Babo-a, Babo-b, and Babo-c. Our previous results showed that Babo-a is the functional receptor for vCrz PCD.

To investigate whether the W573 residue is required for internalization of the Babo-A for vCrz PCD, we substituted this residue with Ala (W573A) in Babo-a and generated UAS-babo-aWA transgenic flies (Fig. 4-6A). Remarkably, ectopic expression of this mutant form in vCrz acts as a dominant negative form and partially blocks the PCD at 6 h APF with four vCrz neurons surviving (Fig. 4-6B). To further explore its role in cell death, the mutant babo-aW573A is combined with MARCM assay to rescue *babo* mutant. As shown in our previous results, Babo-a can completely rescue vCrz PCD defects in *babo* mutant clone using ectopic expression (Fig. 3-1H), whereas Babo-a-WA mutant cannot (Fig. 4-6C & D, n=24) do with 42% of sample bearing GFP labeled homozygous clone. This finding strongly indicates that endocytosis of activated Babo is a key TGF- β signaling event for PCD of vCrz.

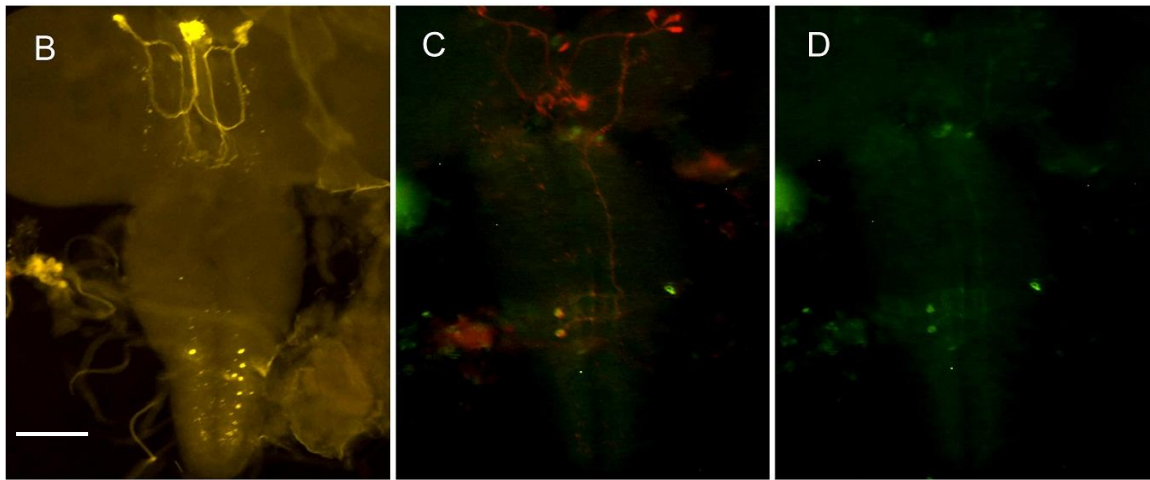
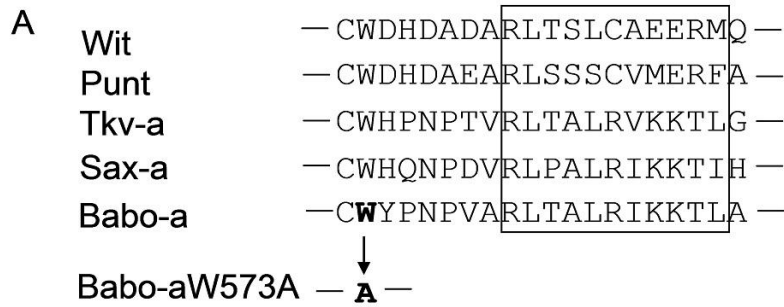


Figure 4- 6. Babo-aW573A is defective in cell death of vCrz.

