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Characterization of DATA-X in the *Drosophila* Embryonic Central Nervous System

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CHARACTERIZATION OF *D*ATR-X IN THE *DROSOPHILA* EMBRYONIC CENTRAL NERVOUS SYSTEM

Xuetao Sun

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements for the degree of
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in
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Abstract

The *Drosophila* Jing zinc finger plays a role in the survival of specialized cells at the midline of the central nervous system (CNS) and of tracheal cells. A screen for gain-of-function (GOF) enhancers of *jing* GOF in the eye identified the *Drosophila* homolog of the human disease gene alpha-thalassemia/mental retardation X-linked (ATR-X). The ATR-X gene encodes an SNF2 family Helicase/ATPase protein with chromatin remodeling activity. *D*ATR-X has a very similar genetic structure but is smaller than its vertebrate counterparts due to the absence of a zinc finger domain. *D*ATR-X transcripts and protein are widely expressed throughout embryogenesis and enriched in the CNS. *D*ATR-X localizes to nuclei in *Drosophila* embryos and its N-terminus is sufficient for its nuclear localization. Alterations in *D*ATR-X and Jing levels specifically in CNS neurons or glia by over-expression and RNA interference (RNAi), suggested that *D*ATR-X and *jing* have a common function, as they both affect repulsion of longitudinal glia (LG), neurons and longitudinal axons from the CNS midline. With a variety of CNS cell type markers and polyclonal anti-*D*ATR-X antibodies, the function of *D*ATR-X within specific subtypes of neurons (MP2 pioneer neurons) and glia (LG) were examined due to their essential involvement in axon guidance. Analysis of *D*ATR-X deficient embryos and those with targeted RNAi-induced reductions points to *D*ATR-X as an essential player in the formation of longitudinal axons and glial survival and positioning in the developing *Drosophila* embryonic CNS. Furthermore, truncated *D*ATR-X proteins missing the Helicase C domain interfered with longitudinal glial repulsion, but at a reduced level compared with wild-type *D*ATR-X. Together, these results establish that

proper *D*ATR-X levels and function in both glial and neuronal nuclei are required for the construction of precise axonal architecture in *Drosophila* embryos.

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List of Abbreviations

AC	anterior commissure
ac/sc	*achaete-scute*
arm	*armadillo*
BDGP	Berkeley *Drosophila* Genome Project
β-gal	β-galactosidase
BHLH-PAS	basic-helix-loop-helix-PAS
Blast	Basic Local Alignment and Search Tool
BMP	bone morphogenic protein
btl	*breathless*
Cas	*Castor*
CME	central midline elements
comm	*commissureless*
CNS	central nervous system
DAPI	4', 6'-diamidino-2-phenylindole hydrochloride
(*D*)ATR-X	(*Drosophila*) α-thalassemia and mental retardation X-linked
Daxx	death domain-associated protein

Dl	Dorsal
Dpp	decapentaplegic
dsRNAi	double-stranded RNA interference
EGFR	epidermal growth factor receptor
EP	enhancer/promoter
Fas II	Fasciclin II
gcm	*glial cell missing*
Gh	*Grainyhead*
GMC	ganglion mother cell
GMR	glass multiple reporter
GOF	gain of function
GST	Glutathione-S-transferase
HA	Hemagglutinin
Helicase C	Helicase conserved C-terminal domain
HP	heterochromatin protein
htl	*heartless*
Hb	*Hunchback*

ind	*intermediate neuroblast defective*
IR	inverted repeat
JNK	Jun-N-terminal kinase
Kr	*Kruppel*
LC	longitudinal connectives
LG	longitudinal glia
LOF	loss of function
MGA	midline glia anterior
MGM	midline glia medial
MGP	midline glia posterior
MR	Mental retardation
msh	*muscle segment homeobox*
NB	neuroblast
NLS	nuclear localization signal (or sequence)
PBS	Phosphate buffered saline
PC	posterior commissure
PI	propidium iodide

PML-NBs	promyelocytic leukemia nuclear bodies
pnt	*pointed*
PNS	peripheral nervous system
prd	*paird*
pros	*prospero*
repo	*reversed polarity*
RNAi	RNA interference
robo	*roundabout*
RT	room temperature
S2 cell	Schneider 2 cell
Sema	Semaphorin
Shh	Sonic Hedgehog
sim	*single-minded*
SMART	Simple Modular Architecture Research Tool
sog	*short gastrulation*
trh	*trachealess*
tgo	*tango*

ttk	*tramtrack*
UAS	upstream activation sequence
VNC	ventral nerve cord
vnd	*ventral nervous system defective*
VNE	ventral neuroectoderm
VUM	ventral unpaired median neurons
XLMR	X-linked mental retardation
XNP	X-linked nuclear protein

Authorization

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Sun, X.T., Morozova, T. and Sonnenfeld, M.J. 2006. Glial and neuronal functions of the *Drosophila* homolog of the human SWI/SNF gene ATR-X (DATR-X) and the jing zinc-finger gene specify the lateral positioning of longitudinal glia and axons. *Genetics*. 173(3):1397-415.

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Chapter 1

INTRODUCTION

1.1. Selective gene expression

Proper regulation of gene expression plays a key role during multi-cellular organism development. Cells are the basic structural and functional unit of metazoans and their variety contributes to tissue development. For example, neurons and glia are the two major cell types in the central nervous system (CNS). These cells perform different functions because they are made differently due to selective gene expression. Cells have to know when and where to turn on and off specific genes. Therefore, the proper orchestration of the regulation network for the information to be stored, delivered, interpreted and achieved is essential. One can expect that misregulation of gene expression may perturb the biological functions of a particular gene which can result in severe developmental abnormalities and pathologies.

1.1.1. Transcription factor: Regulation of transcription in eukaryotes is a result of the combined effects of structural properties of packaged DNA and the interactions of transcription factors (Lee and Young, 2000). Transcription factors are a group of regulatory proteins that bind to DNA regulatory elements and activate or repress transcription. Transcription factors can be classified according to their three-dimensional structure, including basic helix-loop-helix, leucine zipper, helix-turn-helix, and zinc finger proteins (Littlewood and Evan, 1995; Vinson et al., 2002; Wintjens and Rooman, 1996; Laity et al., 2001). These different structural motifs bring transcription factors the specificity for the consensus DNA sequences to which they recognize and bind (Levine and Tijian, 2003). Transcription factors are necessary for regulating gene expression; however, they are not sufficient. The functions of chromatin remodelers, a group of ATP-dependent protein

complexes which modulate histone-DNA interactions in chromatin, are also required to ensure proper regulation (Näär et al., 2001).

1.1.2. Chromatin remodeling protein complexes: The eukaryotic genome is packaged into a periodic nucleoprotein complex called chromatin, which presents a barrier to transcription and other cellular processes that require access to DNA (Wolffe, 1992). The primary repeating unit of chromatin is the nucleosome, which consists of a core histone octamer wrapped by ~146 bp of DNA about two times around (Luger et al., 1997). Neighbouring nucleosomes are separated by short segments of linker DNA. Such chromatin structure blocks the accessibility of the *cis*-acting elements within the promoter region to *trans*-acting regulatory factors, rendering the genes silent by default in a restrictive ground state (Cairns, 2001). In order to overcome the chromatin barrier, the transcriptional machinery is facilitated by factors called chromatin remodelers which always exist as multi-protein complexes (Narlikar et al., 2002). They all possess an ATPase subunit of the SWI2/SNF2 family of ATPases and can be classified into several subfamilies based on their sequence features outside of their ATPase domains (Eisen et al., 1995). These chromatin remodelers use the energy of ATP-hydrolysis to alter nucleosome position relative to a segment of DNA and increase the accessibility of nucleosomal DNA (Becker and Hörz, 2002). Therefore, chromatin remodelers are involved in all processes which deal with chromatin as a substrate including replication, repair, recombination and nucleosome assembly. Many of the chromatin-based regulatory mechanisms are evolutionarily conserved (Schulze and Wallrath, 2007).

1.2. *Drosophila* as a model organism

Genetically tractable model organisms provide more than an entry point to better understand how selective gene expression is carried out. Many of the biological mechanisms elucidated using model organisms are evolutionarily conserved and therefore can serve as a foundation for studies of the homologous processes in humans at multiple levels and related abnormalities which play a role in development of disease. Of these, one main model organism is *Drosophila melanogaster*, commonly known as the fruit fly.

Drosophila has been the subject of research into a wide spectrum of biological phenomena, like mechanisms of inheritance, body pattern formation (Castanon and Baylies, 2002), the function of nervous system, tumour formation (Potter et al., 2000) and modeling human diseases including Alzheimer's and Parkinson's disease (Reiter et al., 2001; Wittmann et al., 2001; Feany and Bender, 2000). The advantages of using *Drosophila* derive from rich genetic and molecular resources including a fully sequenced *Drosophila* genome (Berkeley *Drosophila* Genome Project; Adams et al., 2000), stocks of mutations and conditional mutations in nearly every gene (Bloomington *Drosophila* Stock Center, Vienna *Drosophila* RNAi Center) and many *in vivo* gene expression tools (*Drosophila* Genomics Resource Center). What's more, the short life cycle, low cost to maintain and ease to handle under normal laboratory conditions are also advantages of using *Drosophila* as a model organism.

1.2.1. Modeling human mental retardation (MR) in *Drosophila*

Mental retardation is a common form of cognitive disorder with a prevalence of 1-3% in industrialized countries (Roeleveld et al., 1997). It is characterized by an overall intelligence quotient (IQ) below 70 with deficits in adaptive behaviour that onset before the age of 18 (APA, 1994). MR can be caused by genetic or environmental factors (Chelly et al., 2006). Genetic causes such as chromosomal abnormalities and gene mutations totally account for 25-50% of severe MR (McLaren and Bryson, 1987). The inherited MR is commonly categorized into two major groups: 1) syndromic MR when it occurs in association with other phenotypic features and 2) nonsyndromic (or non-specific) when MR is the only key feature. However, the mutations in several of the MR genes can result in both non-specific and syndromic MRs (Frints et al., 2002).

About 300 human genes have been known to cause MRs and the number is estimated to increase according to the database entries and literature searches (Inlow and Restifo, 2004). These genes are distributed over a broad range of functions, indicating that disruption of any particular molecular process may impair brain development or function then leading to MR. Molecular functions of MR genes include enzymes (51%), mediators of signal transduction (12%), transcription regulators (7%), binding proteins (8%), transporters (8%), and smaller numbers of cell adhesion molecules, structural molecules, motor proteins, tRNAs, apoptosis regulators, chaperones and enzyme regulators (Inlow and Restifo, 2004). These MR genes cover a wide spectrum of biological functions, including nuclear functions (chromosome structure, DNA repair, transcription regulation and rRNA processing), many metabolic and signalling pathways, macromolecular synthesis and modification, cell-cell communication and intracellular

transport. MR genes are involved in neurogenesis and neuron migration during brain development; synaptic plasticity and adult brain neurogenesis; functional and structural organization of synaptic networks (Vaillend et al., 2008).

The analysis of these genes and the mechanisms underlying MRs is usually either through the examination of the cells/tissues expressing these genes or by the investigation of the mutations of the MR genes. This is limited by the availability of the MR mutations. Modeling these genes in more genetically approachable organisms can help us to better understand these MRs. *Drosophila* is one of those excellent animal models we can utilize to study human disease. In particular, 87% of known MR genes have at least one *Drosophila* homolog and 76% have a candidate functional ortholog (Inlow and Restifo, 2004). Comparably, 75% of about 1400 human disease genes have *Drosophila* homolog (Reiter et al., 2001). This suggests that *Drosophila* can be used to study MR and reveal molecular and cellular mechanism of nervous system development relevant to MR (Inlow and Restifo, 2003).

1.2.2. The *Drosophila* life cycle

Drosophila begins its life as an embryo in an egg, which lasts for one day before developing into a larva. The larva spends about six days to pass through three stages, first, second and third instars. Following the molt in the third instar, the larva stops moving and becomes an immobile pupa. *Drosophila* stays in the pupa for about five days, undergoing metamorphosis to develop adult structures such as eyes, wings and legs. When the adult emerges from the pupa, it is fully formed and becomes fertile within about ten hours. The whole life cycle takes about twelve days at room temperature.

1.2.3. Gene manipulations in *Drosophila*

The *Drosophila* genome has been sequenced and is around 5% of the size of mammalian genomes (Adams et al., 2000). Most gene families and pathways in *Drosophila* can find their counterparts in mammals. This is a solid basis to not only seek a genetic interpretation in *Drosophila* for theoretically any biological events at molecular, cellular, developmental and even behavioural levels, but also to enable *Drosophila* biology to directly impact on our understanding of human health at these multiple levels. Progress in gene manipulation tools and resources constantly occurs to assist fly researchers. As a result, a comprehensive range of methods is available in aiding the analysis of mutant phenotypes, molecular genetic research and characterization of specific genes. It is not possible to illustrate here how all of these tools and resources are used to address such a wide range of biological questions; however, this thesis can also be considered as an example of the applications of *Drosophila* resources to accomplish specific tasks. This thesis work was initialized to characterize a *Drosophila* homologue of a human disease gene, in other words, the sequence of gene of interest is already known. Therefore we would focus our considerations primarily on the reverse genetic tools by which the function of the gene of interest can be affected. Two of the widely used methods for but not limited to this purpose are P-element mediated transformation (Rubin and Spradling, 1982) and the Gal4/UAS binary system (Brand and Perrimon, 1993), which were both employed in this thesis work.

1.2.3.1. P-element mediated transformation

Transposable elements are movable DNA segments and exist in almost every phylum (Berg and Howe, 1989). The P-element is probably the best characterized and the most widely used *Drosophila* transposon (Ryder and Russell, 2003). P-element mobilisation only occurs in the presence of transposase through a "cut-and-paste" process at the donor and target sites (Kaufman and Rio, 1992). Structurally, the 2.9 kilobase wild-type autonomous P-element contains an internal transposase encoding sequence, a perfect 31 base pair terminal inverted repeat (IR) and an 11 base pair subterminal IR (O'Hare and Rubin, 1983). The IRs are the sites transposases act on for transposition. Therefore, through controlling the means of delivering the transposase, the P-element has been engineered to suit various purposes since it was developed for transformation (Spradling and Rubin, 1982; Rubin and Spradling, 1982). Usually, an artificial P-element based transposable construct retains the parts of the sequence required for transposition but replaces the transposase gene with another gene of interest, often functioning as a marker or reporter. Transposase is supplied by a separate source, either by co-injecting the construct with a transposase-making element which is incapable of moving by itself, or by introducing the construct in an embryo constitutively making transposase (Δ2-3) (Ryder and Russell, 2003).

1) **Insertional mutagenesis:** Forward genetics is the primary approach for gene discovery on the basis of mutant phenotypes. Traditionally, chemical mutagenesis has been broadly used to randomly induce mutations with a high mutation rate and wide target range. The most commonly used chemical mutagen in *Drosophila*, ethyl methanesulphonate (EMS) is an alkylating agent capable of

producing typically point mutations through G/C to A/T transitions (Lewis and Bacher, 1968; Pastink et al., 1991). Since most G/C base pairs are potential targets for EMS mutagenesis, the probability of inducing a mutation in a specific gene is related to the size of the gene. However, this method lacks efficient means to detect mutations. Comparatively, mutants generated through P-element insertion are molecularly tagged. After incorporation into the genome, a P-element construct can be easily mobilised and inserted randomly in the genome through a transposase acting *in trans*. This insertion event may disrupt neighbouring gene function, giving rise to easily detectable phenotypes.

2) **Creating genomic deficiencies by imprecise excision of P-element:** P-elements can also be used to make deletions of flanking DNA through imprecise excision (Voelker et al., 1984; Salz et al., 1987). Excision of a P-element out of the donor site from a chromosome leaves a double-stranded break (DSB) to be processed through DNA-repair mechanisms (Engels et al., 1990). Prior to the repair, a frequency of 1% DSB is enlarged and as a result of repair, a deletion of a few basepairs to several kilobases extending from either or both directions from the original insertion site can be obtained (Daniels et al., 1985). Since P-elements frequently insert into regions between the promoter and protein-coding sequences (Spradling et al., 1995), the deletions can completely destroy gene function by removing coding sequence or important upstream regulatory sequences.

3) **Enhancer trap (gene tagging):** P-element constructs carrying a reporter gene, commonly the *E. coli β-galactosidase* (*lacZ*) gene, associated with a weak basal promoter, are randomly mobilised in the genome. As P-elements frequently insert

near the promoter of an endogenous gene (Spradling et al., 1995), the endogenous enhancer near the insertion site may activate the basal promoter and as a result, the reporter is expressed in a similar pattern to the endogenous gene (O'Kane and Gehring, 1987; Wilson et al., 1989). The method is known as the P-element mediated enhancer trap detection system (Bellen et al., 1989).

1.2.3.2. Gal4/UAS binary system

Gal4/UAS is a revolutionary versatile system to achieve targeted gene-mis-expression in *Drosophila* (Fig. 1) and is essentially a derivative of P element vector based methods. Gal4 is a yeast protein acting as a transcription activator able to bind the upstream activating sequence (UAS), a weak promoter insufficient to activate gene transcription unless in the presence of Gal4 (Fischer et al., 1988). Accumulation of Gal4 protein does not have any apparent effect upon the fly. With genetic manipulations, Gal4 is controlled by an inducible or tissue-specific driver which allows for the expression of a UAS transgene in the same spatial and temporal patterns as the driver. Therefore, any gene can be directed to any desired cell or tissue at almost any developmental stage in *Drosophila* given a suitable Gal4 driver. There are three big publicly available collections of Gal4 drivers: Bloomington *Drosophila* Stock Center, Exelixis *Drosophila* Stock Collection at Harvard Medical School, *Drosophila* Genetic Resource Center (Kyoto).

This method began mainly as a means of targeted ectopic or over-expression of any gene of interest. An extension of the method made notable progress by combining the Gal4/UAS binary system and RNA interference (RNAi)-mediated targeted

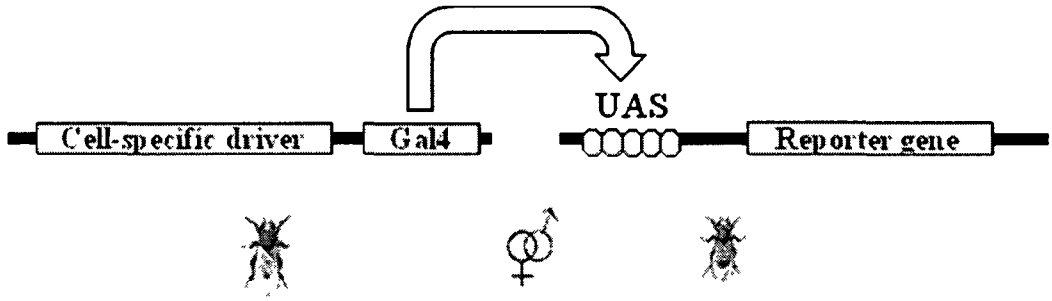


Figure 1. Gal4/UAS binary system. Gal4 is a yeast protein acting as a transcription factor which targets to the upstream activating sequence (UAS), a weak promoter insufficient to activate gene transcription unless in the presence of Gal4 (Fischer et al., 1988). With genetic manipulation, Gal4 or UAS can be integrated into *Drosophila* genomes. Thereafter, one parental fly line with Gal4 can provide a cell-specific driver, and another parental line with UAS can provide reporter genes. Their progeny will have the reporter gene expressed in the same spatial and temporal patterns as the cell-specific driver. (Adapted from Brand and Perrimon, 1993).

gene inactivation to conditionally silence genes post-transcriptionally (Lee and Carthew, 2003). RNAi is an endogenous cellular process by which transcripts are depleted by double stranded (ds) RNA of the corresponding gene, leading to gene silencing (Fire et al., 1998; Meister and Tuschl, 2004). The feasibility of the RNAi approach in knocking down the function of individual genes of interest has been determined in a variety of organisms as a reverse genetic tool (Fraser et al., 2000; Gönczy et al., 2000). Some efforts were made using RNAi in an attempt to investigate genes required for early embryonic development, e.g., in the cardiac system (Kim et al., 2003), and in the nervous system (Koizumi et al., 2007). In these studies, the RNAi was introduced to the embryo by microinjection but the effect exerted on the whole organisms is transient and nonheritable. This is a big limitation especially when spatial and temporal contexts have to be taken into consideration. This shortfall has been overcome with the introduction of UAS-dsRNAi (Lee and Carthew, 2003). A transgene expressing dsRNAi is designed under the control of the UAS element. This UAS-dsRNAi module is responsive to the Gal4 trigger, conditionally producing the silencing dsRNA. Many UAS-transgenic RNAi fly lines have been generated (including this thesis work) and deposited into the fly stock centers due to their ability to deliver cell or tissue-specific single-gene disruption through RNAi-mediated knock-down. The increasing demand for these flies prompted the recent creation of the genome-wide collections of fly RNAi lines (the Vienna *Drosophila* RNAi Center (Vienna, Austria) (http://www.vdrc.at), Dietzl et al., 2007; Fly Stocks of National Institute of Genetics (National Institute of Genetics, Japan) (http://www.shigen.nig.ac.jp/fly/nigfly)).

1.3. *Drosophila* embryonic central nervous system (CNS)

Insects and vertebrates use conserved molecules and mechanisms to assemble their basic nervous system frameworks (Goodman and Doe, 1993). The investigation of the *Drosophila* CNS can help us better understand those of higher complexity in higher organisms which are not as easily genetically accessible.

The CNS consists of neurons and glia. These two major cell types can be generated either by common precursors (neuroglioblasts) or by precursors that are specialized to produce either neurons (neuroblasts) or glia (glioblasts) (Bossing et al., 1996; Schmidt et al., 1997). The *Drosophila* CNS, which is composed of the ventral nerve cord (VNC) and the brain, originates from the neurogenic ectoderm (Fig. 2) which consists of the ventral neuroectoderm (VNE) and the pro-cephalic neuroectoderm. The VNE gives rise to the neuroblasts (NB) of the VNC and the pro-cephalic neuroectoderm generates the brain.

The VNC has been an excellent model to study the molecular genetic mechanisms governing nervous system development due to its relative simplicity compared to the brain. The VNC is segmentally repeated and each segmental unit is called a neuromere. The two sides of a neuromere are structurally bilaterally-symmetric and each side is often referred to as a hemisegment. Each neuromere arises from a sheet of ventral-laterally localized neuroectodermal cells and is composed of three regions: two lateral neurogenic regions separated by a midline region. The neurogenic regions generate neurons and glia and the midline region generates a small number of special midline neurons and glia cells (Fig. 2). The first-born neurons are the closest to the inner surface of the developing

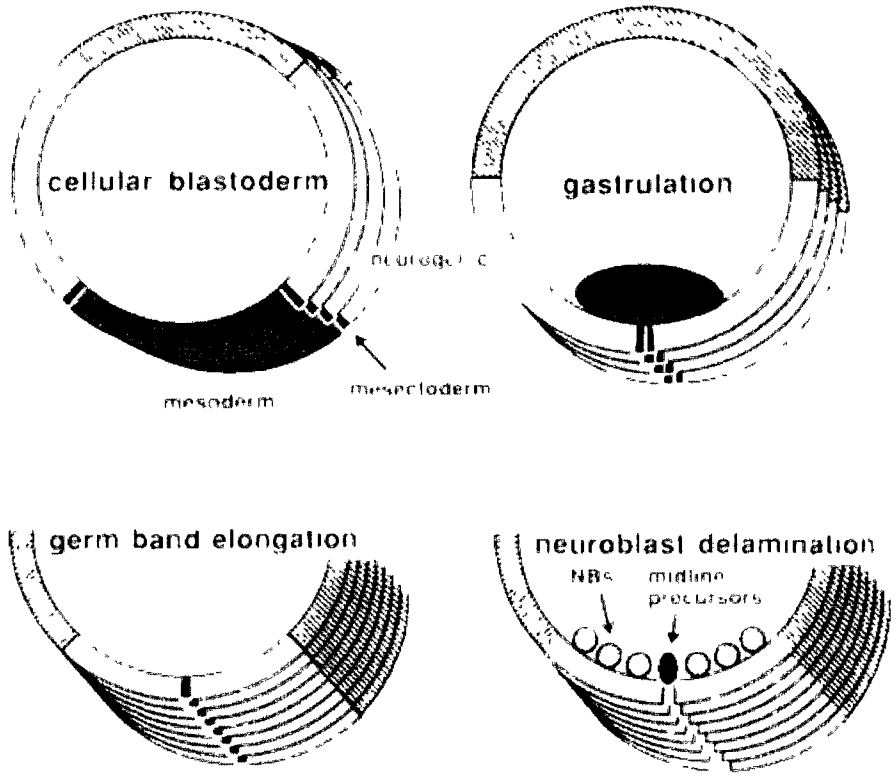

cellular blastoderm
gastrulation
mesoderm
germ band elongation
neuroblast delamination
NBs
midline
precursors

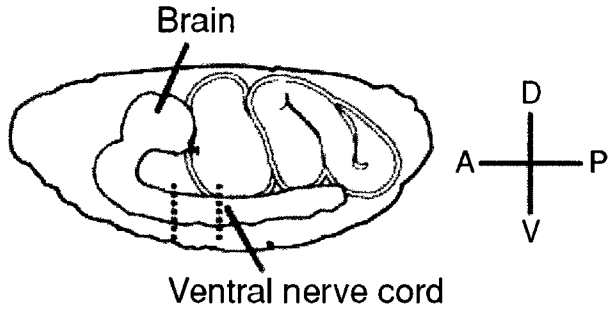
Figure 2. Development of the neurogenic region and ventral midline. Midline development occurs in four successive stages of embryonic development. Dorsal is up. During the blastoderm stage, midline precursor cells are located on either sides of the mesodermal anlage and separate the presumptive mesoderm for the presumptive neurogenic ectoderm. During gastrulation, mesodermal cells invaginate and the two mesectodermal cells meet at the ventral midline. During germ-band elongation, these cells form a row of eight midline precursor cells per segment. Finally, these precursor cells delaminate from the ventral ectoderm into the same layer as the delaminating neuroblasts (NBs) divide and give rise to the neurons and glia of the midline. The transient gap is later filled in by the rearrangement of epidermal cells. (Adapted from Goodman and Doe, 1993).

neuroepithelium and initiate growth cones that commence the formation of the first axon pathways (pioneers). Within each neuromere, the axons develop in a scaffold pattern made up of a pair of bilaterally symmetrical longitudinal axon tracts connected by a pair of commissural axon tracts across the midline anteriorly and posteriorly, and a pair of nerve roots exiting the CNS on each side (Fig. 3). Each major longitudinal tract is made up of three distinct axon bundles, or fascicles (Goodman and Doe, 1993). This will be described later in more detail.

1.3.1. Neuroectoderm formation

The dorsoventral borders of the neuroectoderm are determined in the early embryo by a ventral to dorsal nuclear gradient of the Rel/NF-κB transcription factor, Dorsal (Dl) (Fig. 4A) (Stathopoulos and Levine, 2002). The Dl gradient restricts the expression of decapentaplegic (dpp), the *Drosophila* homologue of vertebrate bone morphogenic proteins (BMPs) (Affolter and Basler, 2007). Low levels of nuclear Dl induce expression of *short gastrulation* (*sog*) in broad bilateral stripes outlining the presumptive neuroectoderm (Francois et al., 1994). Sog is a secreted protein with similarities to vertebrate Chordin (Holley et al., 1995). The role of Sog is to antagonize the dorsalizing effect of Dpp, preventing the neuroectoderm from becoming dorsal epidermis (Ferguson and Anderson, 1992; Francois et al., 1994; Biehs et al., 1996; Ferguson, 1996). The Dpp and Sog morphogen gradients act together with epidermal growth factor receptor (EGFR) signalling to further subdivide the neuroectoderm into three longitudinal domains (Fig. 4B) (Udolph et al., 1998; Von Ohlen and Doe, 2000), each defined by expression of one of the columnar genes, *ventral nervous system defective* (*vnd*),

A


Brain
D
A
P
V
Ventral nerve cord

B

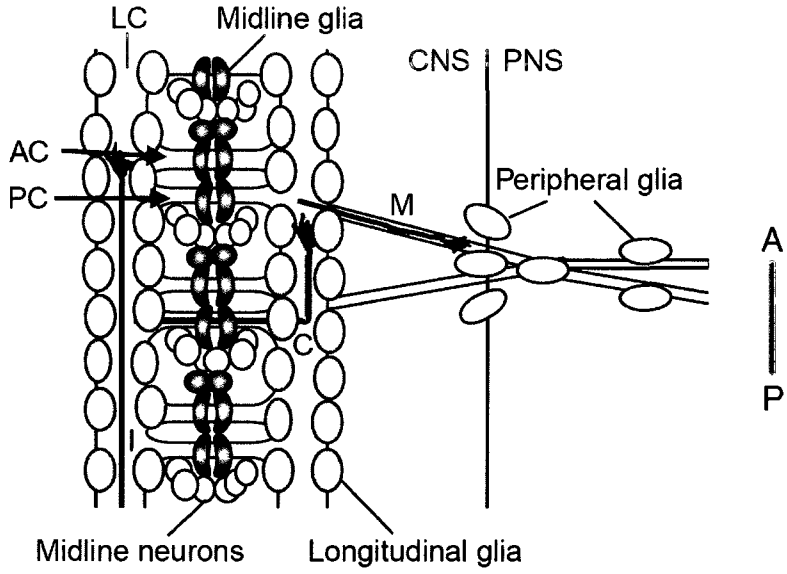

LC
Midline glia
CNS
PNS
AC
PC
M
Peripheral glia
A
P
C
I
Midline neurons
Longitudinal glia

Figure 3. *Drosophila* ventral nerve cord (VNC). (A) A *Drosophila* embryo at the end of embryogenesis. A, anterior; P, posterior; D, dorsal; V, ventral. (B) Ventral view of the ventral nerve cord with anterior up. *Drosophila* embryonic nervous system has a ladder-like structure consisting of longitudinal connectives (LC) and a pair of anterior and posterior commissures (AC and PC). Different glial cell types are found along the axonal pathways. Midline glia are adjacent to the commissures; longitudinal glia are associated with the connectives. Peripheral glial cells set the boundaries between the CNS and PNS and enwrap the peripheral nerves. C, contralateral axons; I, ipsilateral axons; M, motor axons. (Adapted from Goodman and Doe, 1993).

intermediate neuroblast defective (*ind*) or *muscle segment homeobox* (*msh*) (Cornell and Von Ohlen, 2000). The segment polarity genes, which determine segments along the anteroposterior (A/P) axis of the embryo, act in combination with the columnar genes to establish a Cartesian grid-like coordinate system, which effectively subdivides the embryo into a checkerboard of neural equivalence groups (Fig. 4B). A neural equivalence group comprises five to six equally competent neuroectodermal cells, of which only one will become a neuroblast (Skeath and Thor, 2003).

1.3.2. Neurogenesis

1) Neurogenic region

a. Neuroblast formation: Within the neurogenic region of each hemisegment, 30 cells are selected and delaminate as neural precursor cells, or neuroblasts (NB). Two classes of genes are required for NB formation: 1) proneural genes, which control the time of NB formation (Ghysen and Dombly-Chaudiere, 1989), and 2) neurogenic genes, which direct the cell interactions to ensure only one cell within a group of equivalent neuroectodermal cells differentiates into the NB (Lehmann et al., 1983). Genetic studies indicate that all cells in a neural equivalence group acquire neural potential through the expression and function of the *achaete-scute* (*ac/sc*) complex proneural genes: *achaete* (*ac*), *scute* (*sc*) and *l'sc* (Stern, 1954; Garcia-Bellido and Santamaria, 1978; Garcia-Bellido, 1979). These genes all encode basic helix-loop-helix type transcription factors and drive ectodermal cells toward the NB fate (Villares and Cabrera, 1987; Gonzalez et al., 1989). In each equivalence group, the cell which expresses the *ac/sc* genes to the highest level

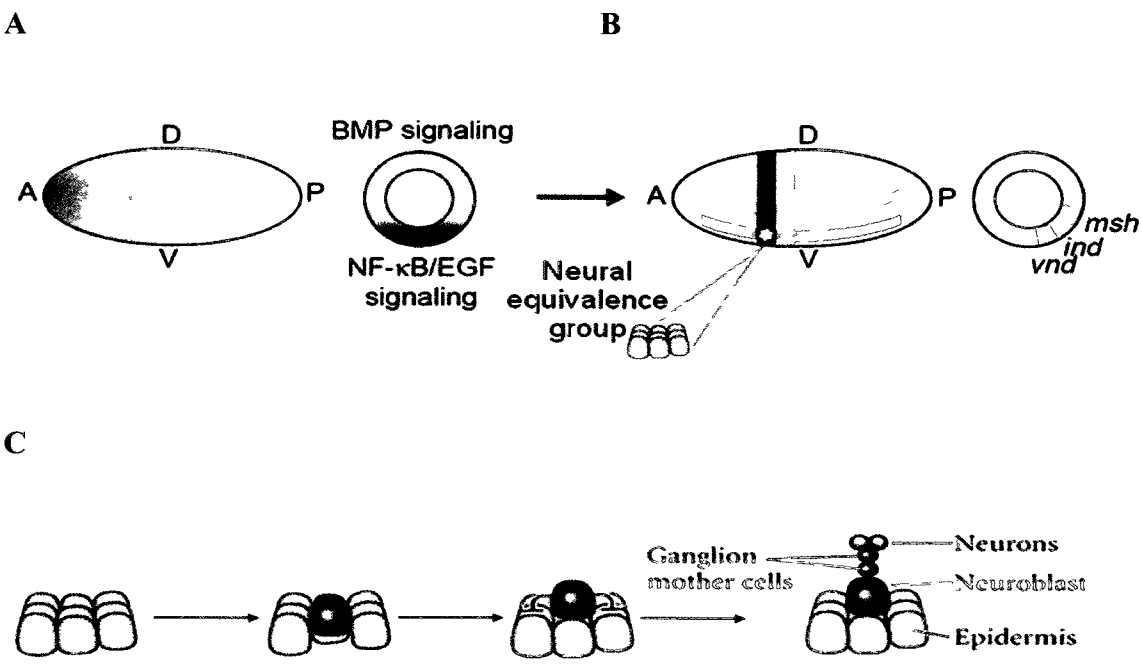


Figure 4. Neuroectoderm specification and neuroblast formation. (A) Complex cascades of patterning genes act in gradients along the AP and DV axes. (B) The segment-polarity (black-gray) and columnar (green-yellow-blue) genes subdivide the neuroectoderm into neural equivalence groups, each of which expresses a unique combination of regulatory genes. (Adapted from Skeath and Thor, 2003). (C) In each neural equivalence group, all of the cells initially express the *ac/sc* complex. Lateral inhibition mediated by Notch and Delta generates one NB by suppressing the expression of *ac/sc* genes in surrounding cells. The NB delaminates and moves internally from the external surface of the ectoderm. The remaining cells of each proneural cluster develop towards an epidermal fate. The newly delaminated NB initiates a stem cell lineage to produce a series of GMCs, each of which divides into a pair of neurons. (Adapted from Goodman and Doe, 1993).

will adopt the NB fate (Martin-Bermudo et al., 1991; Cubas et al., 1991; Skeath and Carroll, 1991). The presumptive NB then triggers the evolutionarily conserved Notch pathway to suppress *ac/sc* expression in the remaining cells of the cluster through lateral inhibition and these surrounding cells will adopt the epidermal fate (Fig. 4C) (Skeath and Carroll, 1992).

b. Formation of GMC and neurons: NB identity is determined by the identity of the cells in the equivalence group from which it segregates (Chu-LaGraff and Doe, 1993; Skeath et al., 1995; Udolph et al., 1995). After segregation, NBs divide asymmetrically in a stem cell manner to generate a chain of smaller secondary precursor cells called ganglion mother cells (GMC) (Fig. 4C). Each GMC then divides once to produce two post-mitotic neurons and/or glial cells (Fig. 4C).

The temporal identity of GMC is regulated by a temporal cascade consisting of the sequential expression of five transcription factors: *Hunchback* (*Hb*) → *Kruppel* (*Kr*) → *Pdm* → *Castor* (*Cas*) → *Grainyhead* (*Gh*) (Fig. 5) (Kambadur et al., 1998; Novotny et al., 2002; Brody and Odenwald, 2000; Isshiki et al., 2001; Brody and Odenwald, 2002). GMCs and their neuronal progeny retain expression of the gene that is present in the NB at the time of the GMC's birth. Therefore, Hb is detected in deep layer neurons, with Kr, Pdm, and Cas found in progressively more superficial layers (Kambadur et al., 1998; Brody and Odenwald, 2000; Isshiki et al., 2001)

The temporal transitions in the expression of these transcription factors enable GMCs born at different times in a lineage to acquire different fates. The

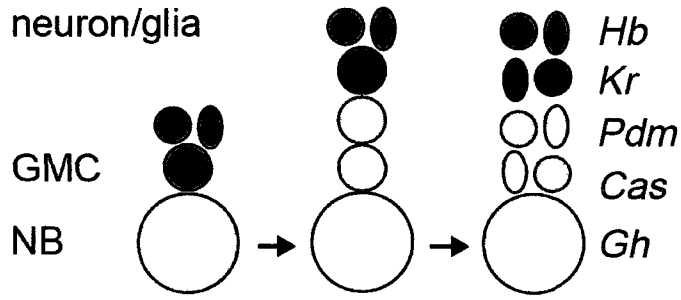


Figure 5. Specification of GMCs. The temporal expression of *Hb* (red) *Kr* (blue) *Pdm* (green) *Cas* (purple) and *Gh* (light blue) leads to diversification of the GMCs generated by each NB. NB/GMC maintains the expression of the gene that is present at the cell's birth. The sequential activation of genes results in a layered pattern of gene expression in the neurons and glia produced by each NB. *Hb*-expressing neurons are located at the basal edge of the VNC and *Gh*-expressing neurons are located at the apical edge, with *Kr*-, *Pdm*- and *Cas*-expressing neurons located in between (Adapted from Skeath and Thor, 2003).

Cartesian coordinate system enables each NB in a hemisegment to acquire a unique identity. Therefore, in each VNC hemisegment, 30 NBs generate a pool of GMCs which in turn make about 400 postmitotic neurons and gila with a ~1:10 ratio for glia/neuron. The specific expression of regulatory genes commits each post-mitotic cell to individual neuronal or glial cell fate (Goodman and Doe, 1993). NBs generally produce a mixed progeny of interneurons, motor neurons and glia (Schmid et al., 1999). In each VNC hemisegment, most of these cells have been individually identified, which means that axon guidance can be studied in *Drosophila* at the level of single cells.

2) Midline region: The midline region separates the two lateral neurogenic regions (Fig. 3) and generates a discrete set of glia and neurons (Klämbt et al., 1991). The midline plays a key part in pathway guidance of commissures and longitudinal axons (Dickson and Gilestro, 2006). After the cellular blastoderm stage, the midline becomes molecularly specialized as a result of the activated transcription of *single-minded* (*sim*), which encodes a basic helix-loop-helix (bHLH)-PAS protein (Crews et al., 1988; Nambu et al., 1990). The midline precursor cells previously located in two parallel strips along the anterior/posterior axis join together at the ventral midline to form a single row. Divisions and differentiation of the cells give rise to the neurons and glia. Sim dimerizes with another bHLH-PAS protein Tango (Tgo), the *Drosophila* homolog of mammalian ARNT and binds to central midline elements (CME) to control CNS midline transcription (Sonnenfeld et al., 1997; Crews, 1998)

1.3.3. Gliogenesis

Each VNC hemisegment contains about 30 glia which can be classified according to their positions and morphology or molecular markers. Based on the regions of the CNS the cells are associated with, three main classes of glia are defined in the *Drosophila* embryonic CNS: the surface, the cortex and the neuropile (Ito et al., 1995). The surface-associated glia include the subperineurial glia (SPG) which lie beneath the outer surface of the VNC, and the channel glia (CG) which are positioned along the dorsovental channels. The cortex-associated glia, whose only subtype in the embryonic VNC is cell-body glia, are those that lie among the neuronal cell bodies in the cortex. The neuropile-associated glia represents those cells which are associated with axonal structures. This category covers the nerve root glia (NG) which includes intersegmental nerve root glia (ISNG) and the segmental nerve root glia (SNG), the interface glia which are associated with the longitudinal connectives and also known as longitudinal glia (LG), and the midline glia (Fig. 3).

Despite these many glial types classified by morphological and positional differences, genetic analysis suggests that there are only two classes of glia in the embryonic CNS: the lateral and the midline glia (Edenfeld et al., 2005). This distinction is accomplished through two non-overlapping genetic cascades. The specification of the lateral glial cells is initiated by the transcription factor Glial Cells Missing (Gcm) (Hosoya et al., 1995; Jones et al., 1995), while the development of midline glial cells is dependent upon the epidermal growth factor (EGFR) signalling pathway (Scholz et al., 1997).

1) **Midline glial cells:** Three pairs of midline glial cells (MGA, MGM and MGP) were identified by their enwrapping of the embryonic axon commissures (Jacobs and Goodman, 1989). These glia are important in the establishment of the normal pattern and shape of the axon commissures (Jacobs, 2000). Midline glial cells are specified by Single-minded/Tango transcription factor heterodimers (Crews, 1998). Midline glia differentiation is controlled by the *Drosophila* epidermal growth factor (EGF) receptor homologue (DER) (Scholz et al., 1997; Crews, 1998).

2) **Lateral glial cells:** Once Gcm initializes glial cell development, the execution of glial differentiation depends on the Gcm target genes. Gcm directly regulates the expression of the BTB-Zn finger protein Tramtrack (Ttk) (Giesen et al., 1997), the ETS transcription factor Pointed (PntP1) (Klaes et al., 1994) and the homeodomain protein Reversed polarity (Repo) (Halter et al., 1995). Ttk maintains stable glial differentiation by repressing neuronal development and controlling glial proliferation (Giesen et al., 1997; Badenhorst, 2001). Repo and PntP1 promote glial differentiation (Klaes et al, 1994; Halter et al., 1995; Yuasa et al, 2003). Repo also cooperates with Trk to suppress neuronal fates (Yuasa et al., 2003). Targets of this cascade are regulators of the G-protein signalling protein Loco and the transcription factor Dead ringer/Retained (Klaes et al., 1994; Granderath et al., 1999; Granderath et al., 2000; Shandala et al., 2003; Yuasa et al, 2003).

1.3.4. Axon pathways in the *Drosophila* embryonic CNS

Information processing performed in the CNS cannot become possible without connections between neurons. One of the key aspects of CNS development is the establishment of the precise wiring of the neurons into functional circuits. During this process, neurons extend their axons along the stereotyped pathways through an environment with cell surface, extracellular matrix and secreted molecules which act as attractive or repulsive guidance cues (Tessier-Lavigne and Goodman, 1996). The growth cone, the process tipped at the leading edge of axons, is armed with receptive machinery to receive guidance cues therefore enabling the extension and formation of the axon pathway.

Two strategies are thought to be used to accomplish this complex task. First, the axons have to travel a long distance to reach their targets. The axon trajectories are thus broken into smaller segments between intermediate targets (or guidepost cells or choice points) therefore a distant navigation is disassembled into a series of smaller journeys. The second strategy is that pioneer axons establish scaffolds of axon pathways for the later developing axons to travel along. In order for the axonal trajectories to be formed, four guidance forces are thought to act coordinatively: short and long-range attractions, and short- and long-range repulsions (Tessier-Lavigne and Goodman; 1996). Axons can be guided at long-range in response to chemoattractants and chemorepellents. Axons can also be guided at short-range through contact-attraction and contact-repulsion. Several or all of the four types of guidance cues are presented simultaneously for individual axons to interpret and then make guidance decisions. In the VNC, the axon tracts are organized in an orthogonal manner (Fig. 3B). The longitudinal axon pathways are positioned on the

either side of the midline. Within each segment, two commissural axon pathways crossing the midline at the anterior and posterior connect the two sides. The majority of axons cross the midline within either anterior or posterior commissures while the rest of neurons only extend their axons on one side of midline. The developing axon tracts of the VNC have been a good *in vivo* model to portray how the wiring of the nervous system is achieved (Araújo and Tear, 2003).

1) **Commissural pathways:** The commissures are formed as the midline glia migrate over the ventral unpaired median (VUM) axons (Fig. 6; Klämbt et al., 1991). The formation of the commissures leads to the connection of the two symmetric halves of the nervous system (Goodman and Doe, 1993). This process involves multiple signals from the midline cells to coordinate the guidance of the growth cones (Dickson and Gilestro, 2006). As a result, the axons either cross the midline or stay ipsilateral.

   Netrin/Frazzled and Slit/Robo are the two major regulatory systems for the neurons to make axonal choices of whether to cross the midline or stay ipsilateral (Fig. 7A). Netrins act as attractive ligands to CNS axons expressing the Frazzled receptor to bring them across the midline (Kolodziej et al., 1996). Slit, expressed by the midline glia, is another major factor guiding axons at the CNS midline. Slit is required to prevent axons from inappropriately approaching the midline. They serve two major purposes: 1) to keep all ipsilateral growth cones away from the midline and 2) to ensure that contra-lateral growth cones are prevented from re-crossing the midline (Sánchez-Soriano et al., 2007). Commissureless (Comm)

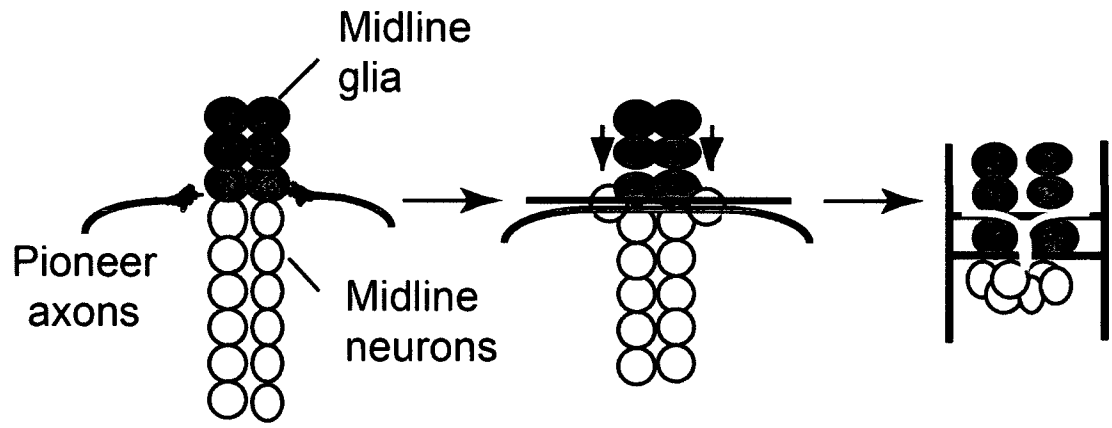


Figure 6. Formation of the commissures. After midline cells have been specified, pioneer growth cones in each hemisegment project towards the most anterior midline neurons, the ventral unpaired median (VUM) neurons, to establish the posterior commissures. VUM neurons then extend axons and pioneer the anterior commissures. The two posteriorly located midline glia migrate between the two anterior and posterior commissural tracts. (Adapted from Hummel et al., 1999).