(A) The Trp, which is conserved in TGF- β family receptors, is substituted with Ala in mutagenesis studies of babo-a. The boxed region is the NANDOR box. (B) Overexpression of babo-aW573A partially block cell death (4.4 ± 0.50 , $n=5$). (Genotype: Crz-gal4/UAS-Babo-aW573A). (C, D) ectopic expression of babo-aW573A did not restore the defect of cell death in *babo*^{Fd} mutant homologous clone. (Genotype: hs-FLP, UAS-mCD8GFP/Y; FRT^{G13}, tubP-gal80/FRT^{G13}BaboFd4; Crz-gal4,UAS-babo-aW573A/+). Scale bars = 100 μ m.

V. Discussion:

Membrane trafficking underpins the fundamental need for cells to maintain cellular homeostasis. For example, actin and microtubular cytoskeletons undergo specific structural changes upon initiation of cell death. A wealth of evidence implicates the importance of membrane trafficking with regards to mitochondrial function and cell death (Leadsham and Gourlay, 2008). However, we provided evidence that membrane trafficking is required for cell death by regulating of signal transduction. Endocytosis and its regulatory signal TGF- β play essential roles in neuron cell death during metamorphosis. Using the advanced genetic analysis tools, we found that the clathrin mediated membrane internalization and Rab5 dependent endocytosis is required for Smad2 nucleus accumulation through distinct mechanism. The Clathrin and Dynamin dependent endocytic trafficking exert their regulation through controlling receptor dependent Smad2 phosphorylation, whereas Rab5 enriched early endosome acts as a platform to regulate the interaction of Smad2 and its cytosolic retention factor in vCrz PCD.

Regulation of signal transduction by endocytosis

Recent studies implicated that endocytic organelles can play a more active role in signal propagation and amplification than was recognized before. The classical mode of signaling presumes that activation of membrane receptor by binding of extracellular ligand occurs at the cell surface. Endocytosis is known as a means to terminate signaling via degradation of activated receptor complexes after their internalization from the cell surface (Ceresa and Schmid, 2000). However, it has become clear that a high order of regulation has been put

forward by exerting the compartmentalization and functional specialization of the endocytic pathway, such as where and how long the signal is emitted (Miaczynska et al., 2004). One instinctive role of endocytosis in signal transduction is to provide temporal and spatial regulation by controlling the duration of signaling at specific organelles. Each endosomes have their unique properties such that they possess different type of endosomal signaling. For example, owing to an enrichment in phosphatidylinositol 3-phosphate (PtdIns3P), early endosome serve as a platform where various proteins including FYVE domain and PX domain-containing proteins are assembled as signaling molecules (Sasaki et al., 2007). In the case of TGF- β signaling, it is reported that the presence of FYVE domain containing proteins, SARA and Hrs in early endosome control the receptor dependent phosphorylation of Smad2 (Miura et al., 2000). Furthermore, late endosome has an acidic environment, which especially favors activity of proteolytic enzymes and therefore preferentially terminated the signal transduction through degradation of signaling molecules.

In this report we show that inhibition of early endosome and its preceding endocytic event lead to the defect of cell death in vCrz. Downstream signal transducer analysis using genetic rescue experiment indicates that the death signal regulated by endocytosis is TGF- β signaling. The clathrin dependent receptor internalization and its succeeding endocytosis pathway serve to regulate the Smad2 subcellular localization during vCrz cell death. In particular, early endosome is major platform where several FYVE domain containing proteins reside and interact with Smad2. Based on our previous data and the data of others, SARA functions to recruit Smad2 to the TGF- β receptor at the plasma membrane (Runyan et