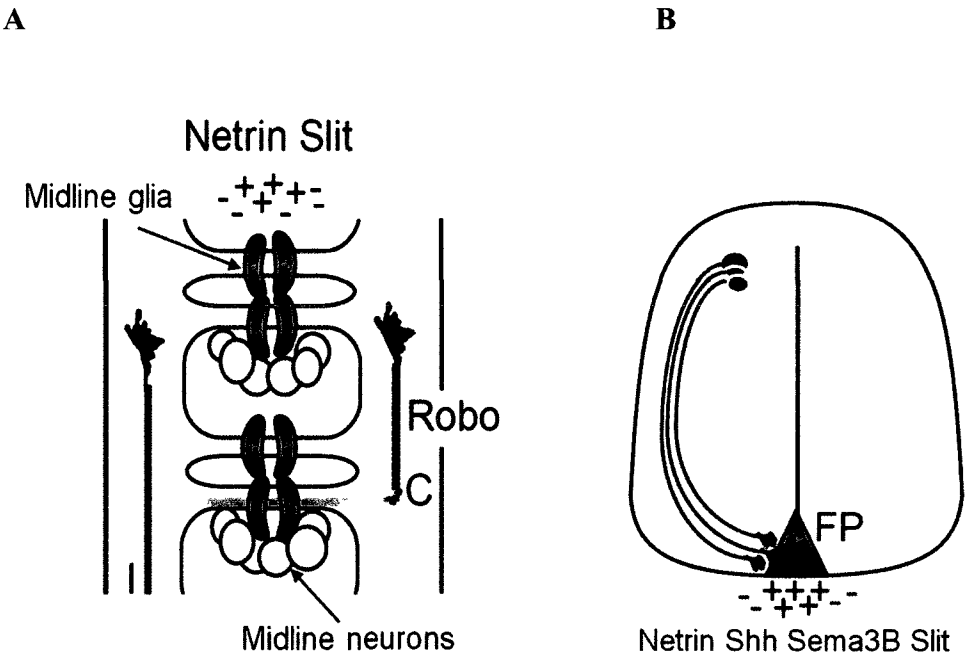


Figure 7. Guidance signals in the *Drosophila* ventral nerve cord and vertebrate spinal cord. (A) Both midline neurons and glia provide the attractive guidance cue Netrin. Glia also secret Slit, which acts as a repellent guidance cue. Netrin attracts commissural axons to the midline; Slit prevents Roundabout (Robo)-expressing ipslateral axons from crossing and contralateraly projecting axons from re-crossing. (Adapted from Kidd et al., 1999). (B) Glia-like cells in the floor plate (FP) of the vertebrate spinal cord act as guidepost cells for commissural axons. They are the source of two attractive guidance cues, Netrin and Sonic Hedgehog (Shh), and the repellent cues Slit and Semaphorin (Sema) 3B. (Adapted from Charron et al., 2003).

is the key regulator of Robo activity during midline crossing in the *Drosophila* VNC. Comm acts in commissural axons to prevent Robo from reaching the surface of the growth cone, depriving the crossing neurons of responsiveness to the Slit repellent before and during midline crossing (Kidd et al., 1998, 1999). After the crossing, Comm is down-regulated and therefore growth cones regain sensitivity to the Slit repellent and do not recross the midline (Georgiou and Tear, 2002).

In the vertebrate spinal cord, glia-like cells in the floor plate play a similar role to guidepost cells at the ventral midline using conserved midline guidance cues (Fig. 7B; Dickson and Gilestro, 2006). Vertebrate commissural neurons are born in the dorsal spinal cord and their axons project to the ventrally located floor plate, and then to the contralateral side. Floor plate cells produce two attractive guidance cues to commissural axons, Netrin-1 and the morphogen Sonic Hedgehog (Shh), which not only control neuronal and glial differentiation, but also act as chemoattractants (Serafini et al., 1996; Charron et al., 2003). Similar to *Drosophila* midline cells, floor plate cells also produce repellent cues, such as Slit-1, 2, 3 and Semaphorin (Sema) 3B (Brose et al., 1999; Long et al., 2004; Sabatier et al., 2004; Zou et al., 2000).

2) **Longitudinal pathways:** In the *Drosophila* embryonic CNS, the first longitudinal axon pathway is initialized by a few early interneurons also called pioneer neurons, MP1, dMP2, vMP2 and pCC (Goodman and Doe, 1993). These cells pioneer a longitudinal axon scaffold which acts as a tract for the following ipsi- and contra-lateral axons to navigate along anteriorly or posteriorly. The

descending growth cones of MP1 and dMP2 meet two ascending growth cones of pCC and vMP2 between two adjacent segments (Fig. 8; Goodman and Doe, 1993). The longitudinal glia serve as guidepost during this process as the intimate contact between them and the four interneurons is required for the normal pioneer neurons' pathfinding and subsequent establishment of connectives (Hidalgo et al., 1995; Hidalgo and Booth, 2000). At the end of embryogenesis, three major axonal fascicles are formed on either side of the midline underlying longitudinal glia which can be visualized with anti Fas II antibodies (Hidalgo and Booth, 2000).

1.3.5. Glial cell migration

Most glial cells migrate significant distances from their precursor area to their specific target sites (Freeman and Doherty, 2006). In most cases, glial migration depends on axons. In the *Drosophila* embryonic VNC, midline glia migrate along the axons of the VUM neurons to their appropriate position, allowing them to separate and ensheath the anterior and posterior commissures properly (Goodman and Doe, 1993). However, glial cells are also able to interpret guidance cues that are normally utilized by axonal growth cones. For example, longitudinal glia transiently express the guidance receptor Robo, become responsive to Slit provided by midline glia and therefore are kept some distance away from the midline and correctly positioned when interacting with axons pioneering longitudinal fascicle formation (Kinrade et al., 2001).

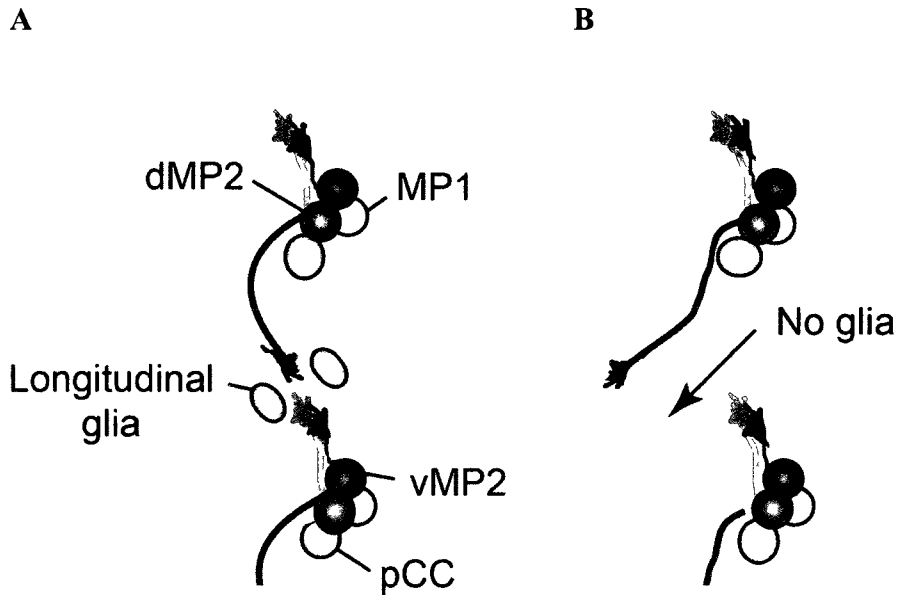


Figure 8. Formation of the longitudinal connectives. Longitudinal glia in the *Drosophila* embryonic CNS function as guidepost cells for pioneer growth cones during the earliest stages of longitudinal tract formation. (A) In each hemisegment, MP1 and dMP2 pioneer neurons extend axons posteriorly, whereas pCC and vMP2 axons project anteriorly. All growth cones encounter two glia at a choice point. (B) If these glia are missing, growth cones fail to meet. (Adapted from Hidalgo and Booth, 2000).

1.4. *jing*

The *Drosophila* gene *jing*, which encodes a zinc finger transcription factor, has been implicated in a role in controlling the differentiation and survival of cells in the CNS midline as well as in the ovary and trachea during embryogenesis (Liu and Montell, 2001; Sedaghat et al., 2002; Sonnenfeld et al., 2004). The *jing* gene was originally identified in two genetic screens for regulators of border cell migration in the ovary and for midline cell development during embryogenesis (Liu and Montell, 2001; Sedaghat et al., 2002). During embryogenesis, *jing* transcripts accumulate in the CNS midline, adjacent neuroectoderm, brain and trachea (Sedaghat et al., 2002; Sonnenfeld et al., 2004). Loss- and gain-of-function *jing* were shown to be associated with defects in CNS axon and tracheal tubule patterning (Sedaghat et al., 2002; Sonnenfeld et al., 2004). Reductions in neuronal and glial gene expression and inappropriate apoptosis in the CNS midline and trachea in *jing* homozygous mutant embryos established the essential role of *jing* for the survival of the specialized cells at the CNS midline and in the trachea (Sedaghat et al., 2002).

1.4.1. Jing CNS midline function

In homozygous *jing* mutant embryos, CNS midline neurons and glia do not differentiate properly (Sedaghat et al., 2002). Embryos double mutant for *jing* and *sim* phenocopy *sim* homozygotes in CNS, suggesting that *jing* functions downstream of *sim* (Sedaghat et al., 2002). *sim* plays an essential part in controlling gene expression in the midline (see above) (Crews et al., 1988; Nambu et al., 1990). Midline cells are reduced in homozygous *jing* mutants, which results in loss of longitudinal connectives and

improper separation of commissures (Sedaghat et al., 2002). More apoptotic glia cells are found in *jing* homozygous mutant than in wild-type embryos, leading to the loss of some of the midline gene expression in *jing* mutants (Sedaghat et al., 2002).

The differentiation of CNS midline cells is regulated by the epidermal growth factor (EGFR) pathway (Nambu et al., 1990; Jacobs, 2000). Several pieces of evidence suggest an involvement of *jing* in EGFR signalling in midline glia: 1) MAPK activity and *Egfr* expression is reduced in *jing* mutant embryos; 2) *jing* over-expression effects phenocopy gain-of-function phenotypes in *Egfr* pathway; 3) midline cell death can be partially rescued by activation of *Egfr* pathway in *jing* mutants; 4) *Egfr* pathway genes and *jing* show dominant genetic interactions in the midline (Sonnenfeld et al., 2004).

1.4.2. Jing function in the trachea system

Jing function is also required for the proper development of the tracheal (respiratory) system, the branched network for the delivery of oxygen in *Drosophila* (Sedaghat et al., 2002; Sonnenfeld et al., 2004). *jing* affects branching morphogenesis and cellular survival in the tracheal system (Sedaghat et al., 2002). Tracheal patterning is regulated by EGFR (Wappner et al., 1997). *jing* and *Egfr* pathway mutants have similar tracheal phenotypes (Sedaghat etal., 2002; Bier et al., 1990; Gabay et al., 1997a,b). Similar to the CNS midline, *jing* and *Egfr* pathway genes also show dominant genetic interactions in the trachea (Sonnenfeld et al., 2004).

1.4.3. Interactors of *jing*

It has been shown that *jing* plays an important role in controlling cell survival and differentiation in both the CNS midline and trachea through the EGFR signal pathway

(Sedaghat et al., 2002; Sonnenfeld et al., 2004). However, the transcriptional mechanisms underlying Jing function are not known. Therefore, in order to unravel the function and molecular mechanisms of *jing*, a genetic screen was carried out in the photoreceptor neurons of the *Drosophila* eye using gain-of-function (GOF) assays (Sun et al., 2006). Dispensable for the fly survival, its compound eye structurally consists of ~750 identical small eyes or ommatidia (Wolf and Ready, 1993). Any defects in shape, location and/or number of the cells can result in an easily detectable rough eye phenotype (Fig. 9D) compared with the wild-type eye (Fig. 9A). The screen was performed with a collection of 591 EP lines each of which carries a P element containing UAS sites proximal to an endogenous gene individually on the third chromosome (Rørth, 1996; Rørth et al., 1998; Sun et al., 2006). Seven genes were found to specifically interact with *jing* during eye formation (Table 1; Sun et al., 2006). Of the seven identified genes, *D*ATR-X, also called X-linked nuclear protein (XNP), is the homologue of the human α-thalassemia and mental retardation X-linked (ATR-X), which is a member of the SWI2/SNF2 family of chromatin remodeling proteins (Picketts et al., 1996).

1.5. The ATR-X gene (α-thalassemia and mental retardation X-linked)

Alpha-thalassemia is a form of anaemia due to reduced production of α-globin that makes up haemoglobin (the iron-containing oxygen-transport metalprotein in the red blood cell). The association of α-thalassemia and mental retardation was first reported in Weatherall et al. (1981). Subsequent observations indicated that there are cases with and without detectable deletion of genes including the α-globin gene (Wilkie et al., 1990a,

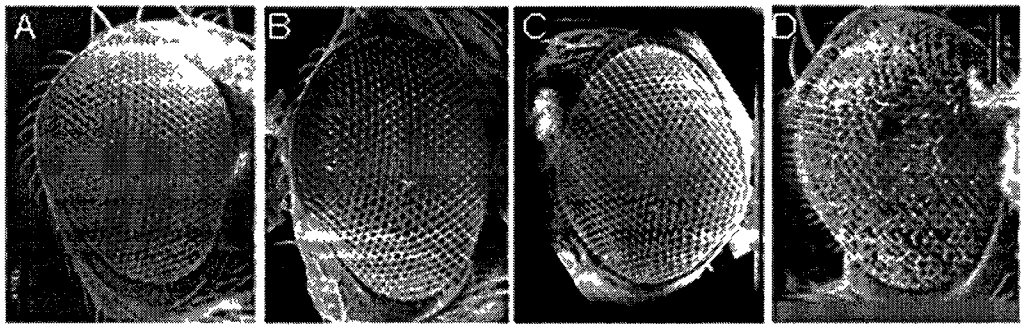


Figure 9. Gain-of-function assay to identify *jing*-interactors in the *Drosophila* eye. UAS-containing transgenes were targeted to the eye by *GMR*-Gal4 (Moses and Rubin; 1991; Hay et al., 1994). The compound eyes are shown in scanning electron micrographs. Genotype, *GMR*-Gal4/+ (A); *GMR*-Gal4/+; UAS-*jingE*/+ (B); *GMR*-Gal4/+; EP(3)0635/+ (C); *GMR*-Gal4/+; UAS-*jingE*/+;EP(3)0635/+ (D). (A) Wild-type like eye structure. (B-C) Over-expression of either *jing* (B) or EP(3)0635 (C) showed a normal eye phenotype. (D) A rough eye phenotype was found when EP(3)0635 was co-overexpressed with *jing* in the eye, suggesting a genetic interaction. Scale bar, 100 μm. (Adapted from Sun et al., 2006).

Table 1. EP lines that enhanced *jing* gain-of-function

EP line	Gene	Predicted Function
EP(3)0635	*D*ATR-X	Chromatin remodeling
EP(3)0473	*D1*	Chromatin remodelling
EP(3)3145	*Dataxin-2*	Translational regulation
EP(3)3060	*lap*	Receptor mediated endocytosis
EP(3)3705	CG32137	Microtubule binding (cell cycle)
EP(3)3354	CG17383 (JIGR-1)	Transcriptional regulation
EP(3)3084	CG15507	unknown

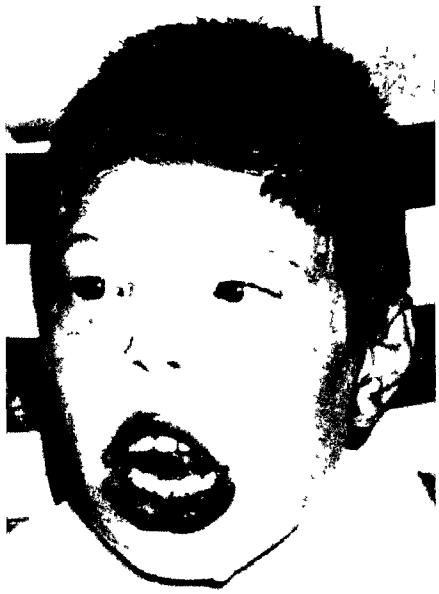
(Adapted from Sun et al., 2006)

1990b). In the cases without detectable molecular abnormalities of the α-globin complex, the wide spectrum of phenotypes (described below) results from mutations in an X-encoded factor, the ATR-X gene which is a putative regulator of gene expression (Picketts et al., 1996). ATR-X has been shown to be the disease gene of several mental retardation syndromes including α-thalassemia X-linked mental retardation syndrome (ATR-X syndrome) (Gibbons et al., 1995b; Villard et al., 1996a,b; Abidi et al., 1999; Lossi et al., 1999; Stevenson et al., 2000a; Abidi et al., 2005).

1.5.1. Clinical description of the ATR-X syndrome

ATR-X syndrome (OMIM no. 301040) is characterized by distinctive craniofacial features, genital anomalies, and severe developmental delays with hypotonia and mental retardation (Fig. 10) (Gibbons et al., 1995a; Stevenson et al., 2000b; Badens et al., 2006). Craniofacial abnormalities include small head circumference, telecanthus or ocular hypertelorism, small nose, tented upper lip, and prominent or everted lower lip with coarsening of the facial features over time. Genital anomalies range from hypospadias and undescended testicles to severe hypospadias and ambiguous genitalia, to normal-appearing female genitalia. The severe developmental impairment and mental retardation are the most important clinical manifestations. Developmental milestones are globally and markedly delayed from the outset. Speech and ambulation occur late in childhood. Some affected individuals never walk independently or develop significant speech. Hypotonia is a hallmark of the condition, contributing to the facial manifestations, drooling, and developmental retardation. Seizures occur in approximately one-third of individuals (Gibbons et al., 1995a). The neurobehavioral phenotype has not been extensively described.

A



B

Clinical finding	Total[a]	%
Profound mental retardation	137/144[b]	95
Characteristic face	116/124	94
Skeletal abnormalities	110/121	91
HbH inclusions	106/122	87
Neonatal hypotonia	78/92	85
Genital abnormalities	103/129	80
Microcephaly	9/117	76
Gut dysmotility	76/104	73
Short stature	63/96	66
Seizures	46/131	35
Cardiac defects	24/135	18
Renal/urinary abnormalities	19/136	14

Figure 10. Clinical features of ATR-X syndrome. (A) A thirteen year-old boy with ATR-X syndrome showing the typical facial appearance. (B) Clinical findings in 145 patients with ATR-X syndrome. (Adapted from Gibbons and Higgs, 2000).

1.5.2. Molecular genetics of ATR-X

ATR-X, located on the X chromosome (Xq13), spans approximately 300kb of genomic DNA and contains 36 exons (Gibbons et al., 1992; Picketts et al., 1996). The predicted ATR-X protein is a member of the SNF2 chromatin remodeling protein family, containing a SWI2/SNF2 helicase/ATPase motif and a plant homeodomain (PHD) zinc finger domain (Picketts et al., 1996). Helicase proteins are involved in various cellular processes ranging from chromosome segregation, e.g. lodestar (Girdham and Glover, 1991), DNA repair, e.g. RAD 16 (Schild et al., 1992) and ERCC6 (Troelstra et al., 1992), DNA recombination, e.g. RAD 54 (Emery et al., 1991), and transcription regulation, e.g., SWI2/SNF2 (Abrams et al., 1986), MOT1(Davis et al., 1992), and Brahma (Tamkun et al., 1992), in which chromatin has to be structurally modified. PHD zinc fingers are a common feature of chromatin related proteins and are involved in protein-protein interaction (Bienz, 2006). This motif may also play a role in DNA binding (Cardoso et al., 2000). Although patient mutations have been identified throughout ATR-X, more than 80% of those reported are missense mutations in the PHD zinc finger and helicase domains (Fig. 11; Gibbons et al., 2008).

1.5.3. ATR-X function

ATR-X transcripts are expressed ubiquitously and are particularly abundant in human and mouse brain (Cardoso et al., 1998). ATR-X expression at early stages of mouse embryogenesis was also detected as well as at late developmental stages and at birth (Stayton et al., 1994). ATR-X is a nuclear protein that localizes to both pericentromeric heterochromatin and promyelocytic leukemia nuclear bodies (PML-NBs)

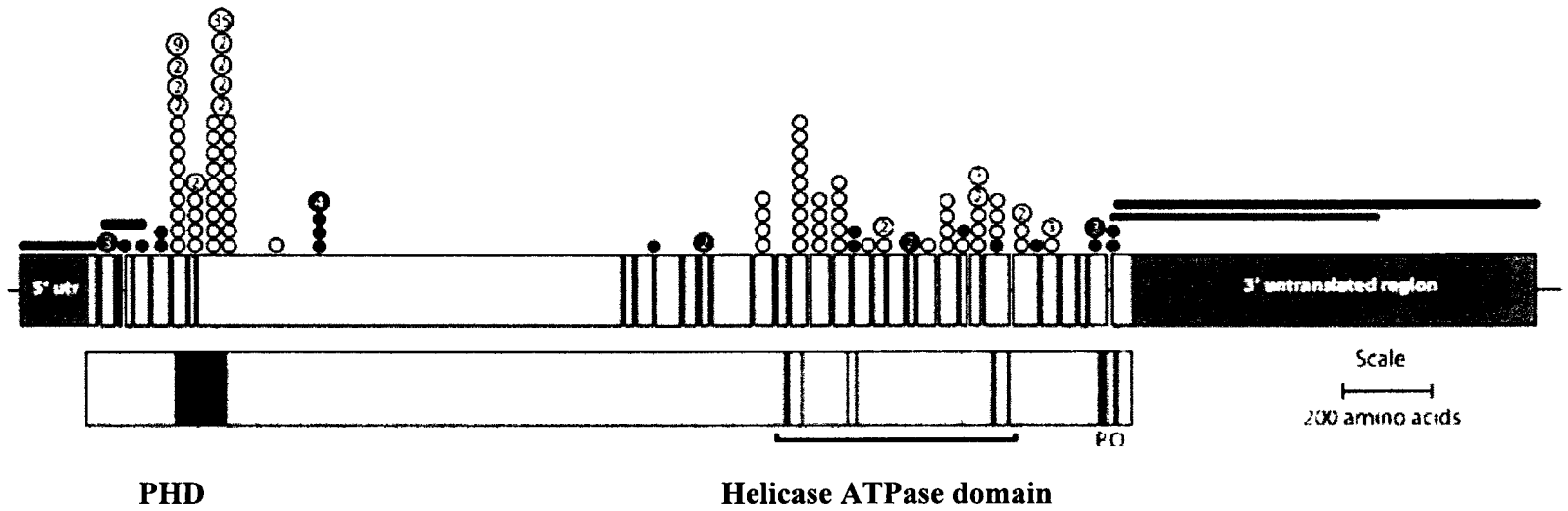


Figure 11. Schematic diagram of the human ATR-X gene. Boxes represent the exons; thin horizontal lines represent introns (not to scale). The PHD zinc finger motif and the helicase domain are noted, as are the P box (P) and a glutamine-rich region (Q). The positions of patient mutations are shown by circles. Filled circles represent mutations that cause protein truncation; open circles represent mutations that maintain the reading frame; deletions are indicated by horizontal lines. (Adapted and modified from Gibbons et al., 2008).

in a cell cycle and phosphorylation dependent manner (McDowell et al., 1999; Ishov et al., 2004). The ATRX protein has been shown to be recruited by heterochromatin protein HP-1 to heterochromatin (Kourmouli et al., 2005), consistent with the previously reported ATR-X's colocalization and direct interaction with HP-1 (McDowell et al., 1999; Lechner et al., 2005). ATR-X also interacts with other heterochromatin-localized proteins, EZH2 and MeCP2 (Cardoso et al., 1998; Nan et al., 2007). ATRX displays ATP-dependent translocase activity and at PML-NBs, ATR-X forms a chromatin-remodeling complex with death domain-associated protein (Daxx) (Ishov et al., 2004; Xue et al., 2003; Tang et al., 2004). A recent study suggests that ATR-X protein is required for normal chromosome congression, cohesion and segregation in human cultured cells (Ritchie et al., 2008).

1.5.4. Animal model

Transgenic mice over-expressing the ATR-X protein presented growth retardation, neural tube defects and a high incidence of embryonic death, implicating an important role of ATR-X dosage in normal development and cortex organization (Bérubé et al., 2002). Conditional knockout of ATR-X during early embryogenesis resulted in an increase in neuronal apoptosis in mouse cortex leading to a reduced forebrain size, suggesting that ATR-X is a critical mediator of cell survival during early neuronal differentiation and that neuronal loss may contribute to the mental retardation observed in ATR-X syndrome patients (Bérubé et al., 2005).

1.6. *D*ATR-X

At the time this thesis work was started, *Drosophila* ATR-X was only reported in two ectopic-misexpression-based screens (Pena-Rangel et al., 2002; Nicolaï et al., 2003). It was shown that thoracic over-expression of *DATR-X* modified the thorax structure and over-expression of *DATR-X* resulted in fewer neurons in the mushroom body (Pena-Rangel et al., 2002; Nicolaï et al., 2003). Recent studies showed that over-expression of *DATR-X* increases apoptosis in the developing eye, wings and neurons via the Jun-N-terminal kinase (JNK) pathway (Lee et al., 2007; Hong et al., 2009; Dhanasekaran and Reddy, 2008). *D*ATR-X has also been shown to co-localize and interact with *Drosophila* heterochromatin protein 1 (dHP-1), similar to its mammalian homologue (Bassett et al., 2008).

1.7. Rationale of the thesis work

The CNS midline role of Jing in the regulation of tyrosine kinase signalling in axon guidance during embryogenesis has been described (Sedaghat et al., 2002; Sonnenfeld et al., 2004). It was clear that Jing regulates gene expression including that of the EGFR but the associated mechanism was unknown. In an attempt to address this issue, *D*ATR-X was chosen from a gain-of-function genetic screen (Fig. 9; Sun et al. 2006) as a candidate for functioning with Jing. Its ability to synergistically enhance *jing* GOF effects in adult neurons raised the possibility that *D*ATR-X may be involved in regulating chromatin structure at Jing genomic target sites. Although the role of Jing in midline guidance of axon pathways had been established (Sedaghat et al., 2002; Sonnenfeld et al.,

2004), its role in other CNS neurons and glia was not known. Similarly, studies of *D*ATR-X during embryogenesis had not been conducted. Therefore, this necessitated a comprehensive analysis of both Jing and *D*ATR-X in embryonic neurons and glia. Since neuro-glia interactions are essential for the formation of longitudinal and commissural axon pathways (see section 1.3.4.), this model system could lend itself to also determining the role of Jing and *D*ATR-X in axon guidance.

The repeatability of the VNC (Fig. 3) which allows many easily quantifiable features as descriptive tools provides a high degree of precision to our observations; therefore, the fine detail of cellular function for *D*ATR-X in the *Drosophila* embryonic CNS would be expected and likely informative about the etiology of human ATR-X syndrome. Meanwhile, the function of *jing* can be better illuminated by the identification of its interacting partners.

1.8. Statement of the hypothesis and objectives

The hypothesis for this study was: the *jing* interactor *D*ATR-X controls axon guidance mechanisms in *Drosophila* embryos. The hypothesis was tested with the following objectives:

Objective 1: analyse the embryonic expression pattern of *DATR-X* with *in-situ* hybridization and reporter *lacZ* assays.

Objective 2: raise an antibody against *D*ATR-X to determine the CNS cell types expressing *D*ATR-X and to identify its subcellular localization.

Objective 3: targeted gain-of-function and loss-of-function assays to determine *D*ATR-X function in embryonic CNS neurons and glia during the formation of axon networks.

Objective 4: assess the relationship of *D*ATR-X and *jing* in embryonic CNS neurons and glia and *in vitro*.

MATERIALS AND METHODS

2.1. Fly Stocks

Flies were raised on standard cornmeal-molasses media and maintained at 25°C (Ashburner, 1989). The following fly stocks were obtained from Bloomington Stock Centre (Bloomington, IN): w^{1118}, MP2 neuronal driver*15J2*-Gal4 (Hidalgo and Brand, 1997), *prd*-Gal4, pan-neuronal driver *Elav*-Gal4 (on X chromosome) (Robinow and White, 1988; Yao and White, 1994), and Df(3R)BSC522/TM6C, Sb^1 (DATR-X^{Df}) (FBst0025050). The collection of 3rd chromosome transgenic enhancer/promoter (EP) strains, generated and described by Rørth (1996), was obtained from the Hungarian Szeged stock center. The EP(3)0635 line carries UAS element immediately upstream to the endogenous DATR-X gene (Rørth, 1996; Rørth et al., 1998; Pena-Rangel et al., 2002). GCM-Gal4 drives expression in all CNS glia expressing the *glial cells missing* (*gcm*) gene (Hosoya et al., 1995; Jones et al., 1995; Freeman et al., 2003) and was obtained from Dr. M. Freeman (MRC laboratory of Molecular Biology, UK). The longitudinal glial driver *htl*-Gal4 (Shishido et al., 1997) was from Dr. Donald J. Van Meyel (McGill University Centre for Research in Neuroscience, Montreal, QC). UAS-*jing* was provided by Dr. M. Sonnenfeld (Sedaghat et al., 2002; Sonnenfeld et al., 2004). The three *DATR-X* excision lines generated through imprecise excision, *DATR-X1*, *DATR-X2* and *DATR-X3* (Bassett et al., 2008), were gifts from Dr. Andrew Travers (MRC Laboratory of Molecular Biology, Cambridge, UK). Individually, the long isoform of *DATR-X* is absent in *DATR-X3*; both isoforms are reduced in *DATR-X2*; and neither isoform is present in *DATR-X1* (Bassett et al., 2008). Essentially *DATR-X1* is also a deficiency but smaller than *DATR-*X^{Df} (Bassett et al., 2008). *DATR-*X^{Df} and *DATR-X* excisions were balanced with TKG5 GFP (TM3, Kr-GFP, Sb) (Casso et al., 1999) (gift

from Dr. David Park, University of Ottawa, Ottawa, ON) in order for the embryos (older than stage 9) homozygous for the deletion/mutation of *DATR-X* to be identified by their lack of GFP signal and sorted from their siblings.

In all Gal4/UAS studies, each element is heterozygous in the resultant embryos analysed. *DATR-X* and *jing* were over-expressed in CNS neurons or glila by crossing homozygous *Elav*-Gal4 or *gcm*-gal4 with homozygous UAS-*jing*, EP(3)0635 or UAS-*DATR-X*. For co-expression experiments flies carrying *Elav*-Gal4 and UAS-*jing* were crossed with flies homozygous for EP(3)0635. Controls included *Elav*-Gal4/+, UAS-*jing*/+, and EP(3)0635/+. For double RNAi, flies carrying *Elav*-Gal4 and UAS-*jing* RNAi were crossed to flies carrying UAS-*DATR-X* RNAi.

2.2. Molecular genetic analysis of *DATR-X*

Genomic and cDNA sequences encoding the *Drosophila D*ATR-X homolog were identified by searching the Berkeley *Drosophila* Genome Project (BDGP) database for the EP(3)0635 associated gene (Adams et al., 2000; Rubin et al., 2000). The location of the EP element in EP(3)0635 was confirmed by PCR using inverted repeat primers and flanking genomic *DATR-X* sequence (CGACGGGACCACCTTATGTTATTTCATCAT G) followed by DNA sequence analysis. *DATR-X* cDNAs (expressed sequence tags (ESTs) SD07188 and LD28477) were obtained from Open Biosystems. *DATR-X* EST SD07188 contains 2,382 base pairs of *DATR-X* coding sequence beginning at base pair 1968 with respect to full-length LD28477 cDNA sequence. *DATR-X* clones SD07188 and LD28477 were subjected to DNA sequence analysis and compared to that in the

Gadfly database (Celera genomics, Adams et al., 2000). Homology searches were done using BLAST (http://www.ncbi.nlm.nih.gov). Full-length *DATR-X* sequence (accession AAL13821; Flybase) was aligned with that of C. *elegans* (AAD55361), mouse (AAC08741) and human (AAB49970) using Clustal X (1.83) (Thompson et al., 1997). Protein sequences were compared using BL2SEQ (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) (Tatusova and Madden, 1999). Protein domains were analysed using SMART and PROSITE (Letunic et al., 2004; Hulo et al., 2004).

2.3. Making of UAS transgenic lines

The *DATR-X* full length 3933 base pair (bp) or partial (first 2760 bp for truncated version) coding sequence was amplified from cDNA clone LD28477 (Berkeley *Drosophila* Genome Project, Open Biosystems) by PCR using a reverse primer containing the Hemagglutinin (HA) and ligated in frame into the *EcoR I/Kpn I* sites of the pUAST vector (Brand and Perrimon, 1993). The transgenic flies were generated by P element mediated transformation (Spradling and Rubin, 1982; Genetic Services, Inc, Sudbury, MA). Expression of the transgene carrying full length or truncated *DATR-X-HA* was confirmed by the pair-rule striped pattern when stained with antibodies against *D*ATR-X and the HA tag respectively after over-expression was triggered with *prd*-Gal4.

2.4. *in situ* hybridization

Whole-mount *in situ* hybridization was carried out using digoxigenin-labelled RNA probes according to Janody et al., (2000). The *DATR-X* riboprobe was generated by linearizing the SD07188 EST with *HaeII*. Sense probes were synthesized using the T7 promoter and anti-sense using Sp6 according to manufacturers' specifications (Roche Diagnostic Corporation, Indianapolis, IN). Probe specificity was tested in gain-of-function *DATR-X* embryos carrying the *prd*-Gal4 driver and EP(3)0635 or UAS-*DATR-X* transgenes. For post-embryonic analysis, third instar larval nerve cords were dissected in PBS and fixed in 4% formaldehyde for 20 minutes. Fixed nerve cords were rinsed three times in fresh PBS and washed for 1 hour. Nerve cords were then subjected to *in situ* hybridization as described above. Digoxigenin stained nerve cords and embryos were mounted in 80% glycerol and examined on a Zeiss Axioskop 2 at 20X and 40X magnifications and images were captured with a Nikon DXm1200 digital camera using Nikon ACT-1 (Versioin 2.62) software. All the images were edited with Adobe Photoshop CS2.

2.5. Reporter gene construction

593 base pairs were obtained by PCR from the 5' *DATR-X* region surrounding the EP element insertion site. This fragment was cloned into the *EcoR1* site in pCaSper in order to fuse it with *lacZ* coding sequence (Thummel et al., 1988). To generate transgenic strains, the pCaSper plasmids were co-injected together with a plasmid encoding Δ2-3 transposase into w^{1118} embryos by P element mediated transformation

(Genetic Services, Cambridge. MA) (Rubin and Spradling, 1983). Multiple strains were analyzed for *lacZ* expression in embryos stained with monoclonal anti-β-Galactosidase.

2.6. RNA interference (RNAi)

For *DATR-X* RNAi, a 697 base pair sequence was generated by PCR from base pair positions 3181-3837 using LD28477 cDNA as a template. For RNAi of *jing*, a 597base pair sequence was generated by PCR from base pair position 2289 from the ATG on the LD36562 cDNA (Open Biosystems). The DNA fragments were individually subcloned sequentially, in opposite directions, into the *AvrII* and *NheI* sites of the pWIZ plasmid (a gift of R. Carthew) except that the inverted sequence was directionally cloned using *Xba1* and *Nhe1* restriction enzymes (Lee and Carthew, 2003). The final inverted repeat containing vectors, UAS-*DATR-X* RNAi and UAS-*jing*-RNAi were confirmed by sequence analysis. To generate transgenic strains, the UAS-*DATR-X* RNAi or UAS-*jing*-RNAi plasmids, were co-injected together with a plasmid encoding Δ2-3 transposase into w^{1118} embryos by P element mediated transformation (Genetic Services, Cambridge. MA) (Rubin and Spradling, 1983). Several independent UAS-*DATR-X* RNAi transformant lines were bred to homozygosity and mapped. To confirm down-regulation of mRNA expression, embryos carrying *armadillo*-Gal4 (Ahmad and Henikoff, 2001) and UAS-*DATR-X* RNAi were subjected to *in situ* hybridization using *D*ATRX riboprobes. The number of stage 13 embryos with reduced expression in transgene and *Gal4* driver carrying embryos was compared with that in control embryos carrying either the driver or the transgene alone.

2.7. Plasmid constructs

The *DATR-X* full length or partial cDNA (corresponding to 1-137, 138-328, 329-361, 266-449, 1-449, 450-920 and 921-1311 amino acid residues respectively) was amplified by PCR using a reverse primer containing the HA tag from *DATR-X* cDNA LD28477 and ligated in frame into the *EcoR I/Kpn I* sites of expression vector pPAC (gift from Dr. James Woodgett, Ontario Cancer Institute within the Princess Margaret Hospital, Toronto, ON). pPAC-Jing full-length was initially made but failed to be expressed. Then plasmids carrying two truncated pPAC-*jing* versions (corresponding to 1-839 and 840-1486 amino acid residues respectively) were generated with a C-terminal HA tag and cloned into *Xba I/Xho I* sites of pPAC. Sequences of all constructs were confirmed by DNA sequencing at StemCore Laboratories, OHRI (Ottawa, ON).

2.8. Production of GST-*D*ATR-X rabbit antiserum

To produce an antibody against *D*ATR-X, a 474 base pair (3331-3804) fragment from *DATR-X* cDNA was amplified by PCR and subcloned in frame into the *EcoR I/Xho I* sites of GST vector pGEX-4T1 (Amersham). This sequence corresponds to a region of the putative *D*ATR-X protein without any conservation to other *Drosophila* proteins. *E.coli* BL21 cells transformed with the plasmid encoding GST-*D*ATR-X were grown until the optical density at 600 nm reached 0.6. Then the fusion protein was induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h. The harvested cells were resuspended in PBS with Complete Protease Inhibitor Cocktail (Roche) and lysed by sonication (Branson Sonifier 450). Then Triton X-100 was added to a final concentration of 1% before centrifugation and Glutathione Sepharose 4B beads (Amersham) were

added into the supernatant. Next, the beads were washed with PBS and finally the GST fusion proteins were eluted with reduced glutathione (Amersham). 4 milligrams of GST-*D*ATR-X fusion protein was injected into two rabbits three times for each at an interval of ~19 days to produce the GST-*D*ATR-X antiserum (Cedarlane, ON).

2.9. Affinity purification of *D*ATR-X antibody

GST and GST-*D*ATR-X fusion proteins were coupled to CNBr activated sepharose (Amersham) to generate affinity columns according to the manufacturer's protocol. The eluted protein was first subjected to dialysis in coupling buffer (0.1M$NaHCO_3$, 0.5M NaCl, pH8.3) overnight at 4°C and then incubated with the sepharose (prepared in 1mM HCl) overnight at 4°C followed by another incubation in 0.1M Tris buffer for 6h at room temperature. Then the resin was washed once in 0.1M Acetate, 0.5M NaCl (pH 4.0), once in 0.1M Tris-HCl, 0.5M NaCl (pH 8.0), twice in PBS and then stored in PBS with 0.01% sodium azide at 4°C.

The antiserum against GST protein was removed by diluting 5 ml of serum with 5 ml of PBS/Tween (1 X PBS, 0.2% Tween-20, 0.01% sodium azide) and incubating with 5 ml of GST-affinity column in PBS/Tween for 2h at 4°C. Then the effluent was loaded onto GST-*D*ATR-X affinity columns and incubated overnight at 4°C. The supernatant was removed and the column was washed with 10 ml PBS/Tween each for 5 times and twice with PBS. The beads were resuspended in PBS and then transferred to a Bio-Rad column and eluted with 10ml of 0.2 M Glycine (pH 2.8). The eluted fractions containing antibody were combined and concentrated by centrifuging with Amicon Ultra Centrifugal

Filter (Millipore). Given the working hypothesis that the antibody is recognizing *D*ATR-X, the specificity was tested by Western analysis and immunostaining.

2.10. Immunostaining of *Drosophila* embryos and microscopy

For fixation, embryos were first dechorinated in 50% Lavo–6 bleach (Sodium Hypochloride 6%) (Lavo Inc., Montréal, QC) for 5 min and washed thoroughly with dH_2O. Then embryos were fixed in a glass scintillation vial with gentle shaking in (1:1) PBS containing 4% formaldehyde:heptane for 30 min. Then, the lower formaldehyde phase was removed with a Pasteur pipette. 15 ml of methanol was added and the vial was shaken vigorously for 30 sec to remove the vitelline membranes. Embryos falling to the bottom of the vial were transferred to a fresh tube for additional three washes with methanol for 5 min each. Then the embryos were washed in PBT (1X PBS with 0.1% Triton X-100) three times for 10 min each before blocking with PBTN (1X PBT with 5% normal goat serum). Then, the embryos were incubated overnight with primary antibodies in PBTN at 4°C. After that, the embryos were washed with PBT six times for 10 min each before incubating with appropriate secondary antibodies for 2 h at room temperature. Lastly, the embryos were washed 6 times for 10 min each and mounted in Vectashield (Vector Laboratories).

The following antibodies were obtained from the Development Studies Hybridoma Bank (DSHB, University of Iowa, Iowa City, IA): BP102 (Carney et al., 1997), 1:5; 22C10 (Zipursky et al., 1984), 1:10; 8D12 mouse anti-Repo (Alfonso and Jones, 2002), 1:10; 1D4 mouse anti-Fas II (Hummel et al., 2000), 1:10; mouse anti-

Prospero (Campbell et al., 1994), 1:10; 13C9 anti-Robo (Kidd et al., 1998), 1:10. Other antibodies included: rabbit anti-*D*ATR-X, 1:40; rabbit anti-Repo (gift from Dr. Joachim Urban, Universität Mainz, Mainz, Germany) (Halter et al., 1995), 1:500; rabbit anti-HA (Sigma), 1:100, rabbit anti-ß-Galactosidase (Promega), 1:100. Secondary antibodies were, Texas red and FITC-conjugated secondary antibodies (1:100), Alexa Fluor 488 goat anti-rabbit (1:100), Alexa Fluor 594 goat anti-mouse (1:100) (Invitrogen) or horseradish peroxidise (HRP)-conjugated antibodies (1:100) (Promega), 1:100. For propidium iodide nuclear staining, the samples were pretreated with 400 µg/ml RNAse for 2 h before incubating with 10 µg/ml propidium iodide in PBS for 20 min at room temperature. For HRP detection, the embryos were incubated in diaminobenzedine for 5 min before reaction with H_2O_2. HRP-stained embryos were dehydrated through an ethanol series, mounted in methyl salicylate, and examined with a Zeiss Aixoskop 2 microscope fitted with a Nikon DXm1200 digital camera using Nikon ACT-1 (Version 2.62) software. Immunofluorescent images were taken with a Zeiss LSM 5 Pascal Laser Scanning Microscope. All the images were edited with Adobe Photoshop CS2.

2.11. S2 cells transfection and immunofluorescence

The *Drosophila* Schneider 2 cell (S2) line was originally derived from primary cultures of late-stage *Drosophila* male embryos and is a highly versatile expression system which can be used to analyze gene function (Schneider, 1972; Benting et al., 2000; Towers and Sattelle, 2002). S2 cells were maintained at room temperature in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% FBS, 50 units/ml of penicillin

and 50 μg/ml streptomycin. Cells were transfected using Cellfectin as instructed by the manufacturer (Invitrogen). After at least 48 h, the cells were transferred to Lab-Tek chamber slides coated with poly-L-lysine and subsequently settled using IEC Centra 8 table top centrifuge. Cells were fixed in PBS with 4% formaldehyde for 10 min and washed in PBSB (1X PBS with 0.2% Triton X-100, 0.5% BSA) three times for 10 min each. Slides were incubated with primary antibodies for 1 h at room temperature. Dilutions for primary antibodies were mouse anti-HA (Sigma), 1:500, rabbit anti-*D*ATR-X, 1:40. Slides were washed in PBSB three times for 10 min each and incubated with the Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 594 goat anti-mouse (Invitrogen) in a dilution of 1:100 for 1 h at room temperature. After washing in PBSB, DNA was stained with 1 μg/ml 4', 6'-diamidino-2-phenylindole hydrochloride (DAPI) for 2.5 min. Coverslips were then mounted on slides in Vectashield (Vector Laboratories). Fluorescence images were captured on a Zeiss Axiovert 200M inverted microscope equipped with an AxioCam HRm digital camera using AxioVision 4.6 (Carl Zeiss MicroImaging GmbH).

2.12. Immunoprecipitation

S2 cells were harvested 48 h after transfection with expression constructs by centrifuging at 2000 rpm for 5 min. The cells were washed once with ice-cold PBS and then resuspended in IP buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 mM $MgCl_2$, 1% NP-40 and Complete protease inhibitor cocktail (Roche)). The lysate was passed 5 times through a BD 21 gauge needle attached to a BD sterile

plastic syringe, then incubated on ice for 20 min followed by centrifuging at 15, 000 rpm for 5 min at 4°C. Then the supernatant was collected as whole cell protein extract and the protein concentration was determined with BCA protein Assay Kit (Pierce). The appropriate amount of supernatant containing at least 0.5 mg proteins were transferred to a cold fresh tube and the reaction volume was finalized to 1 ml with IP buffer. The sample was incubated with 1 μg of rabbit anti-*D*ATR-X at 4°C overnight before an addition of 20 μl of washed Protein A/G beads (Santa Cruz) and incubation at 4°C for 1 h. Then the beads were pelleted by centrifuging at 2500 rpm for 5 min at 4 °C following three washes with 1ml IP buffer for each. Then 40 μl of Laemmli Buffer was added before boiling at 95°C for 5 min. The supernatant was collected by centrifugation and subjected to SDS-PAGE and probed with appropriate antibodies.