al., 2005). The following dissociation of SARA and Smad2 has been observed under two prerequisites: phosphorylation and endocytic regulation. Endocytosis exerts its roles of separation of Smad2 from its cytosolic retention factor, SARA, by reducing their affinity between each other. Based on structural analysis of SARA and Smad2 complex, SARA is capable to directly interact with the β sheet and the three-helix bundle of Smad2 through its SBD (Smad2 Binding Domain) (Wu et al., 2000). The receptor dependent phosphorylation places negative charge on c-terminal motif of Smad2, reducing its binding affinity to SARA. In our proposed model, ligand dependent activation of receptor initiates protein complex internalization. The following endocytic event will recruit the cytosolic retention factor SARA to early endosome through the FYVE domain. The specific property owned by early endosome favors the dissociation of Smad2 from SARA. Comparing with cytosol, the slightly acidified environment of early endosome could cause negatively charged Smad2, a reminiscent of phosphorylation dependent alteration, therefore trigger the dissociation of SARA and Smad2. Also, a complex network of Rab5 regulator and effectors is found to be clustered in early endosome (Zerial and McBride, 2001). The combinatorial use of Rab5, its effector and other membrane proteins that create the specificity of that particular membrane environment could favor the dissociation of Smad2 and its retention factor. Furthermore, we found another cytosolic retention factor, Hrs. It has a redundant role in regulating Smad2 subcellular localization. Overexpression of Hrs and SARA could over saturate the binding specifically to phosphatidylinositol 3-phosphate (PI(3)P), and cause their cytoplasmic localization, where high affinity protein-protein interaction occurs. Therefore, the signal

transducer Smad2 will be retained in the cytoplasm and unable to undergo nucleus translocation. Taken together, endocytosis is very crucial for signal propagation from cell surface to nucleus. As a major regulatory organelle, early endosome governs the nucleus translocation of Smad2 by regulating the dissociation between Smad2 and cytosolic retention factor (Fig. 4-7).

Beyond Dynamin's role in endocytosis

As a family of large mechanochemical GTPases, Dynamin is well known to be involved in a diversity of cellular processes, including endocytosis, protein trafficking, and organelle partitioning (Hinshaw, 2000; Danino and Hinshaw, 2001). However, recent study point more clearly than ever toward a novel role for this protein in cell death. Ubiquitously expressed isoform dynamin-2 in HeLa cell activate the transcription factor p53 and induces cell death (Fish et al., 2000). Inhibition of Drp1, a dynamin-related protein by overexpression of a dominant negative mutant prevents staurosporine-induced cell death in HeLa cells (Frank et al., 2001). Here we presented evidence that the neuronal isoform dynamin-1 is also capable to induce cell death. Surprisingly, our results implicate that dynamin control cell death by internalizing of TGF- β receptor. This novel result raises the possibility that we could couple the role of dynamin in endocytosis with signal transduction in cell death. For instance, another role for dynamin is modulating of actin cytoskeleton. Vps1p, one of yeast dynamin-related protein is required for normal actin cytoskeleton organization. In higher eukaryotic cells, remodeling of actin and microtubular cytoskeletons is an essential event of destruction of plasma membrane integrity followed by membrane

blebbing. Considering actin cytoskeleton participating in endocytosis, it is intuitive that the function of dynamin I endocytosis might also depend on their roles as actin cytoskeleton regulators. In the present study, inhibition of dynamin activity lead to a more severe phenotypic blocking effect than other protein and ectopic expression of Smad2 only partially retrieve the defect of cell death caused by blocking of Shibire function. It might points to deeper levels of understanding how cell death is executed by coordinating various regulatory events.