2.13. SDS-PAGE electrophoresis and Western analysis

Proteins were separated by 8% SDS-PAGE gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad) in transfer buffer (48 mM Tris, 39 mM Glycine, 0.037% SDS, 20% methanol). The blots were blocked with 5% non fat dry milk in TBST buffer at room temperature for 1 h before incubating with mouse anti-HA (Sigma, 1:4000) or rabbit anti-*D*ATR-X (1:2000) overnight at 4°C. Then the blots were washed three times with TBST and incubated with HRP-conjugated anti-mouse or rabbit IgG (Promega) in a dilution of 1:10000 in 5% non-fat milk for 1 h at room temperature. After three times wash with TBST, signals were visualized with SuperSignal West Pico Substrate (Pierce).

RESULTS

3.1. Molecular genetics of *DATR-X*

The *DATR-X* gene, which corresponds to the Berkeley *Drosophila* Genome Project (BDGP) annotated sequence CG4548, is located at cytological interval 96E1-2 on the right arm of the third chromosome (Adams et al., 2000; http://www.flybase.org, Flybase ID: FBgn0039338). Database searches with genomic sequence flanking the EP(3)0635 P element confirmed that the transposon in the strain was inserted in the 5′-untranslated region of the gene, 470 bp from the start of the open reading frame (Fig. 12). EP(3)0635 carries a special P element containing a UAS element (Blair, 2003). This promoter faces outward at the end(s) of the P element, so that it can activate the expression of *DATR-X* under the control of Gal4 (Fig. 1; McGuire et al., 2004).

The *DATR-X* gene encodes a protein of 1311 amino acid residues. There are two isoforms of *DATR-X* and the second methionine start codon in the protein is localized at the amino acid 266 (Fig. 12; Bassett et al., 2008). The predicated *D*ATR-X protein contains two conserved domains, SNF2_N domain and Helicase conserved C-terminal domain (Helicase C) (SMART: http://smart.embl.de/; Letunic et al., 2004; Fig. 13A). The SNF2 family domain is found in proteins typically involved in processes requiring chromatin remodeling including, transcription regulation, DNA repair and recombination (Eisen et al., 1995). The Helicase conserved C-terminal domain possibly acts as an integral part of the helicase and is found in helicases and helicase related proteins. The DEAD-like helicase superfamily (DEXDc) consists of proteins involved in ATP-dependent, nucleic-acid unwinding (Jankowsky and Jankowsky, 2000).

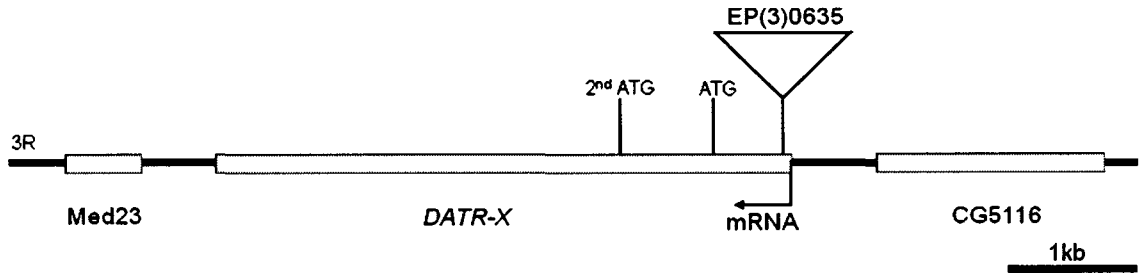


Figure 12. *DATR-X* genomic region. *DATR-X* is located on the right arm of the third chromosome at cytological region 96E1-2. Genes are shown as arrows and the start point of the mRNA and protein are indicated (ATG). The start codon at amino acid 266 is marked as 2nd ATG. The insertion site of EP(3)0635 is indicated.

3.2. *D*ATR-X homologues

The *D*ATR-X protein sequence showed 49.4% overall similarity to that of human ATR-X by alignment using the Clustal X algorithm (Thompson et al., 1997) (Fig. 13). ATR-X homologues are found in human, mouse, *C. elegans* and *Drosophila* (Fig. 13A) (Villard et al., 1999). Most of the sequence conservation between human and *D*ATR-X is confined to the helicase and SNF2 domains. The SNF2 domains of *C. elegans*, mouse and human ATR-X show 54.9%, 66.4% and 65.8% amino acid identity with that of *D*ATR-X, respectively (Fig. 13A). The ATPase domains of *C. elegans*, mouse and human ATR-X show 58.9%, 63% and 63% amino acid identity with that of *D*ATR-X, respectively (Fig. 13A). ATR-X has nucleosome-stimulated ATPase activity that is used to modify chromatin structure and that is a common site of patient mutations (Xue et al., 2003; Tang et al., 2004; Gibbons et al., 1995b). Mutations associated with mental retardation are in invariant residues and the amino acids at the sites of two human mutations, designated Mu K1600R and Ped23 D2035V, are conserved in *D*ATR-X (Fig. 13B).

There are some differences in the amino acid sequences of vertebrate and invertebrate ATR-X. Human and mouse ATR-X have a zinc finger DNA-binding domain consisting of three multicysteine zinc finger motifs (C_2-C_2) that is not present in invertebrate ATR-X proteins (Fig. 13A) (Cardoso et al., 2000). These zinc fingers are required for nuclear localization and DNA binding by ATR-X (Cardoso et al., 2000). Therefore, it is possible that invertebrate ATR-X proteins require site-specific DNA targeting in order to carry out their ATPase or other nuclear activities. The C-terminal of ATR-X has a region rich in poly-glutamine repeats (Fig. 13A) proposed to be involved in

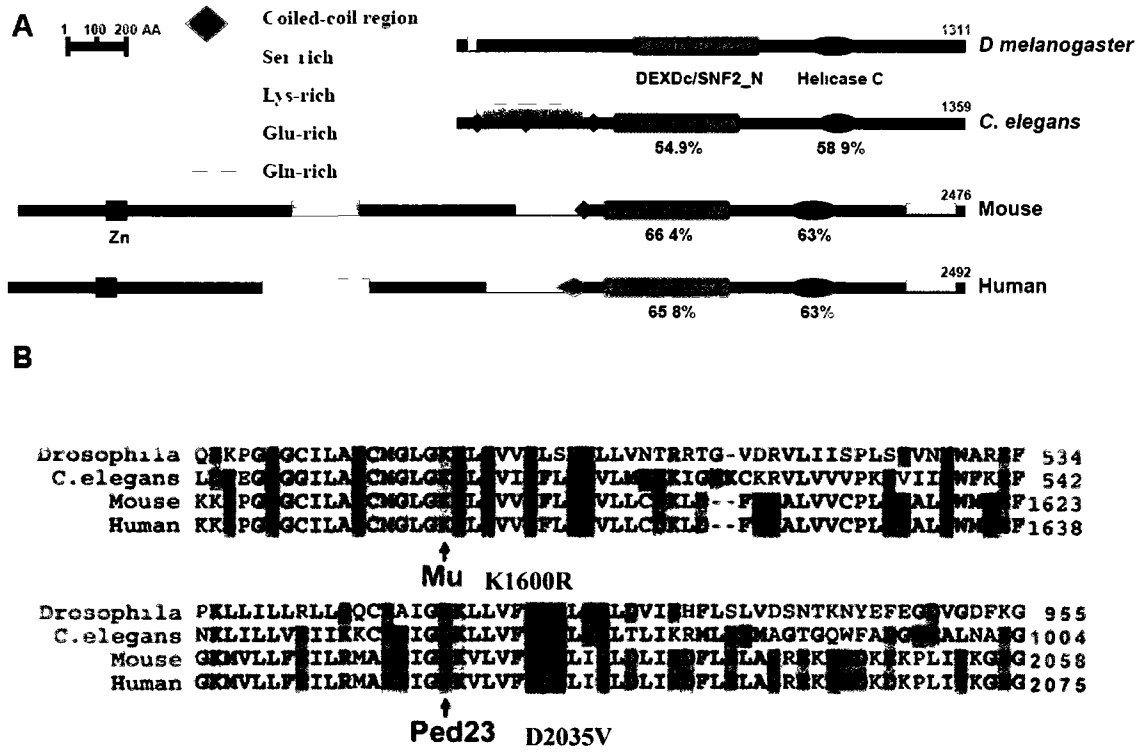


Figure 13. ATR-X homologues. (A) Comparison of *D*ATR-X protein domain architecture as predicted using SMART and PROSITE. Invertebrate ATR-X is truncated in comparison with mouse and human but shares high identities in the C-terminal amino acids with the latter proteins. Percent identity between the SNF2 and Helicase C domains of *C. elegans*, mouse and human are in comparison to those of *Drosophila melanogaster*. (B) Comparison of amino acid identity over the regions containing mutations found in the ATPase domain of ATR-X from human patients with ATR-X syndrome. The mutations include Mu K1600R and Ped23 D2035V both in highly conserved regions. (Sun et al., 2006).

protein-protein interactions that also is not present in either *D. melanogaster* or *C. elegans* ATR-X (Picketts et al., 1996).

Based on the protein sequence comparisons of *D*ATR-X and the *Drosophila* homologue of RAD54 (Kooistra et al., 1997), and these two from other species, a phylogenetic tree was created using the program ClustalX (Thompson et al., 1997; Fig. 14). The closest homologues of insect *D*ATR-X are the human ATR-X and mouse ATR-X proteins.

3.3. Expression patterns of *D*ATR-X during *Drosophila* embryogenesis

The expression patterns of *D*ATR-X during development of the *Drosophila* embryonic nervous system were sought by three approaches: 1) *in situ* hybridization to detect *DATR-X* transcripts; 2) *DATR-X*-lacZ reporter assays; 3) immunological detection using an antibody against *D*ATR-X raised in rabbits to monitor the distribution of *D*ATR-X protein.

3.3.1. *DATR-X* transcripts are present in *Drosophila* CNS during early stages of embryogenesis

Whole-mount control embryos were hybridized with digoxigenin-labelled anti-sense and sense *DATR-X* riboprobes (see Materials and Methods). Ectopic activation of EP(3)0635 was detected in *prd*-expressing stripes after hybridization of *prd*-Gal4/EP(3)0635 embryos with anti-sense *DATR-X* probes confirming that *DATR-X* is

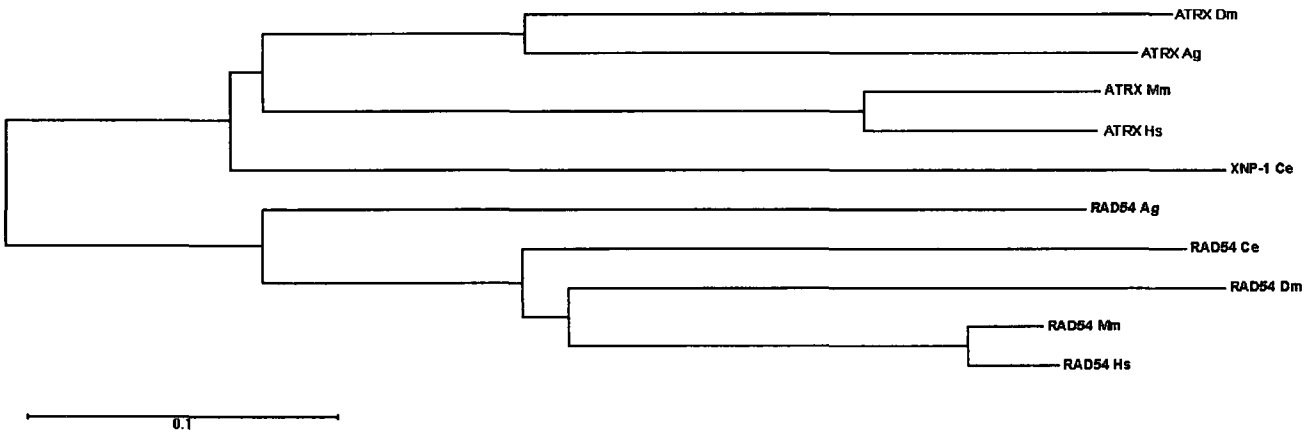


Figure 14. Schematic diagram of the human ATR-X gene. Boxes represent the exons; thin horizontal lines represent introns (not to scale). The PHD zinc finger motif and the helicase domain are noted, as are the P box (P) and a glutamine-rich region (Q). The positions of patient mutations are shown by circles. Filled circles represent mutations that cause protein truncation; open circles represent mutations that maintain the reading frame; deletions are indicated by horizontal lines. (Adapted and modified

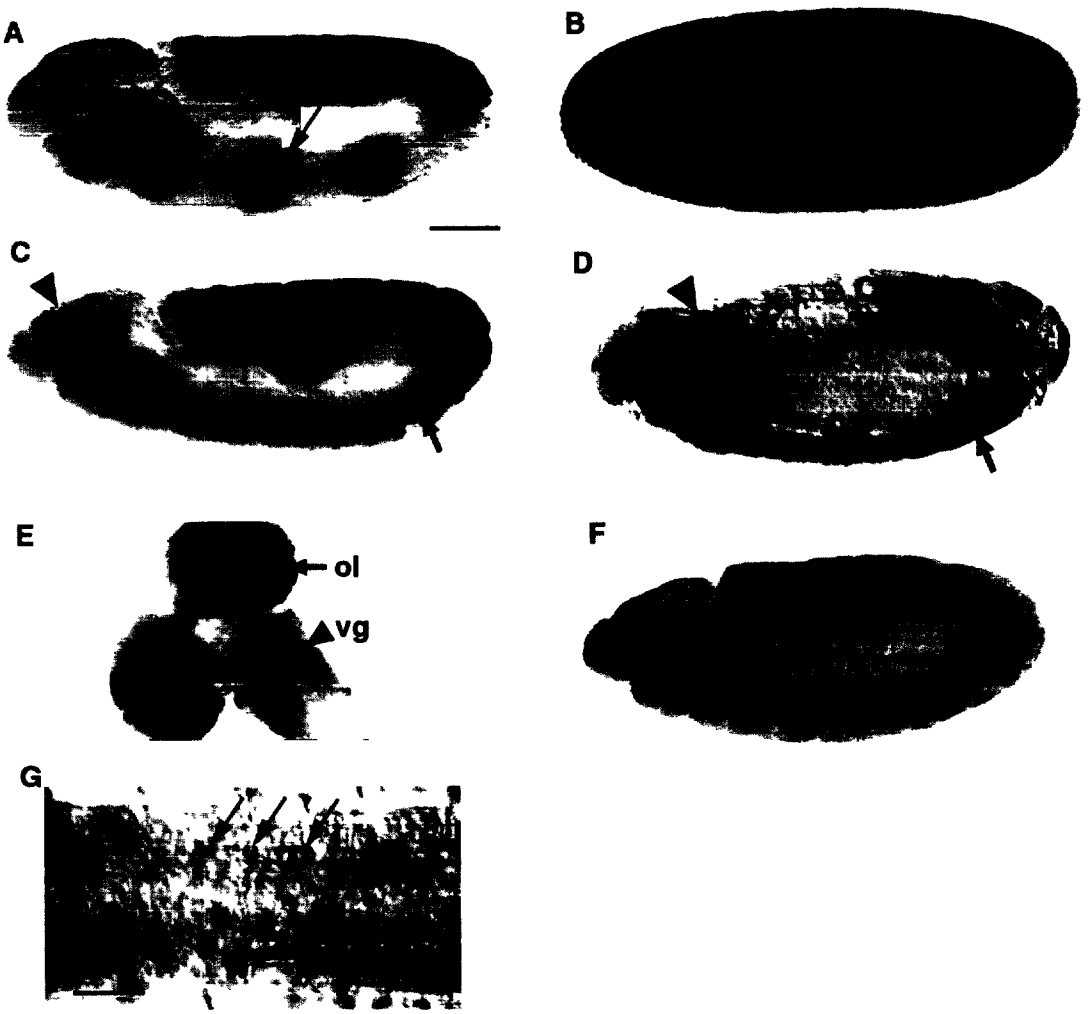

A
B
C
D
E
ol
vg
F
G

Figure 15. *DATR-X* expression in the CNS as shown by *in situ* hybridization to whole-mount embryos. (A) To validate the probe specificity, an embryo ectopically expressing *DATR-X* in ectodermal strips (arrow) of the *paired* gene was hybridized with a *DATR-X* riboprobe. Genotype, *prd*-Gal4/EP(3)0635. (B) *DATR-X* transcripts are present in cellular blastoderm embryos. (C) A 5.5-6 hr-old embryo showing *DATR-X* transcripts in supraesophageal ganglion (arrowhead) and neuroectoderm (arrow). (D) A stage 15 embryo showing elevated *DATR-X* transcripts in the supraesophageal ganglion (arrowhead) and ventral nerve cord (arrow). (E) Third instar larval CNS expressing *DATR-X* in the brain hemispheres (arrow) and ventral ganglion (arrowhead). *DATR-X* is expressed most strongly in the ventral region of the ganglion (vg, arrowhead) and optic lobe (ol, arrow). (F) Negative control. Anterior is to the left for all. (G) *DATR-X* expression in a stage 15 ventral nerve cord (ventral view). Strong expression is observed in cells lining the longitudinal connectives (arrows) and in lateral cells (arrowhead). (A), (C), (D) and (F) are in lateral view. Scale bar, (A-F) 200 μm; (G) 30 μm. (Sun et al., 2006).

induced by UAS and properly recognized by the probe (Fig. 15A). In wild-type embryos, *DATR-X* transcripts are present throughout cellular blastoderm embryos (Fig. 15B) and during gastrulation (Fig. 15C). In wild-type stage 15 embryos, *DATR-X* transcripts become enriched in the neuroectoderm and supraesophageal ganglion (brain) (Fig. 15D). In the mature ventral nerve cord, *DATR-X* mRNA is expressed in cells along the position of the longitudinal axon tracts (Fig. 15G, arrows) and in more laterally located cells (Fig. 15G, arrowhead). Similar CNS expression patterns were not observed in embryos hybridized with sense *DATR-X* riboprobes (Fig. 15F). *DATR-X* transcripts are also present throughout the brains of third instar wild-type larvae but are predominant in the optic lobe region (Fig. 15E).

3.3.2. *D*ATR-X-*lacZ*

To better understand the pattern of expression, a segment of 593 bp from the 5' *D*ATRX region surrounding the EP element insertion site was subcloned into the pCa*S*per plasmid which carries the *lacZ* reporter (Thummel et al., 1988) (Fig. 16). Then transgenic strains carrying pCa*S*per-*DATR-X-lacZ* plasmids were generated by P element mediated transformation (Genetic Services, Cambridge. MA) (Rubin and Spradling, 1983).

The embryos carrying *DATRX-lacZ* reporter gene were co-stained with anti-β-Galactosidase and the glial-specific anti-Repo antibody (Alfonso and Jones, 2002). Analysis by confocal microscopy revealed that *DATRX-lacZ* is expressed in both glial and neuronal lineages. In addition, many of the *DATRX-lacZ*-expressing glia are present in positions characteristic of the longitudinal glia (Fig. 17). Other *DATRX-lacZ*-

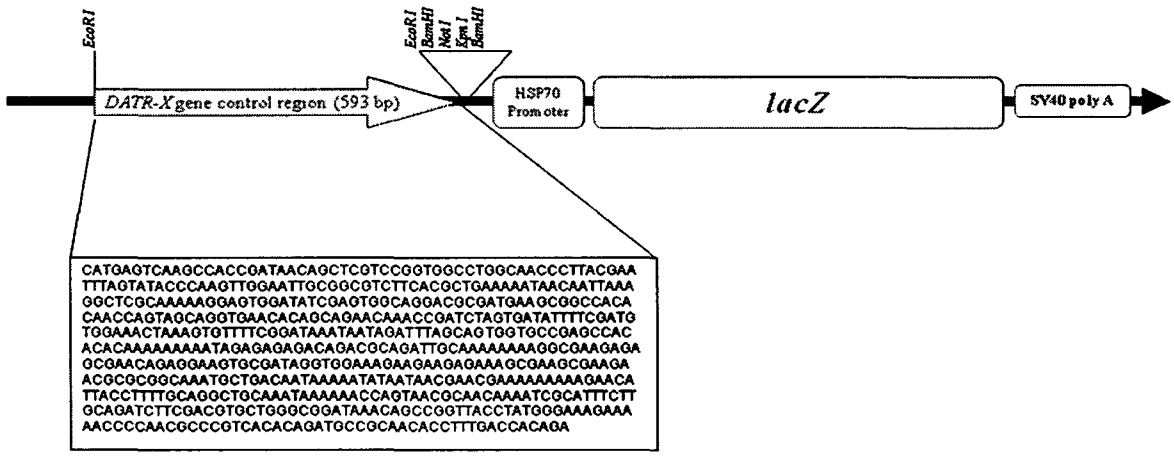


Figure 16. The *DATR-X-lacZ* construct. 593 bp from 5' *DATR-X* genomic sequence surrounding the EP element insertion site was subcloned into the pCaSper plasmid carrying the *lacZ* reporter.

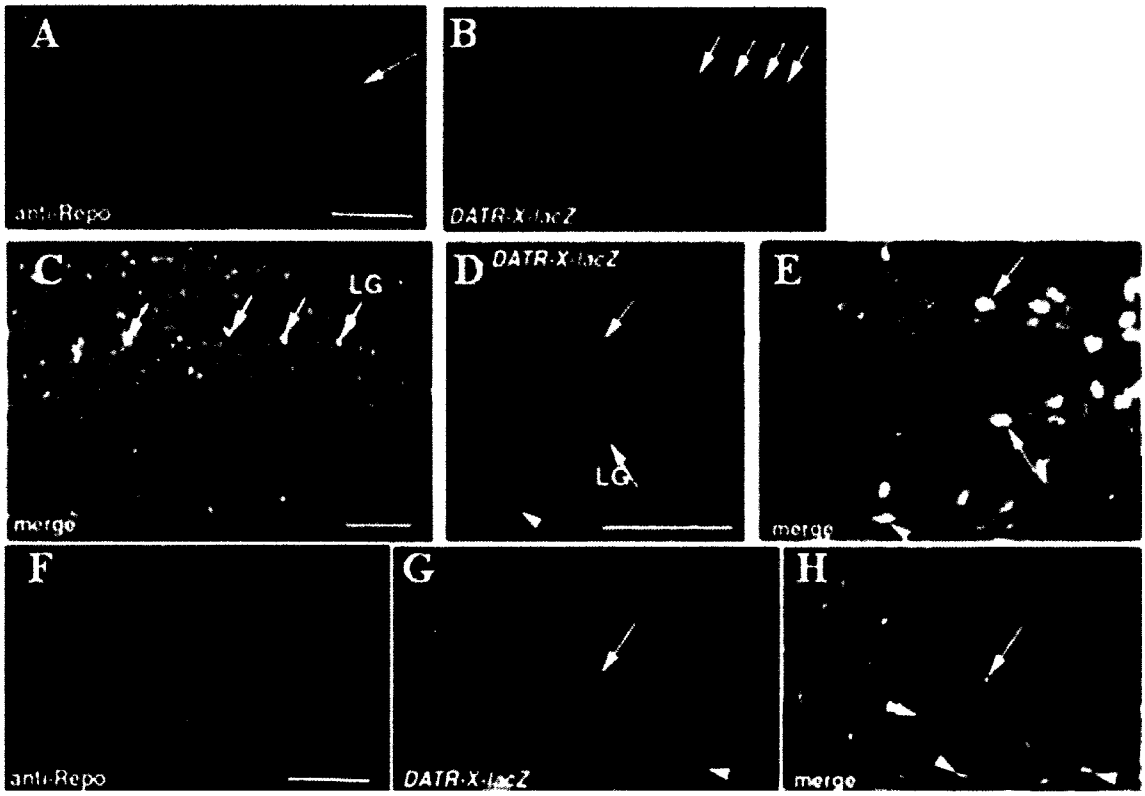


Figure 17. *DATR-X* expression in neurons and glia as revealed by *lacZ* assay.

Confocal images were captured from whole-mount stage 15 embryos stained with polyclonal rabbit anti-β-galactosidase (FITC-conjugated secondary antibody, green) and glial-specific anti-Repo (Texas Red-conjugated secondary antibody, red). (A-H) Focal planes of *DATR-X-lacZ* expression in the longitudinal glia (LG) (arrows). (G and H) Lateral views to show that *DATR-X-lacZ* is expressed in ventral glia (arrowhead) and the dorsal longitudinal glia (arrow). *DATR-X-lacZ* is also expressed in neurons (double arrowhead). Scale bar: 50 μm. (Sun et al., 2006).

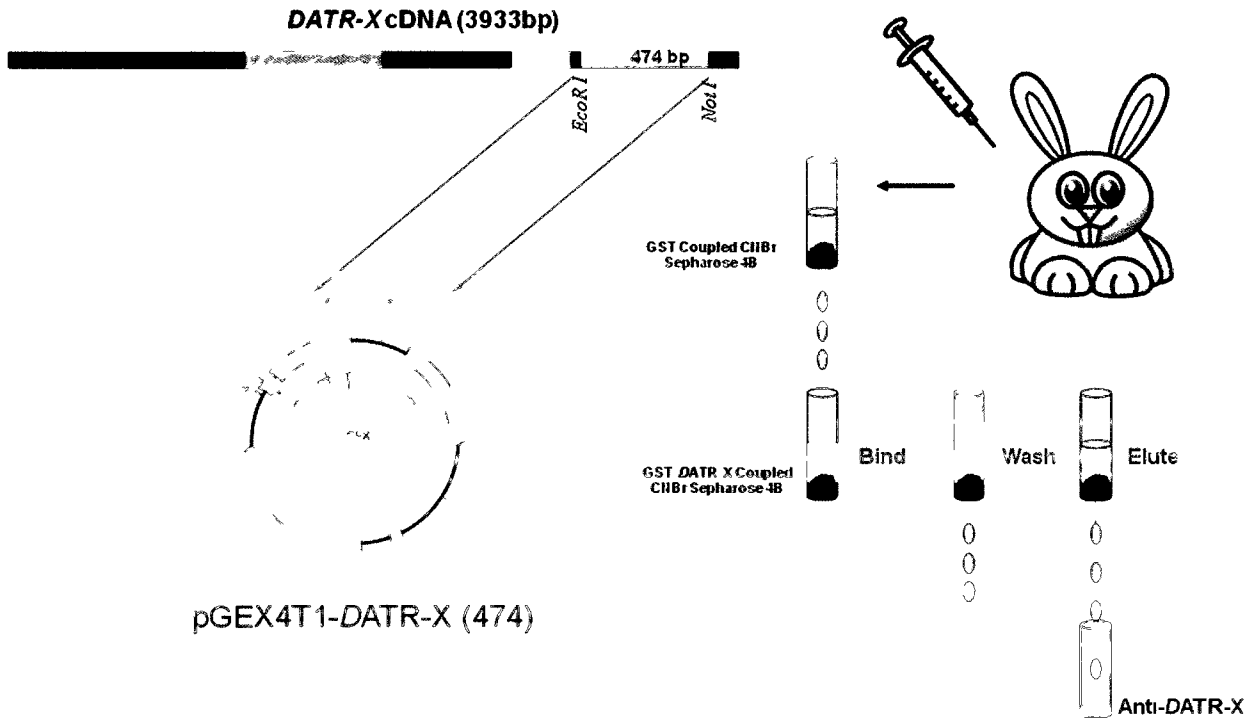
expressing glia occupy more lateral (Fig. 17, D and E, arrowheads) or ventral regions (Fig. 17, G and H).

3.3.3. *D*ATR-X is ubiquitously distributed and in cell nuclei during embryogenesis

In *Drosophila* embryos, the early ubiquitous expression of *DATR-X* transcripts and later enrichment in the CNS have been demonstrated (Fig. 15 and 17; Sun et al., 2006). To characterize the distribution pattern of *D*ATR-X protein, a polyclonal antibody against *D*ATR-X was raised in rabbits and affinity purified (Fig. 18A). The antigenic region of anti-*D*ATR-X is localized to the C-terminus and shared by both long and short isoforms (Bassett et al., 2008). Both versions of *D*ATR-X proteins were detected by anti-*D*ATR-X by Western analysis of protein extracts from the untransfected *Drosophila* S2 cells (Fig. 18B) and those transfected with HA-tagged *D*ATR-X (Fig. 18C). Specificity of the antibody was also confirmed in flies ectopically expressing *D*ATR-X (*prd*-Gal4/UAS-*D*ATR-X-HA) by searching for the unique pair-rule pattern of segmentally repeating stripes (Fig. 32).

Given the working hypothesis that the antibody is recognizing *D*ATR-X, immunostaining of wild-type *Drosophila* embryos with the polyclonal anti-*D*ATR-X antibody showed that *D*ATR-X protein is widely distributed in cellular blastoderm embryos (Fig. 19A) and later is plentiful throughout the CNS (Fig. 19B and C, arrow), similar to the distribution of *DATR-X* transcripts reported previously (Fig. 15; Sun et al., 2006). Co-staining of wild-type embryos with anti-*D*ATR-X and the nuclear marker, propidium iodide (PI), confirmed that *D*ATR-X is confined to cell nuclei (Fig. 19A-A″, B-B″ and C-C″).

A


DATR-X cDNA (3933bp)
474 bp
EcoR I
Not I
pGEX4T1-DATR-X (474)
GST Coupled CIBr Sepharose 4B
GST DATR X Coupled CIBr Sepharose 4B
Bind
Wash
Elute
Anti-DATR-X

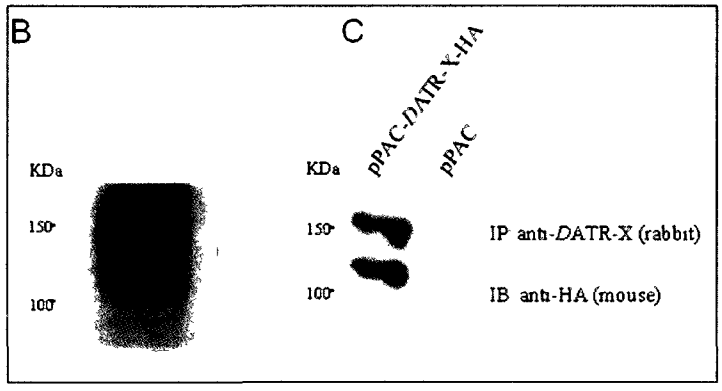

B
KDa
150
100
C
pPAC-DATR-X-HA
pPAC
KDa
150
100
IP anti-DATR-X (rabbit)
IB anti-HA (mouse)

Figure 18. Generation of polyclonal antibody against *D*ATR-X. (A) Scheme of the generation of the polyclonal *D*ATR-X antibody. To produce antibody against *D*ATR-X, a 474 bp (3331-3804) fragment corresponding to a region of the putative *D*ATR-X protein without any conservation to other *Drosophila* proteins was amplified by PCR and subcloned in frame into the *EcoR I*/*Xho I* sites of pGEX-4T1 (Amersham). Total 4 mg GST-*D*ATR-X fusion protein was extracted using the Glutathione Sepharose 4B beads (Amersham) and injected into two rabbits to produce the GST-*D*ATR-X antiserum (Cedarlane, ON). Affinity purification columns of the antiserum were generated by coupling GST or GST-*D*ATR-X fusion proteins to CNBr activated sepharose (Amersham). The antiserum against GST protein was removed by first absorbing against GST-affinity column. The effluent is then loaded onto a GST-*D*ATR-X affinity column. Eluted fractions containing antibody were combined and concentrated by centrifuging with an Amicon Ultra Centrifugal Filter (Millipore). (B-C) The rabbit anti-*D*ATR-X was examined by Western analysis and immunoprecipitation. Non-transfected *Drosophila* S2 cells were lysed for Western analysis and blotted by using rabbit anti-*D*ATR-X (B) or S2 cells transfected with pPAC-*D*ATR-X-HA or empty pPAC vector were lysed for immunoprecipitation (C). Proteins were pulled down with rabbit anti-*D*ATR-X and probed with mouse anti-HA. The antigenic region of anti-*D*ATR-X was C-terminally localized and both long and short forms of *D*ATR-X were recognized as shown (C). IP, immunoprecipitation; IB, immunoblotting.

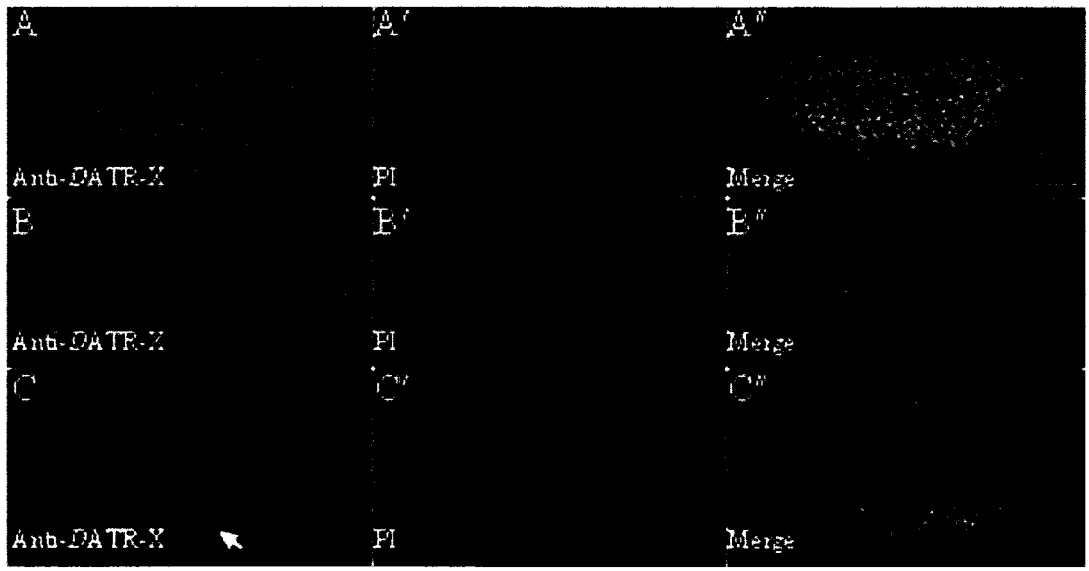


Figure 19. *D*ATR-X is a ubiquitous nuclear protein. Wild type *Drosophila* embryos were stained with both polyclonal anti-*D*ATR-X (green) (A, B, C) and the nuclear marker propidium iodide (PI) (red) (A′, B′ and C′). The overlapped fluorescence (A″, B″ and C″) revealed the nuclear localization of *D*ATR-X. Anterior is left for all. (A) A blastula stage embryo showing high levels of *D*ATR-X ubiquitously distributed. (B) Ventral view of an embryo with CNS structure becoming more defined during embryogenesis. (C) The lateral view of (B) with dorsal up. Arrow points to the CNS VNC where *D*ATR-X protein is abundant. Scale bar, 50 μm.

3.4. Gain of function assay

Co-expression of *jing* and *DATR-X* strongly affected adult neuronal development (Fig. 9; Sun et al., 2006). Since *DATR-X* and *jing* were both present in the embryonic CNS (Fig. 15, 17 and 19; Sedaghat et al., 2002; Sonnenfeld., 2004), it was necessary to determine whether their co-expression had similar effects in embryonic neurons. Using the Gal4/UAS binary system which provides a useful tool to target gene expression in a controlled manner (Fig. 1; Brand and Perrimon, 1993), *jing* and *DATR-X* were expressed in all postmitotic neurons with *Elav*-GAL4 (Robinow and White, 1988; Yao et al., 1994). Their CNS axons were monitored with antibody BP102 (Carney et al., 1997). In the wild-type VNC, axon scaffolds are organized in an orthogonal manner as a pair of bilaterally symmetrical longitudinal axon tracts are connected by a pair of commissural axon tracts within each segment (Fig. 3 and 20A) (Sun et al., 2006). Expression of either *jing* or *DATR-X* alone resulted in subtle defects in the CNS axon scaffold as observed with BP102 staining. These defects included thinner longitudinal connectives (Fig. 20, B and C, arrowheads) and reduced spacing between the anterior and posterior commissures (Fig. 20, B and C, arrows) (Sun et al., 2006). Stronger effects were observed after expression of two copies of either *jing* (Fig. 20E) or *DATR-X* (Fig. 20F) where commissural axons were not properly separated (Fig. 20, E and F, arrows) (Sun et al., 2006).

Pan-neural co-expression of *jing* and *DATR-X* resulted in more severe defects in axonal patterning than expression of one or two copies of either transgene alone. An average of 65% of segments had no longitudinal connectives after *jing* and *DATR-X* co-expression (Fig. 20D, arrowhead; Fig. 20G) in comparison with expression of two copies

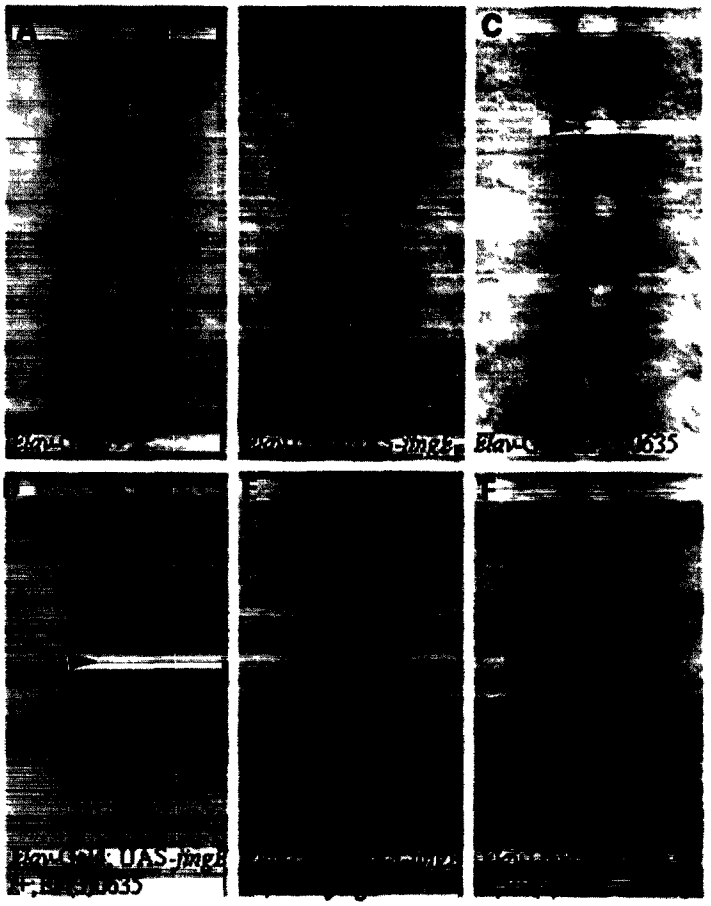

C
G
Percentage of segments with longitudinal loss

Figure 20. Pan-neural expression of *DATR-X* and *jing* in embryonic CNS neurons synergistically affects axon patterning. Ventral views of the ventral nerve cord (VNC) of stage 15 embryos stained with BP102 (A-F) and shown with anterior up. (A) A control embryo heterozygous for *Elav*-Gal4 and w^{1118}. Thick longitudinal connectives connect each hemisegment (arrowhead). The arrow denotes the space between the anterior and posterior commissural axons. (B and C) After over-expression of *jing* (B) or *DATR-X* (C) in post-mitotic CNS neurons, longitudinal axons (arrowheads) are very thin and the space between commissural axons is smaller (arrows). (D) Co-expression of one copy of EP(3)0635 and UAS-*jingE* in post-mitotic neurons has synergistic effects. Note the absence of longitudinal connectives (arrowhead). (E and F) Additive effects of over-expressing two copies of *UAS-jingE* (E) or EP(3)0635 (F). Note that commissural axons are often not separated (arrows) and longitudinal connectives are very thin (arrowheads). (G) Quantitative analysis of longitudinal connective formation. Scale bars, 50 μm. (Adapted from Sun et al., 2006).

of UAS-*jing* (5% of segments, n=80) and EP(3)0635 (18% of segments, n= 55) (Fig. 20G) (Sun et al., 2006). Therefore, *jing* and *DATR-X* co-expression has synergistic effects specifically in embryonic CNS neurons.

3.5. Loss of function assay

To characterize the requirement for *D*ATR-X function in the *Drosophila* embryonic CNS, two approaches were taken for the loss-of-function assay: 1) phenotypic analysis of homozygous *DATR-X* mutants generated by imprecise P-element excision and 2) cell-specific *DATR-X* disruption conditionally through RNA interference (RNAi).

3.5.1. Mutant analysis of *DATR-X* function in the embryonic CNS

The requirement for regulation of proper *DATR-X* levels has been demonstrated by the effects of ectopic mis-expression of *DATR-X* on *Drosophila* embryonic CNS (Fig. 20; Sun et al., 2006). To understand the requirement for *D*ATR-X function in the *Drosophila* embryonic CNS, an analysis of homozygous *DATR-X* mutants was carried out. For this purpose, fly strains were obtained that carried *DATR-X* deletions generated through imprecise P-element excision (Bassett et al., 2008). P elements are sequences of DNA capable of removing themselves from the chromosome and reinserting elsewhere only in the presence of the enzyme transposase (Δ2, 3), which sometimes excises some of the adjacent chromosomal DNA in addition to the P element itself, leaving a deficiency (Voelker et al., 1984; Salz et al., 1987). With this procedure, EP(3)0635 was used to generate *DATR-X* excision mutants (Bassett et al., 2008). The obtained excision fly lines, *DATR-X1*, *DATR-X2* and *DATR-X3* expressed zero or reduced levels of *DATR-X* (see

Materials and Methods; Bassett et al., 2008), allowing for functional analysis of *DATR-X* in the *Drosophila* embryonic CNS.

The axons or glial biology of homozygous *DATR-X* mutations were monitored with individual antibodies by image scanning. In the wild type embryonic CNS, Fas II-expressing longitudinal axons are continuous and stay ipsilateral (Fig. 21A). In all homozygous *DATR-X* mutants there were breaks in the longitudinal axon bundles (Fig. 21, C and D, arrows), misrouting of axons across the midline (Fig. 21,B, C and D, arrowheads) and fusion of the axons into a single fascicle (Fig. 21, B and E, arrowheads). The two deficiency lines (*DATR-X1* and *DATR-X*^{Df}) showed higher penetrance and expressivity of axon defects than *DATR-X3*, which lacks the long *D*ATR-X isoform, and *DATR-X2*, which expresses reduced level of both *DATR-X* isoforms (Table 2).

In wild type embryos, longitudinal glia (LG) expressing Repo (stained by 8D12 anti-Repo) are arranged in two rows on either side of the CNS midline (Fig. 21F; Jacobs and Goodman, 1989). However, in homozygous *DATR-X* mutants, gaps were found in these LG rows (Fig. 21, G-J, arrows) and some LG migrated inappropriately to the midline (Fig. 21, G-J, arrowheads), revealing that *D*ATR-X is required for the positioning of these cells. Similarly, the penetrance and expressivity in both *D*ATR-X deficient populations were always higher than in *DATR-X2* and *DATR-X3* except that *DATR-X2* embryos showed higher expressivity (6.4%) of mispostioned glia than in *DATR-X1* (5.1%) (Table 3). It was noted that the breaks of the longitudinal connectives sometimes occurred where the LG were missing (Fig. 21, Q-T) and misrouting axons crossed the midline where LGs were mispositioned (Fig. 21, Q, R and T, arrowheads).

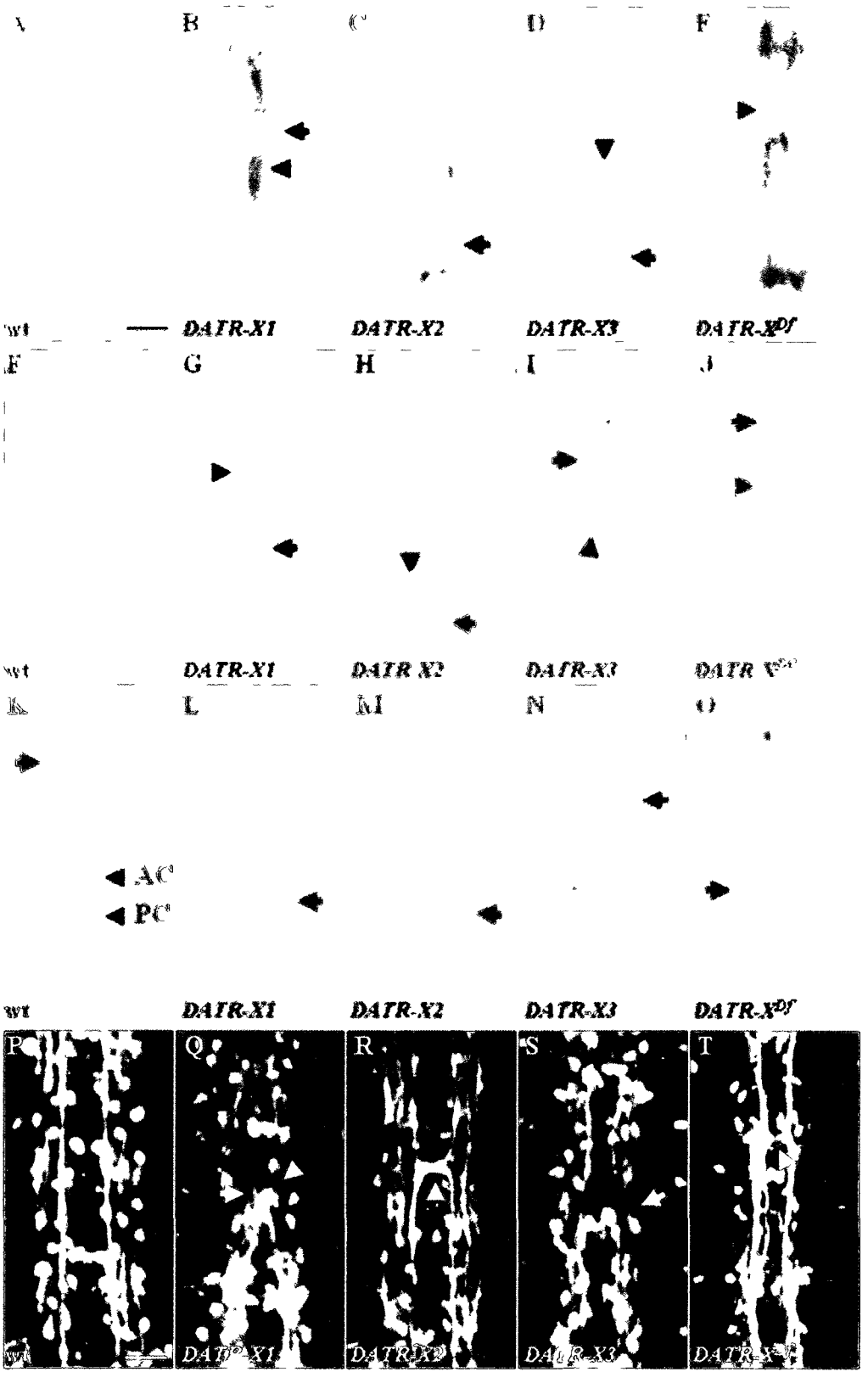

wt
DATR-X1
DATR-X2
DATR-X3
DATR-Xdf
AC
PC

Figure 21. Analysis of longitudinal glia and axons in homozygous *DATR-X* mutants. Embryos were stained with the longitudinal axon marker 1D4 anti-Fas II (A-E and P-T), the glial marker anti-Repo (F-J and P-T) or the CNS axon marker BP102 (K-O). For double staining (P-T), a rabbit anti-Repo (green) was applied to distinguish the axonal marker 1D4 (red) from the glial (Repo) labelling. Ventral views of whole mount wild type and mutant stage 15 embryos are shown with anterior up for all. Scale bar, 10 μm. *DATR-X* homozygous mutant embryos are associated with the longitudinal bundles inappropriately crossing the midline (B-E, arrowheads) and gaps in connectivity (B-D, arrows). Gaps were also found in longitudinal glial (LG) tracts (G-J, arrows) and some LG abnormally migrated to the midline (G-J, arrowheads). Consistently, breaks were also found in follower CNS axons labeled with BP102 (L-O, arrows). (K) Wild-type embryo. Longitudinal connectives (arrow) are positioned on the either side of the midline. Within each segment, anterior and posterior commissural axons (denoted as AC and PC, arrowheads) cross the midline and connect two sides. Double-labeling revealed that breaks in longitudinal axons did not always occur at the places where LGs were missing (Q, S, arrows) and misrouting axons crossed the midline where LGs were mis-positioned (Q, R, T, arrowheads).