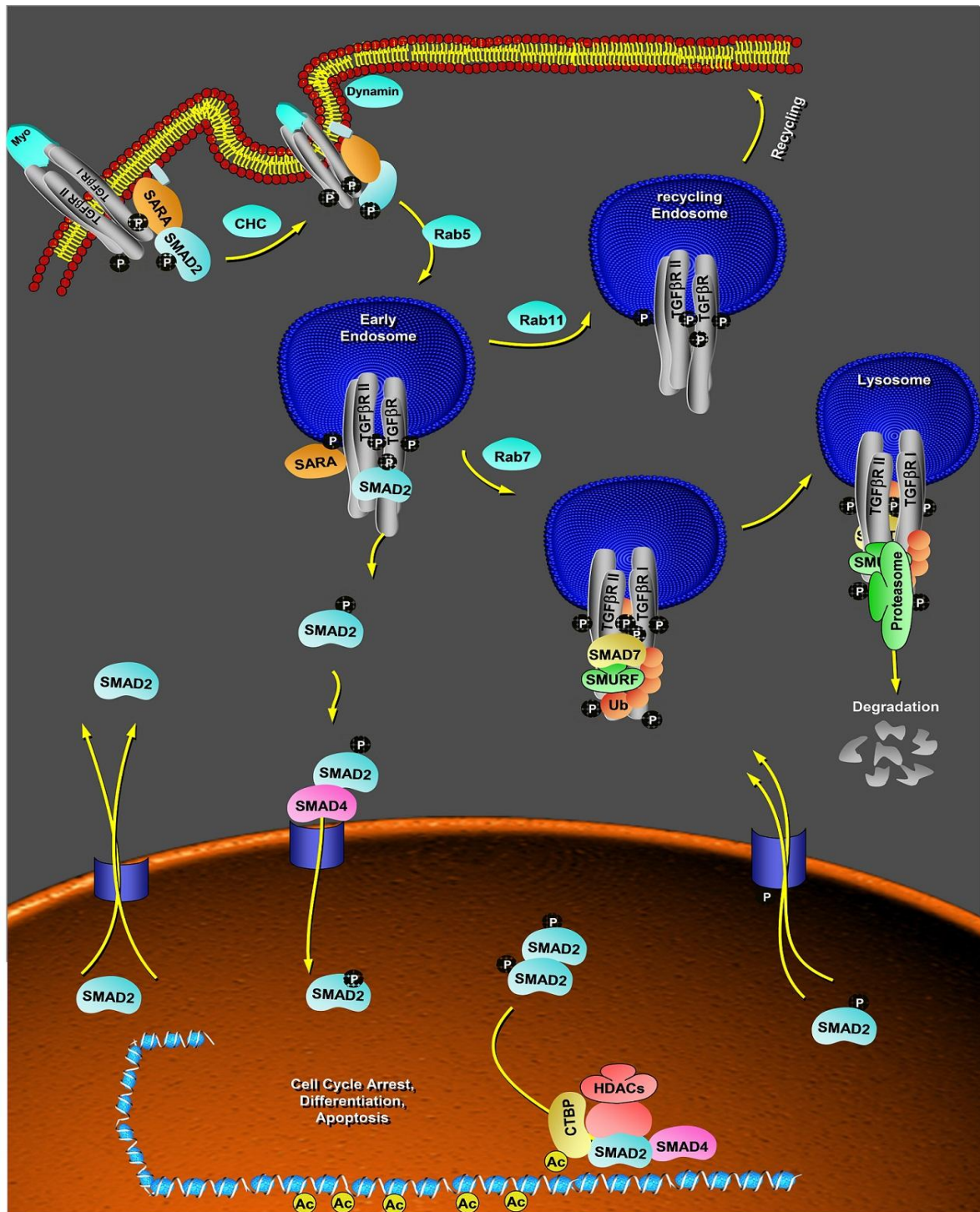


Figure 4- 7. Endocytic regulation of TGF- β signaling in vCrz PCD

Upon ligand binding, the receptors can initiate signaling by activating the Smad pathway through phosphorylation. The concomitant set-up of ligand-bound receptor also triggers the membrane internalization and subsequent endocytic trafficking. The receptor induced Smad2

phosphorylation continues and even maximizes their signaling activity during this process. Therefore, inhibition of activity of CHC and Dynamin will effect the phosphorylation dependent signal transduction. However the major function of Rab5-mediated early endosomes is to release the Smad2 from the SARA-Smad2-receptor complex. Hrs also resides in early endosome and has redundant role of SARA. Thus, early endosome serves as a sorting station, whereby the free phosphorylated Smad2 accompanied by Smad4 will translocated into nucleus, whereas some of the ligand-bound receptor are recycled back to the plasma membrane for re-use, or sorted to late endosomes and lysosomes for degradation.