Table 2. Quantification of longitudinal connectivity in homozygous *DATR-X* mutant embryos

	Penetrance			expressivity		
Genotype	broken	midline crossings	n	broken	midline crossings	n
w^{1118}	0	0	105	0	0	679
DATR-X1	22.3%	50.5%	103	5.6%	15.0%	821
DATR-X2	14.9%	46.5%	101	2.4%	11.0%	827
DATR-X3	23.4%	27.0%	111	3.7%	6.8%	842
$DATR\text{-}X^{Df}$	36.6%	58.4%	101	10.4%	18.2%	791

Longitudinal axons were stained with 1D4 anti Fas II in embryos of stage 15/16. The samples were counted as "broken" when the breakage was found along the longitudinal axons, and as "midline crossings" when these axons misrouted across the midline.

Penetrance is indicated as the percentage of embryos showing phenotype out of the total number of embryos counted. Expressivity is indicated as the percentage of abnormal segments out of the total number of segments examined.

Table 3. Quantification of longitudinal glial positioning in *DATR-X* mutant embryos

	Penetrance			expressivity		
Genotype	gap	cross	n	gap	cross	n
w^{1118}	0	0	110	0	0	851
DATR-X1	21.3%	48.1%	108	5.2%	5.1%	906
DATR-X2	14.3%	44.8 %	105	3.2%	6.4%	872
DATR-X3	14.3%	26.8%	112	1.8%	4.1%	892
$DATR\text{-}X^{Df}$	35.9%	57.3%	103	13.4%	8.6%	848

Glia were stained with anti-Repo in embryos of stage 15/16. "Gap" stands for the broken glia line and "cross" refers to the migration of glia towards the midline.

Penetrance is indicated as the percentage of embryos showing phenotype out of the total number of embryos counted. Expressivity is indicated as the percentage of abnormal segments out of the total number of segments examined.

Follower axons that use pioneer axons and LG for their guidance were also examined using antibody BP102 which labels many CNS axons except pioneer axons after stage 14 (Hidalgo and Booth, 2000). Consistently, subsequent fasciculation of the follower axons was abnormal as revealed by the loss or thinning of the axon bundles in the embryos homozygous for all four *DATR-X* mutations (Fig. 21, L-O, arrows; Table 4). However, disrupted follower axons can not reflect which would be the primary contributing factors, since both the pioneer axons and the LG are equally important for axon scaffold formation and both showed abnormality here. Therefore the axon extension of pioneer neurons were examined in embryos of stage 12 at which pioneer neurons initiate axon extension and LG just move to the position of the future connectives prior to the axonal growth (Hidalgo, 2003).

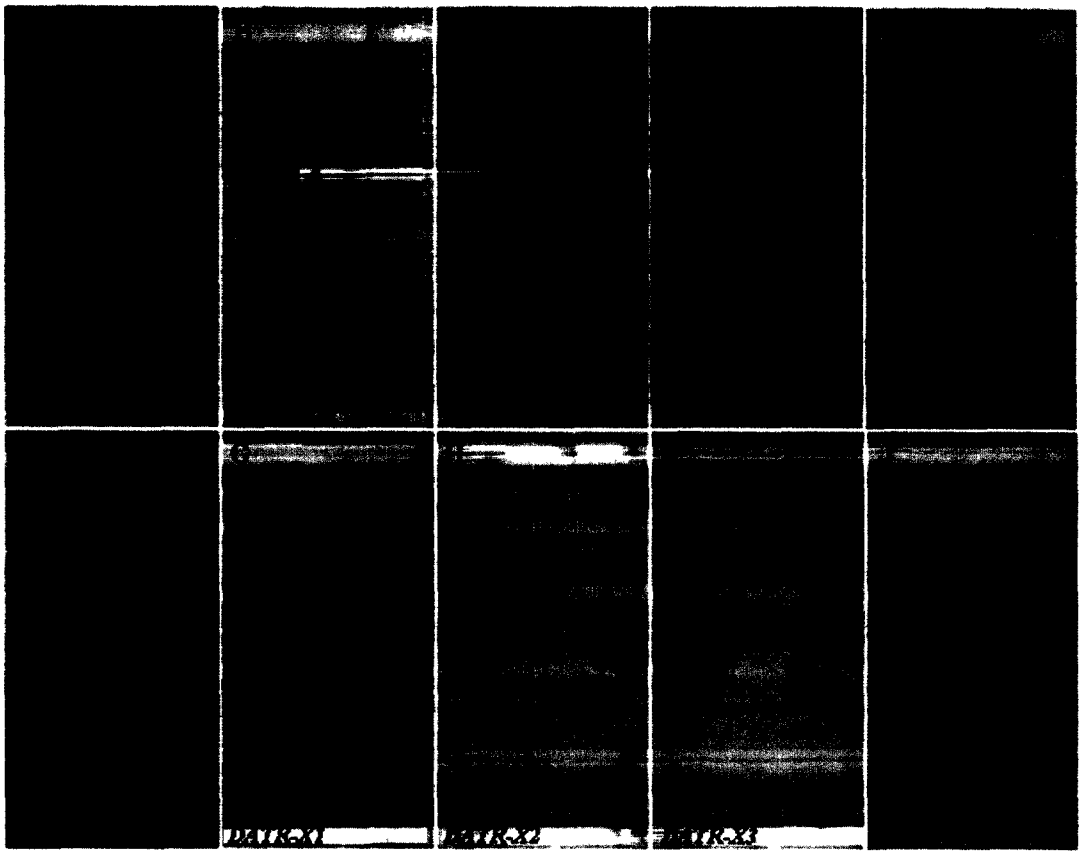
At early stage 12, pioneer pCC neurons extend their growth cones anteriorly (Fig. 22, arrow; Grenningloh et al., 1991). However, this extension was missing or stalled (Fig. 22, B-E, arrows; Table 5) or even directed towards the midline instead of anteriorly (Fig. 22B, arrowhead) in homozygous *DATR-X* mutant embryos, suggesting that *DATR-X* is required for the proper initial extension of pCC axon growth cones and the midline repulsion of pCC axon growth cones that were able to extend. At this stage, the longitudinal glia are arrayed in a characteristic diamond pattern on either side of the midline in each hemisegment (Fig. 22F, arrow; Halter et al., 1995). However, the LG were mispositioned in homozygous *DATR-X* mutant embryos (Fig. 22G-J). Some of these cells migrated toward the CNS midline, suggesting a loss of repulsion (Fig. 22J). These data revealed that abnormal axon behaviour started as early as the pioneering stage (Fig. 20B-E) and that glia mis-positioning occurred during axon pioneering (Fig. 22G-J)

Table 4. Quantification of follower axons in *DATR-X* mutant embryos

	Penetrance			expressivity		
Genotype	broken	thin	n	broken	thin	n
w^{1118}	0	0	110	0	0	764
DATR-X1	37.5%	34.6%	104	9.48%	11.2%	907
DATR-X2	10.8%	24.5%	102	2.6%	7.2%	897
DATR-X3	27.2%	31.1%	103	5.2%	7.0%	981
$DATR\text{-}X^{Df}$	45.7%	41.0%	105	12.1%	9.8%	990

Follower axons were stained with BP102 in embryos of stage 15/16. The abnormality of these axons was scored as "broken" when the longitudinal axons appeared to be missing and "thin" when immunoreactivity along longitudinal axons was reduced.

Penetrance is indicated as the percentage of embryos showing phenotype out of the total number of embryos counted. Expressivity is indicated as the percentage of abnormal segments out of the total number of segments examined.



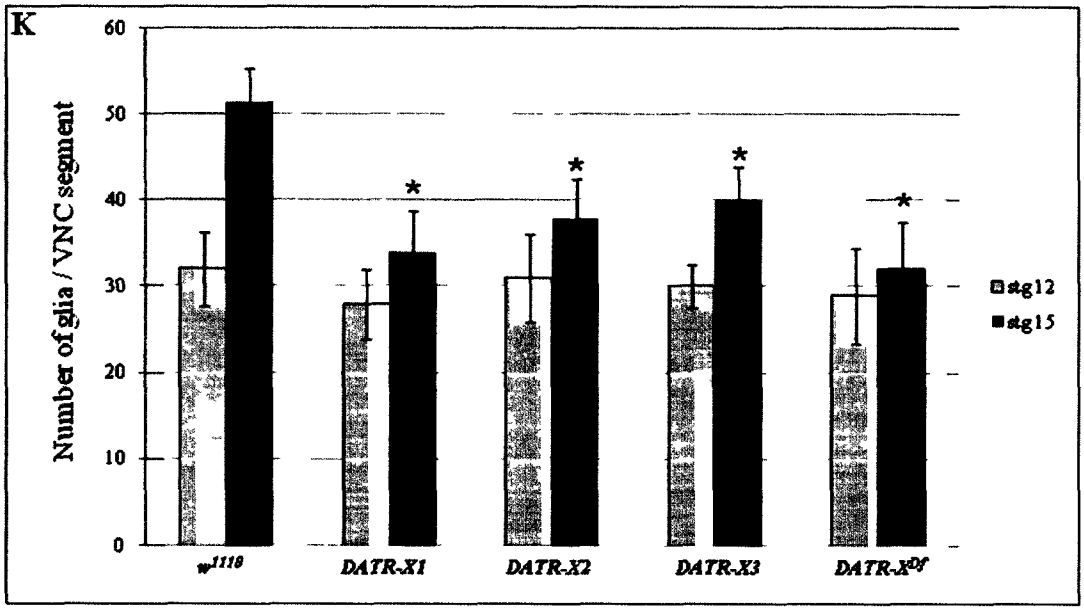

K
Number of glia / VNC segment
60
50
40
30
20
10
0
*
*
*
*
stg12
stg15
w1118
DATR-X1
DATR-X2
DATR-X3
DATR-XDf

Figure 22. CNS phenotypes in *D*ATRX mutants (II). Stage 12 embryos were stained with the longitudinal axon marker 1D4 anti-Fas II (A-E) or the glial marker anti-Repo (F-J). Ventral views of wild type (A and F), *DATR-X1* (B and G), *DATR-X2* (C and H), *DATR-X3* (D and I), and *DATR-X*^{Df} (E and J) are shown. Anterior is up for all. (A) A single growth cone from the pCC pioneer neuron extends anteriorly in wild type embryos. (B-E) pCC anterior extensions were missing (arrows) or diverted to the midline (arrowhead). (F) In wild type embryos, the LG are positioned on either side of the midline in each hemisegment (arrow). (G-J) In homozygous *DATR-X* mutants, the glia were not arranged in a regular pattern. In some embryos, longitudinal glia were missing (G, arrowhead) or migrated inappropriately to the midline (J, arrow). (K) Quantitative analysis of glia during stages 12 and 15 in *D*ATR-X mutants. Differences in the number of glia in each sample were evaluated relative to the corresponding stages of control. (* $P<0.001$, Student's *t*-test, $n\geq780$). Scale bar, 10 μm.

Table 5. Quantification of longitudinal axon phenotypes in *DATR-X* mutant embryos of stage 12

	Penetrance			expressivity		
Genotype	stall	cross	n	stall	cross	n
w^{1118}	0.8%	0	125	0.1%	0	781
DATR-X1	60.0%	10.9%	110	14.8%	1.6%	790
DATR-X2	41.0%	15.2%	105	8.2%	2.9%	788
DATR-X3	28.7%	27.8%	108	5.7%	2.8%	791
$DATR\text{-}X^{Df}$	32.7%	15.0%	113	15.4%	2.2%	772

Longitudinal axons were stained with 1D4 anti Fas II. The samples were counted as "stall" when projection is missing in at least one neuron per segment. Penetrance is indicated as the percentage of embryos showing an abnormal phenotype out of the total number of embryos counted. Expressivity is shown as the percentage of the number of abnormal segments out of the total number of segments counted.

in homozygous *DATR-X* mutant flies. Later during wild-type embryogenesis, Robo is expressed only in axons, which restricts glial movement in Robo-independent mechanisms including axon contact and trophic support (Kinrade et al., 2001). In the mature nerve cord, LG were mis-positioned to the CNS midline of *DATR-X* mutants (Fig. 21G-J). Consistently, longitudinal axons were shifted to the CNS midline as well in these embryos (Fig. 21B-E).

Glial-specific Repo expression can be detected from early stage 11 onwards (Halter et al., 1995). The number of Repo-expressing glial cells increases during the CNS development (Halter et al., 1995). These *DATR-X* mutants showed the wild-type numbers of glia at the stage 12 (Fig. 22K), suggesting that *D*ATR-X is required for proper glial differentiation and not for the division or specification of these cells. However, total glial cell numbers significantly decreased at stage 15 in homozygous *DATR-X* mutant embryos (Fig. 22K), suggesting that *D*ATR-X is required for glial survival during late embryogenesis.

3.5.2. RNA interference (RNAi)

Widespread expression of *DATR-X* necessitates a directed approach to analyse its function. We therefore choose to explore the neural and glial-specific roles of *DATR-X* in regulating axon formation in the *Drosophila* embryonic CNS. To this end, transgenic flies carrying UAS-*DATR-X* RNAi were generated to allow for the conditional silencing of post-transcriptional sequences of *DATR-X* in a spatially and temporally controlled manner (Fig. 23). A 657 base pair sequence from non-conserved regions of *DATR-X* was

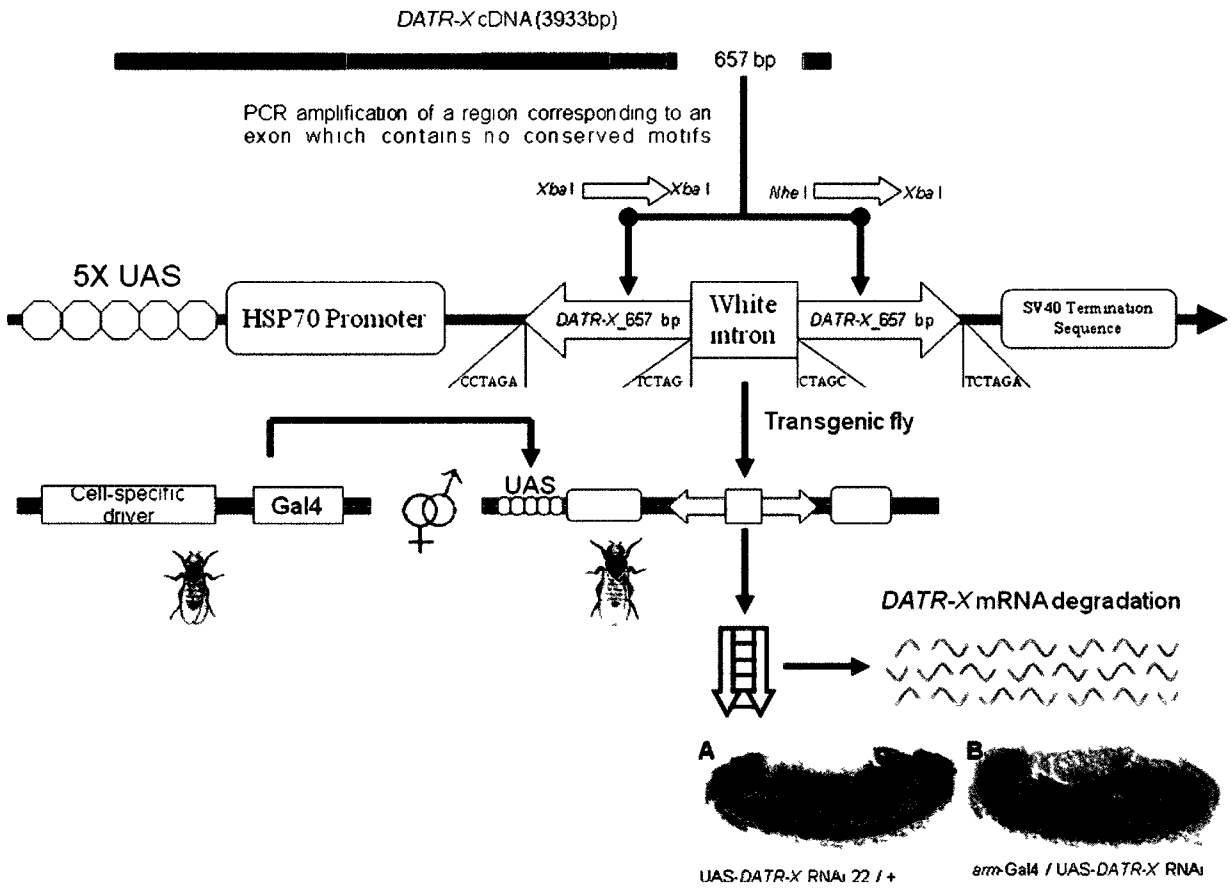


Figure 23. Generation of the *DATR-X* RNA interference (RNAi) transgene. A 657 bp region from *DATR-X* cDNA was selected which corresponded to non-conserved sequence. The segment was cloned twice with inverted orientation before and after the 74 bp *white* intron within the pWIZ vector (Lee and Carthew, 2003). Then the transgenic flies carrying UAS-*DATR-X* dsRNA were generated through embryonic injection. When crossing UAS-*DATR-X* RNAi with fly carrying certain cell-specific Gal4 drivers, the *D*ATR-X can be knocked down in specific fly CNS cell lineages. Knockdown of *DATR-X* expression by RNAi was examined by *in-situ* hybridization. (A) *DATR-X* expression in a control embryo. (B) An embryo expressing the UAS-*DATR-X* RNAi transgene as driven by *arm*-Gal4 (Ahmad and Henikoff, 2001).

subcloned into a pUAST derivative plasmid to produce intron-spliced hairpin RNA (Lee and Carthew, 2003) corresponding to the *DATR-X* gene. The Gal4/UAS system (Brand and Perrimon, 1993) was then used to allow hairpin RNA to conditionally down-regulate *DATR-X* in specific cell lineages which was confirmed by *in situ* hybridization (Fig. 23). Similar procedures were performed to generate UAS-*jing* RNAi transgenic lines (Sun et al., 2006).

3.5.2.1. Neuronal-specific functions of *jing* and *DATR-X* are required for repulsion of longitudinal axons and glia from the CNS midline

In order to discern the neuronal and glial contributions of each gene product to axon scaffold formation, *DATR-X* and *jing* expression was knocked down using conditional RNAi (Hammond et al., 2001; Lee and Carthew, 2003).

Neuronal-specific knock-down of *jing* and *DATR-X* was directed by the *Elav*-Gal4 driver and the longitudinal connective formation was analyzed by staining the embryos with monoclonal antibody 1D4 anti-Fas II (Hummel et al., 2000). In these mutant embryos, FasII-positive longitudinal axons aberrantly cross the CNS midline in stage 16 embryos (Figure 24, B and C, arrowheads) (Sun et al., 2006). In addition, there are breaks in the longitudinal connectives suggesting axon outgrowth defects (Fig. 24, B and C, arrows) (Sun et al., 2006). In embryos with simultaneous neuronal knockdown of *jing* and *DATR-X* all FasII-positive lateral fascicles fuse into a single tract at the CNS midline (Fig. 24D) (Sun et al., 2006). These results establish an autonomous neuronal requirement for *jing* and *DATR-X* function in the outgrowth and lateral positioning of longitudinal axons and a genetic synergy during this process.

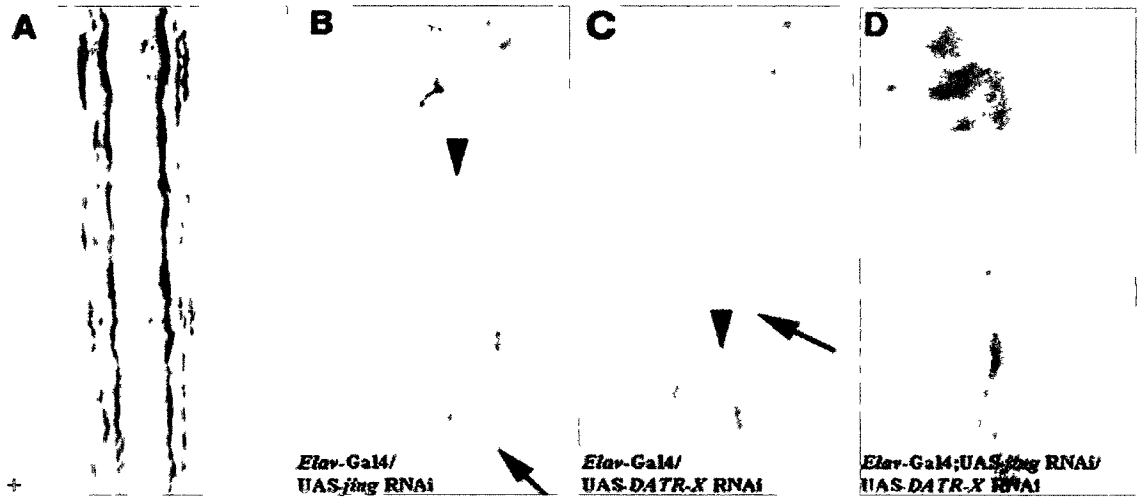


Figure 24. *DATR-X* and *jing* functions are required specifically in CNS neurons for longitudinal connective formation. *DATR-X* and *jing* RNAi transgene expression was driven in neurons by *Elav*-Gal4. Shown are close-up views of the VNC of whole-mount stage16 embryos stained with anti-Fasciclin II monoclonal antibody 1D4. (A) In wild-type embryos, three longitudinal fascicles are clearly delineated. (B-D) In *jing* (B) and *DATR-X* (C) neural RNAi mutants, longitudinal fascicles misroute across the midline (arrowheads) and are broken (arrows). (D) After co-expression of *jing* and *D*ATR-X RNAi transgenes in neurons, longitudinal fascicles fuse into one tract at the midline. Scale bars, 50 μm. (Adapted from Sun et al., 2006).