Chapter Five

Discussion

I. Summary

PCD is essential for the development and homeostasis of the nervous system. The bewildering study of the cell death core machinery during the past several decades has caused the elucidation of the genetic and molecular pathway effecting PCD and has revealed an intrinsic program of degeneration that consists of hundreds of proteins build-in molecular network (Chu and Chen, 2008). Less is known, however, about the upstream regulatory events that selectively control the cell death program in heterogeneous cell population. This is particularly the case for PCD in nervous system. It is estimated that 50% of neuron will undergo cell death in mammalian nervous system and 25% in the VNC of *Drosophila* (Buss et al., 2006; Rogulja-Ortmann et al., 2007). How is cell death program initiated to kill a selected member of the population while ensuring the survival of the others in the population of nervous system? Results of the present investigation, in brief revealed that extracellular modulators, such as TGF- β , lead to either the death or the survival of neuron bearing their specific receptors. Furthermore, the endocytic organelles play an active role in TGF- β signal propagation and amplification.

The complexity of the cell death program began to increase with the notation that the death of cell in different context may thus reflect of survival factor, presence of intrinsic or extrinsic death factors, or both. Genome-wide analysis of dying salivary glands in *Drosophila* revealed that around 1000 gene transcripts exhibited a significant magnitude of

change comparing 6- and 12-hr salivary glands. It includes genes involved in cell death, cytoskeleton remodeling, noncaspase proteolysis, and protein translation (Gorski et al., 2003; Lee et al., 2003). To identify genes that may regulate cell death in nervous system, we underwent screening of candidate genes based on mosaic technique and RNAi-induced gene knockdown. We found that a wide variety of genes are involved in neuron cell death including growth factors and survival factors, cell surface receptors, transcription factors, cytoplasmic protein, nucleus receptors, and protein translation related proteins. It indicates that neuron cell death is not a simple proteolytic cascades based on caspase, but a co-operative action of multiple processes that requires absolute precise coordination of all the developmental genes and signaling cascades involved. In this chapter I will further discuss other possible factors in vCrz PCD.

II. Nuclear receptor

The nuclear receptors comprise the receptors for steroid, thyroid and retinoid hormones, vitamins A and D and many orphan receptors lacking identified ligands (Chawla et al., 2001). They belong to a superfamily of transcription factors that share similar structure modular containing DNA binding domain (DBD) and ligand binding domain (LBD) and control a variety of systemic processes, such as metabolism, maturation, and fecundity (Francis et al., 2003). Mutations of some nuclear receptors have been reported to cause development defect and a variety of human diseases.

In insects, one of NR, Ecdysone receptor (EcR), is particularly well documented during

metamorphosis. For example, genetic analyses have shown that EcR is essential for cell autonomous remodeling of mushroom body neurons and SCP immunoreactive (IR) neurosecretory cells and the class IV dendritic arborization (C4da) neurons (Lee et al., 2000b; Schubiger et al., 2005; Schubiger et al., 1998). Our previous studies have implicated the EcR is also involved vCrz death (Choi et al., 2006). Genetic disruptions of EcR-B and its corresponding signal transduction suppressed vCrz death. Studies from the salivary gland in *Drosophila* have revealed an EcR dependent transcriptional regulatory hierarchy. Upon binding to ecdysone, the heterodimeric receptors (EcR:USP) activate a cascade of signal transduction via the primary response genes including *BR-C*, *E74*, and *E75*, *E93* (Thummel, 1996).

The *Drosophila* genome encodes 18 canonical NRs and they represent six NR subfamilies across the animal kingdom (Laudet, 1997). Compared to the intense study of EcR and USP, the functions of the remaining 16 NRs in neural development have not been well documented. During metamorphosis, a high-titer pulse of ecdysone at L3 wandering stage is reported to regulate EcR and some other NRs as well (Sullivan and Thummel, 2003). This ecdysone pulse triggers lots of neuron cell death in ventral nerve cord (Lee et al., 2011). It indicates the potential role for these NRs. Recently, Lin, et al. (2009) developed a genetic toolkit using miRNA-based RNA interference and systematically surveyed these NRs functions in brain development. They found that *unfulfilled (unf)*, an ortholog of human photoreceptor specific nuclear receptor (PNR), is required for axonal morphogenesis and neuronal subtype identity during the development of mushroom bodies (MBs).

In the present study, we knocked down each of the 18 *Drosophila* NR genes in vCrz using this toolkit. In addition to EcR, USP, we identified another 2 NRs that is required for vCrz PCD, unf, and DHR78 (Table. 5-1). DHR78 has two vertebrate orthologs, TR2 (NR2C1) and TR4 (NR2C2). In vitro study shows that DHR78 is capable of binding to a subset of EcR/USP-binding sites and inhibits ecdysone dependent signal induction (Fisk and Thummel, 1995; Zelhof et al., 1995). DHR78-null mutants display growth defect and die at early L3 stage (Fisk and Thummel, 1998). Taken together, our data indicate that besides EcR, many NRs are required for ecdysone signaling through their protein and protein interaction, therefore maintain appropriate growth during development.

III. Glial function in cell death

The programmed degeneration of neural processes is closely associated with its neighboring cells, glial cells. During metamorphosis, some larval neurons survive but undergo extensive remodeling in order to acquire the adult pattern of projections. Excessive or obsolete axons, dendrites and synapses are eliminated by the engulfing activity of the surrounding glia (Awasaki and Ito, 2004; Watts et al., 2004). Inhibition of glial activity leads to inappropriate adult neural circuits (Awasaki et al., 2006). Similar observations are also made during mammalian brain development. In the neuromuscular junctions, the surrounding Schwann cells are required for the reduction of synapses and axon branches in the course of neural circuit refinement (Bishop et al., 2004). Despite intensive studies of the underlying mechanisms of cell death, little is known about how neighboring cell interact with

Table 5-1. Complete survey of 18 nuclear receptors' function in vCrz PCD by miRNA knockdown

Gene name	Subfamily	Crz-gal4>miRNAi	Documented function
EcR	1H	11.25±0.47, n=4	Cell death defect
USP	2B	16.0, n=6	Cell death defect
FTZ-F1	5A	3.0±1.08, n=4	Cell death defect
UNF	2E	11.5±0.72, n=6	No
DHR78	2C/D	12.2±1.13, n=6	No
DSF	2E	6.4±0.75, n=5	No
ERR	3B	Normal	No
E78	1D/E	Normal	No
HNF4	2A	Normal	No
DHR3	1F	Normal	No
DHR38	4A	Normal	No
DHR96	1I/J	Normal	No
DHR39	5A	Normal	No
DHR4	6A	Normal	No
DHR83	2E	Normal	No
E75	1D/E	Normal	No
SVP	2F	Normal	No
TLL	2E	Normal	No

All those miRNA stocks are obtained from Dr. Tzumin Lee and Crz-gal4^{S2b,T2a} is used in the crossing.

doomed cells and trigger the cell death.

As the primary phagocytes, glial cells are involved in the neural degeneration in the developing and mature brain. Glial cell infiltration and engulfment of the neuronal debris during axon pruning or during Wallerian degeneration in the adult brain is an essential event that facilitates the corresponding degenerative process (Aldskogius and Kozlova, 1998). In *Drosophila*, the engulf receptor, *drpr* is expressed in engulfing cells and required for glial phagocytosis of their targets (Freeman et al., 2003). It is an ortholog of the *C. elegans* CED-1, containing 15 extracellular atypical epidermal growth factor (EGF) repeats, a single transmembrane domain, and a novel intracellular domain. An intracellular NPXY motif in close proximity to transmembrane region endows Drpr an ability of binding with the proteins containing a phosphotyrosine-binding (PTB) domain, which is required for cell corpse engulfment. In addition, *drpr* is also highly expressed in *Drosophila* macrophages, suggesting that immune cells in flies may also use the *drpr/ced-1* pathway for the general cell corpse removal (Freeman et al., 2003).

In order to explore the potential role of glia, we first conditionally inhibited the glial function by the targeted expression of temperature-sensitive allele of *shibire* (*shi^{ts}*). As a dominant-negative at a restrictive temperature, Shi^{ts} inhibit phagocytosis and other membrane-related function (Kitamoto, 2001; Orth and McNiven, 2003). We expressed Shi^{ts} in glia by using the repo-GAL4 driver at a permissive temperature (25°C) and then placed them in the restrictive temperature (29°C) from wandering larvae stage to 6 h APF. Raising *repo>shi^{ts}* animal at the restrictive temperature lead to the cell death defect and 6 pairs of

vCrz are retained at 6 h APF (Fig. 5-1A). Furthermore, because Drpr is required for glial activity, we tested whether *drpr*^{Δ5} null mutants have a defect in vCrz cell death (MacDonald et al., 2006), and found that homozygous *drpr*^{Δ5} null mutants have a comparable phenotype with *repo>shi^{ts1}* animal (Fig. 5-1B), with 4 vCrz neurons survived. These results indicate that reduced glial activity by inhibiting membrane function or decreased *drpr* expression appears to block the vCrz cell death. Interestingly, recent study showed that Drpr is present in dying salivary glands and directly mediate their degradation via activation of autophagy (McPhee et al., 2010). Therefore, how Drpr functions to regulate autophagy specifically in a cell death context remains to be determined.

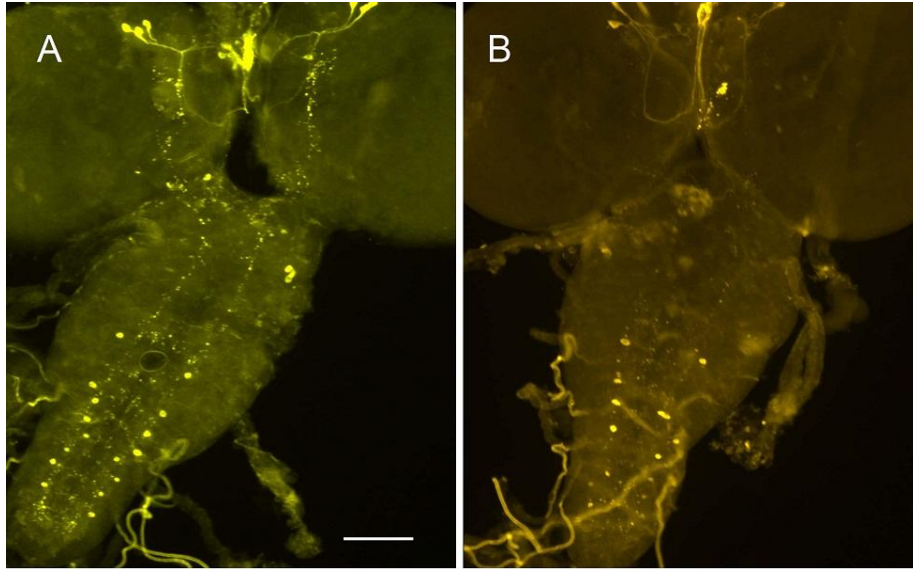


Figure 5- 1. Glial activity and cell death.

(A) When Shi^{ts} is activated in glial cells during early pupal stage (0-6h APF), abnormal cell death occurred with 14 neurons alive, even though the axonal projection is not present (13.66 ± 0.33 , $n=6$). (Genotype: $\text{Repo-gal4/UAS-Shi}^{\text{ts}}$). (B) $\text{drpr}^{\Delta 5}$ null mutant displays uncomplete cell death with 4 neuron surviving (4.3 ± 0.83 , $n=3$).

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