Consistent with these results, pan-neural knockdown of *jing* or *DATR-X* was associated with high levels of Robo at the CNS midline during both stage 12 and stage 16 (Sun et al., 2006). The phenotypes were variable as in the most severe case all Robo-positive axons were present at the midline and in less severe cases midline Robo was observed in fewer hemisegments (Sun et al., 2006). The high Robo levels in *jing* and *DATR-X* pan-neural mutants suggests that these genes do not regulate *robo* expression (Sun et al., 2006). However, the medial axon displacement suggests that these mutations may affect the expression of other genes required for Robo to 'read' the Slit cue. Alternatively, *jing* and *D*ATR-X may regulate longitudinal positioning in a Robo-independent manner (Kinrade et al., 2001).

3.5.2.2. Glial functions of *jing* and *DATR-X* are required for ipsilateral positioning of longitudinal glia (LG), glial survival and patterning of longitudinal axons

Genetic and cell ablation experiments have shown the critical role that neuronal-glial communication plays during pioneering of the longitudinal axon tracts (Hidalgo and Booth, 2000; Booth et al., 2000; Whitington et al., 2004; Hidalgo et al., 2001; Kinrade et al., 2001). Therefore, it might be informative to examine whether *jing* and *DATR-X* play a role in glial guidance of longitudinal axons. Glial development was examined after pan-glial expression of *jing* and *DATR-X* RNAi transgenes under the control of *glial cells missing* (*gcm*)-*Gal4* (Hosoya et al., 1995; Jones et al., 1995) and using anti-Repo as a marker to assess glial fates (Campbell et al., 1994; Halter et al., 1995; Xiong et al., 1994).

Repo-positive LG were already misplaced medially during stage 12 after pan-glial knockdown of *jing* and *DATR-X* (Fig. 25, B and C), similar to those Robo-positive LG

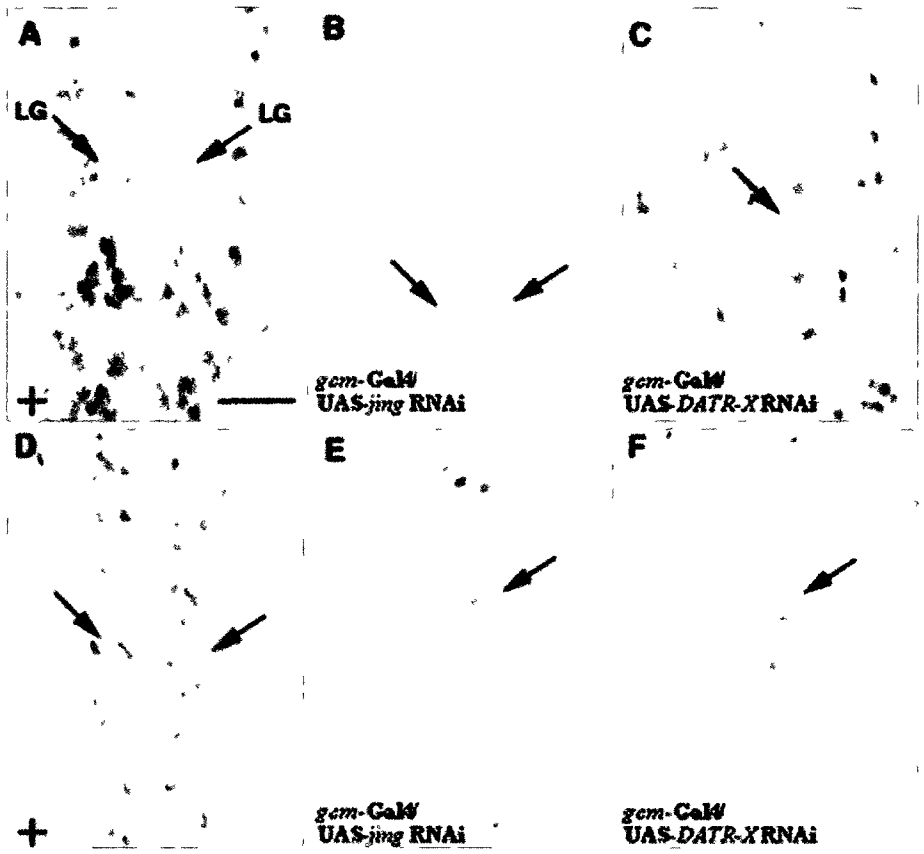


Figure 25. *D*ATR-X and *jing* are required for glial patterning. CNS glia cells were identified in whole-mount embryos stained with anti-Repo. Shown are close-up ventral views of the nerve cord of stage 12 (A-C) and stage 15 (D-F) embryos. (A) In wild-type stage 12 embryos, longitudinal glia (LG) have already migrated medially to their positions to guide pioneer longitudinal axons. (B and C) In contrast, LG inappropriately occupy positions at the CNS midline in those with glial-specific knockdown of *jing* (B) and *DATR-X* (C) as driven by *gcm-Gal4*. (D) In wild-type embryos, the LG occupy a two-cell wide row lining the longitudinal connectives (arrows). (E-F) In *jing* (E) and *DATR-X* (F) glial mutants, glia cells are misplaced across the midline (arrows). Scale bars, 50 μm. (Adapted from Sun et al., 2006).

(Sun et al., 2006). However, the numbers of glia in these mutants at this stage were not significantly changed compared to wild type (Sun et al., 2006), suggesting that *jing* and *DATR-X* in these cells are involved in glial differentiation and not in the division or specification of these cells. Later during wild-type embryogenesis, Robo is present only in axons (Sun et al., 2006) and therefore glial movement is restricted by Robo-independent mechanisms including axon contact and trophic support (Kinrade et al., 2001). In the stage 15 embryos, LG were medially misplaced to the CNS midline after glial knockdown of *jing* and *DATR-X* (Fig. 25, E and F). Consistently, longitudinal axons were shifted to the CNS midline as well in these embryos (Sun et al., 2006). Despite the maintenance of glial-axonal contact, the glial numbers steadily decreased during embryogenesis in *jing* and *DATR-X* glial mutants (Sun et al., 2006). However, glial numbers were unaffected after neuronal-specific *jing* and *DATR-X* knockdown despite a medial misplacement and mispositioning of LG (Sun et al., 2006). Therefore, *jing* and *DATR-X* glial-specific mutations perturb an autonomous survival function that cannot be compensated for by axon contact.

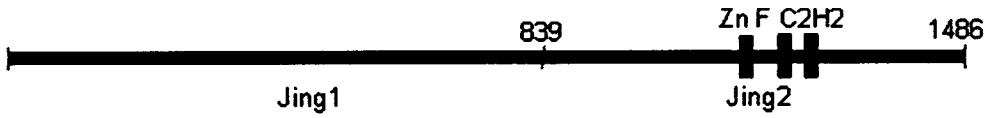
3.6. The relationship of *D*ATR-X and Jing, *in vitro*

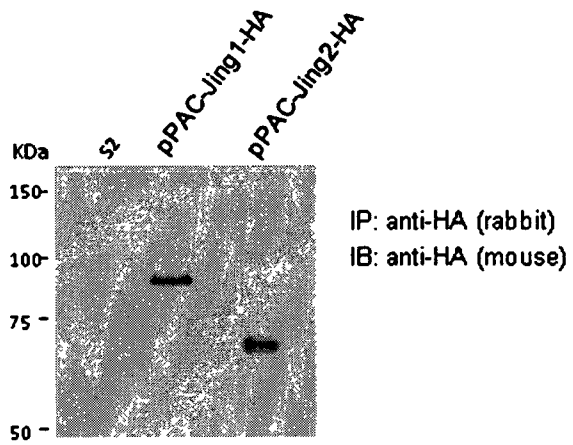
The genetic interactions between *jing* and *DATR-X* in adult and embryonic neurons suggest that Jing and *D*ATR-X functions overlap during axon guidance (Fig. 9 and 20). However, it is not known whether *jing* and *DATR-X* function in the same pathway. An important issue is how *D*ATR-X finds its targets to promote possible changes in chromatin structure and regulate gene expression. Mutations in the zinc finger coding region of human ATR-X (Fig. 13) were shown to disturb its nuclear localization

and to reduce its DNA-binding ability (Cardoso et al., 2000). Though it is unknown whether distinct chromatin remodeling complexes work in the same way, they always exist in the form of multi-protein complexes (Narlikar et al., 2002). Given the absence of a Zn finger motif in *D*ATR-X, one role of Jing may be to provide sequence-specific targeting information for *D*ATR-X. In this case, a direct physical interaction between Jing and *D*ATR-X is possible. Jing contains nuclear localization signals (NLS) and Zn fingers that were shown in its mouse homologue, AEBP2, to bind DNA (Sedaghat et al., 2002; He et al., 1999). Recent evidence shows that Jing can physically associate with the regulatory region of the *Drosophila* fibroblast growth factor receptor (FGFR) (Sonnenfeld et al., submitted). Alternatively, Jing may function in a complex with *D*ATR-X without providing nuclear targeting function. In this case, Jing would co-immunoprecipitate with the *D*ATR-X complex but they may not directly interact.

In order to test this hypothesis, expressing vector pPAC carrying tagged full-length *D*ATR-X and truncated versions of Jing that collectively span the entire protein were generated and used to transfect the *Drosophila* embryonic cell line S2 cells (Schneider, 1972). The expression of these two versions of Jing was first confirmed by western analysis of S2 cells transfected with these two constructs (Fig. 26A). However, co-immunoprecipitation performed using whole cell extracts from S2 cells co-transfected with tagged *D*ATR-X and truncated Jing constructs showed that *D*ATR-X was unable to pull down either truncated versions of Jing (Fig. 26B). This suggests an unlikely direct interaction between *D*ATR-X and the truncated Jing *in vitro*.

A


839
Zn F C2H2
1486
Jing1
Jing2


KDa
S2
pPAC-Jing1-HA
pPAC-Jing2-HA
150
100
75
50
IP: anti-HA (rabbit)
IB: anti-HA (mouse)

B

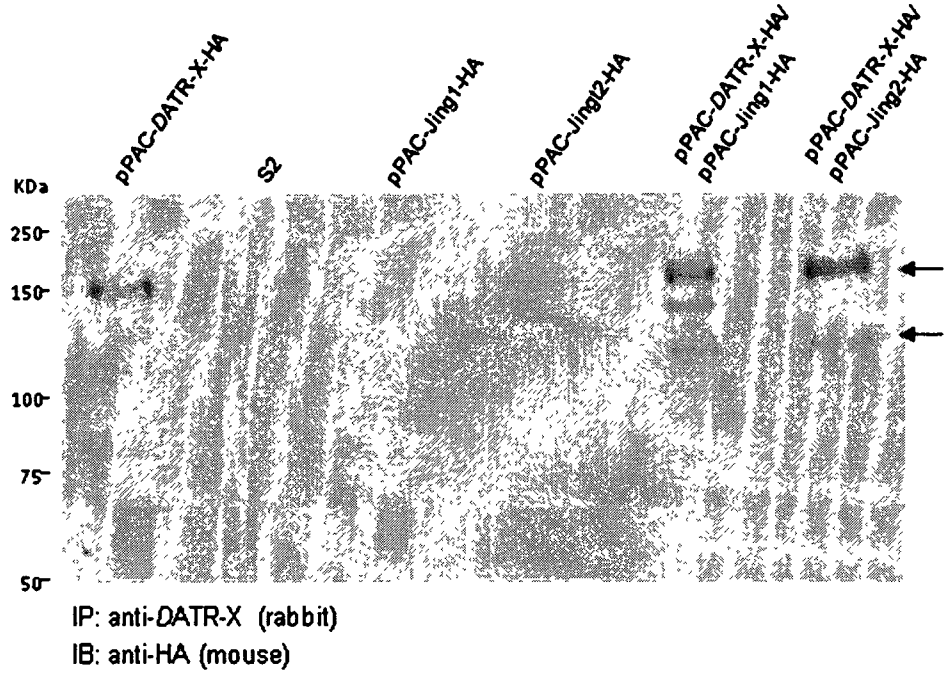

pPAC-DATR-X-HA
S2
pPAC-Jing1-HA
pPAC-Jing2-HA
pPAC-DATR-X-HA
pPAC-Jing1-HA
pPAC-DATR-X-HA
pPAC-Jing2-HA
KDa
250
150
100
75
50
IP: anti-DATR-X (rabbit)
IB: anti-HA (mouse)

Figure 26. *D*ATR-X expressed in *Drosophila* S2 cells failed to pull down either truncated version of Jing. S2 cells transfected with expression plasmids were lysed for western blotting (A) or co-immunoprecipitation (B). The full length *D*ATR-X tagged with HA was subcloned into expression vector pPAC. pPAC-Jing full-length was initially made but failed to be expressed. Plasmids carrying two truncated Jing versions Jng1 and Jing2 (corresponding to 1-839 and 840-1486 amino acid residues respectively) were generated with a C-terminal HA tag and cloned into the *Xba I*/*Xho I* sites of pPAC. (A) Jing1 and Jing2 were detected by western blotting. (B) *D*ATR-X failed to pull down either version of truncated Jing. The two bands (arrows) corresponded to the two isoforms of *D*ATR-X. IP, immunoprecipitation; IB, immunoblotting.

3.7. Cellular function for *D*ATR-X in the *Drosophila* embryonic CNS

Pan-neural and glial functions for *D*ATR-X have been shown (Fig. 24 and 25). Analysis of embryos deficient in *D*ATR-X and with reduced levels (Fig. 21) suggests that cells are probably sensitive to even a small dysregulation of its function. Given the widespread distribution of *D*ATR-X, perturbation of *D*ATR-X activity could result in a variety of problems at the cellular level. To avoid such pleiotropic effects, it was thus necessary to analyse *D*ATR-X function at a single-cell resolution in the *Drosophila* embryonic CNS. With the raised polyclonal antibody against *D*ATR-X (Fig. 18 and 19) and a variety of cell type markers, CNS cells expressing *D*ATR-X were identified and *D*ATR-X function in these cells studied.

3.7.1. *D*ATR-X is expressed in MP2 pioneer neurons and longitudinal glia (LG)

Gain-of-function and loss-of-function assays show that *D*ATR-X regulation is important during embryonic axon guidance (Fig. 20-22, 24 and 25; Sun et al., 2006). However, given that the *Elav* promoter used for pan-neuronal targeting is reported to drive expression in embryonic glia and neural progenitor cells (Berger et al., 2007), the associated phenotype due to mis-expression of *D*ATR-X may result from expression in both neurons and glia (Fig. 20; Sun et al., 2006). In the *Drosophila* embryonic CNS, pioneer neurons form major longitudinal axons to set up the axon scaffold of the ventral nerve cord along with the LG (Hidalgo, 2003). This scaffold is used by the follower neurons to extend their axons towards their targets (Hidalgo, 2003). Cells in wild-type embryos expressing *D*ATR-X proteins were identified with anti-*D*ATR-X and the

neuronal marker 22C10 (Zipursky et al., 1984), and the glial markers anti-Repo (Alfonso and Jones, 2002) and anti-Prospero (Campbell et al., 1994).

In wild-type embryos doubly stained with anti-*D*ATR-X and the neuronal marker 22C10 (Zipursky et al., 1984), the detected florescence revealed that *D*ATR-X is present in MP2 pioneer neurons when these cells emerge at stage 11 and prior to axon extension (Fig. 27A″). The 22C10 antigen is encoded by *futsch*, which is a microtubule-associated protein 1B (MAP1B) – like protein and is localized at the growing axon, the cell body and the dendrite (Hummel et al., 2000). At the stage 11, the first mAB 22C10 reactivity can be detected in the MP2 neurons prior to axonogenesis (Fig. 27A; Hummel et al., 2000). *D*ATR-X is a nuclear protein (Fig. 19). Therefore, the co-localization was revealed with the staining pattern that the green florescence was surrounded with the red 22C10 (Fig. 27A″, arrowhead).

Co-immunostaining of wild-type stage 14 embryos with the glial marker anti-Repo (Halter et al., 1995) and anti-*D*ATR-X also showed the presence of *D*ATR-X in LG and in other CNS glia (Fig. 27B-B″), consistent with the expression pattern depicted by previous reporter *lacZ* assays (Fig. 17; Sun et al., 2006). Repo (also called RK2) is a homeobox protein and is expressed in the nuclei of nearly all PNS and CNS glia except midline gila (Xiong et al., 1994; Campbell et al., 1994; Halter et al., 1995). Thus the co-localization was found in the nuclei (Fig. 27B″). The presence of *D*ATR-X in LG was further confirmed by double staining embryos with anti-*D*ATR-X and the longitudinal glial marker anti-Prospero (Campbell et al., 1994) which only labels the 5-6 anteriormost LG within each hemisegment (Fig. 27C-C″; Doe et al., 1991). Together, these studies establish *D*ATR-X as a glial and neuronal nuclear protein.

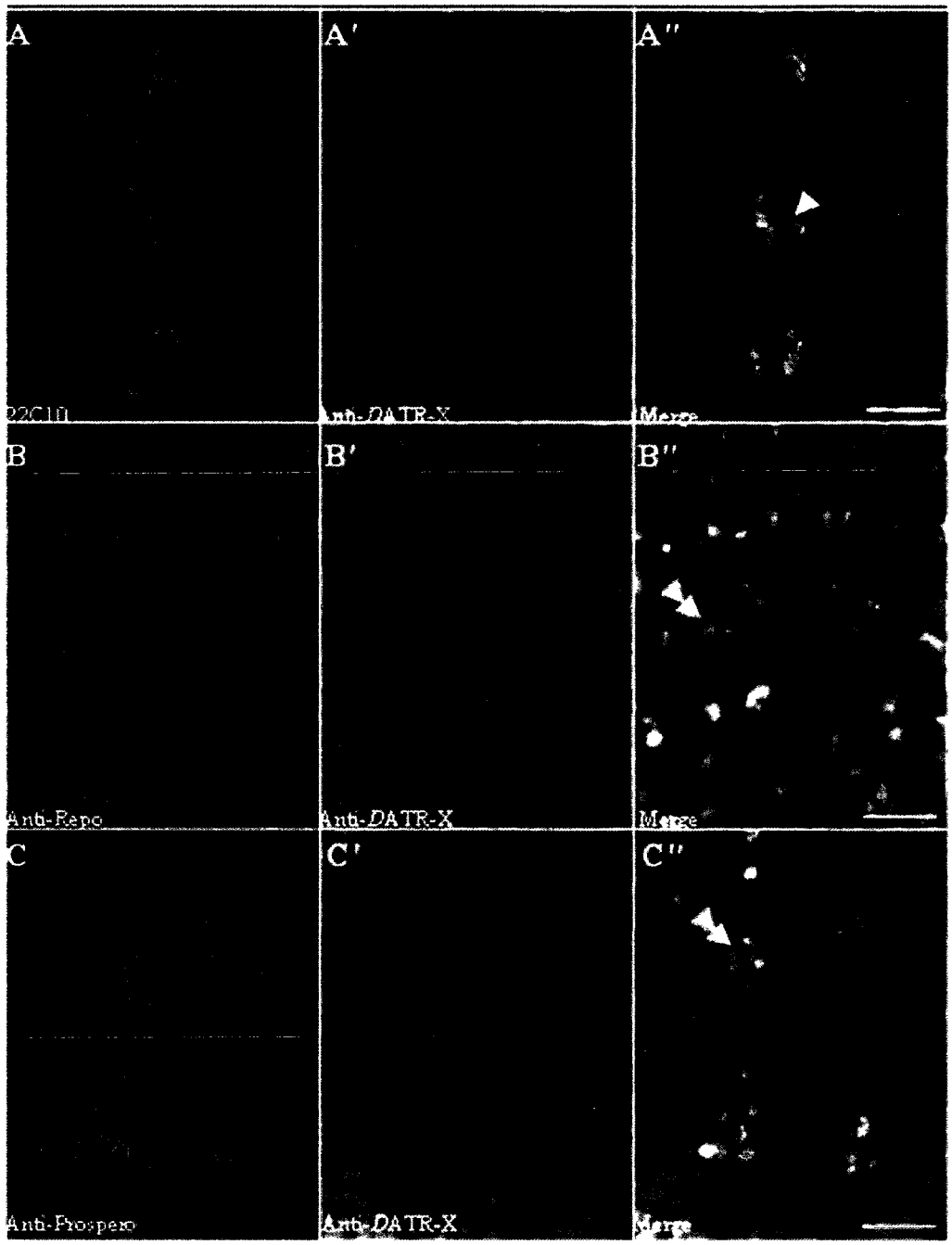

A
A'
A''
22C10
Anti-DATR-X
Merge
B
B'
B''
Anti-Repo
Anti-DATR-X
Merge
C
C'
C''
Anti-Prospero
Anti-DATR-X
Merge

Figure 27. Confirmation of nuclear *D*ATR-X in the MP2 pioneer neurons and longitudinal glia (LG) of the *Drosophila* embryonic VNC. Wild type *Drosophila* embryos were stained with both polyclonal anti-*D*ATR-X (A′, B′ and C′) and 22C10 (A), 8D12 anti-Repo (B) or anti-Prospero (C). Shown are close-up ventral views of VNC with anterior up for all. *D*ATR-X is expressed in MP2 pioneer neurons (arrowhead, A″) and LG (double arrowhead, B″ and C″). (A) The 22C10 epitope is localized at the growing axon, the cell body and the dendrite (Hummel et al., 2000). Anti-*D*ATR-X was detected within nuclei of 22C10 positive MP2 during stage 11 (A″, arrowhead). Anti-Repo marks all the CNS glia except midline glia (B) and anti-Prospero labels 5-6 LG (C) distributed along the connectives per hemisegment. Scale bar, 10 μm.

3.7.2. RNAi assays for *D*ATR-X in CNS cell subtypes

We have shown that the expression of *D*ATR-X is not transient and is maintained throughout the embryonic stages in the nuclei of many cells including both MP2 neurons and LG, implicating a constant requirement of *D*ATR-X in these cells. In order to further disclose the function of *D*ATR-X in these cell types, reductions of *DATR-X* by RNAi were targeted to specific subsets of neurons and glia using more restrictive Gal4 drivers: a MP2 pioneer neuronal driver, *15J2* (Hidalgo and Brand, 1997) and a longitudinal glial driver, *htl*-gal4 (Shishido et al., 1997). *DATR-X*-RNAi has been confirmed as an effective method to generate a CNS phenotype when targeted to all neurons or glia (Fig. 24 and 25) (Sun et al., 2006). An RNAi based screen also identified *DATR-X* as a gene required for *Drosophila* embryonic CNS development (Koizumi et al., 2007).

DATR-X RNAi was targeted to MP2 pioneer neurons using driver *15J2* to assess the function of *D*ATR-X in these cells. Resultant embryos were heterozygous for *15J2* and UAS-*DATR-X* RNAi. To assess the effects on CNS development, the longitudinal axons were examined in these embryos immunostained with 1D4 anti-Fas II and the LG were identified with anti-Repo (Halter et al., 1995). However, after *DATR-X* RNAi was introduced to MP2 neurons, misrouted axons (Fig. 28C, arrow) or mis-positioned glia were found only in less than 5% of embryos during CNS development (Table 6 and 7), suggesting an unlikely direct impact from reduced *DATR-X* expression in MP2 neurons on the longitudinal axon bundles and the longitudinal glial organization.

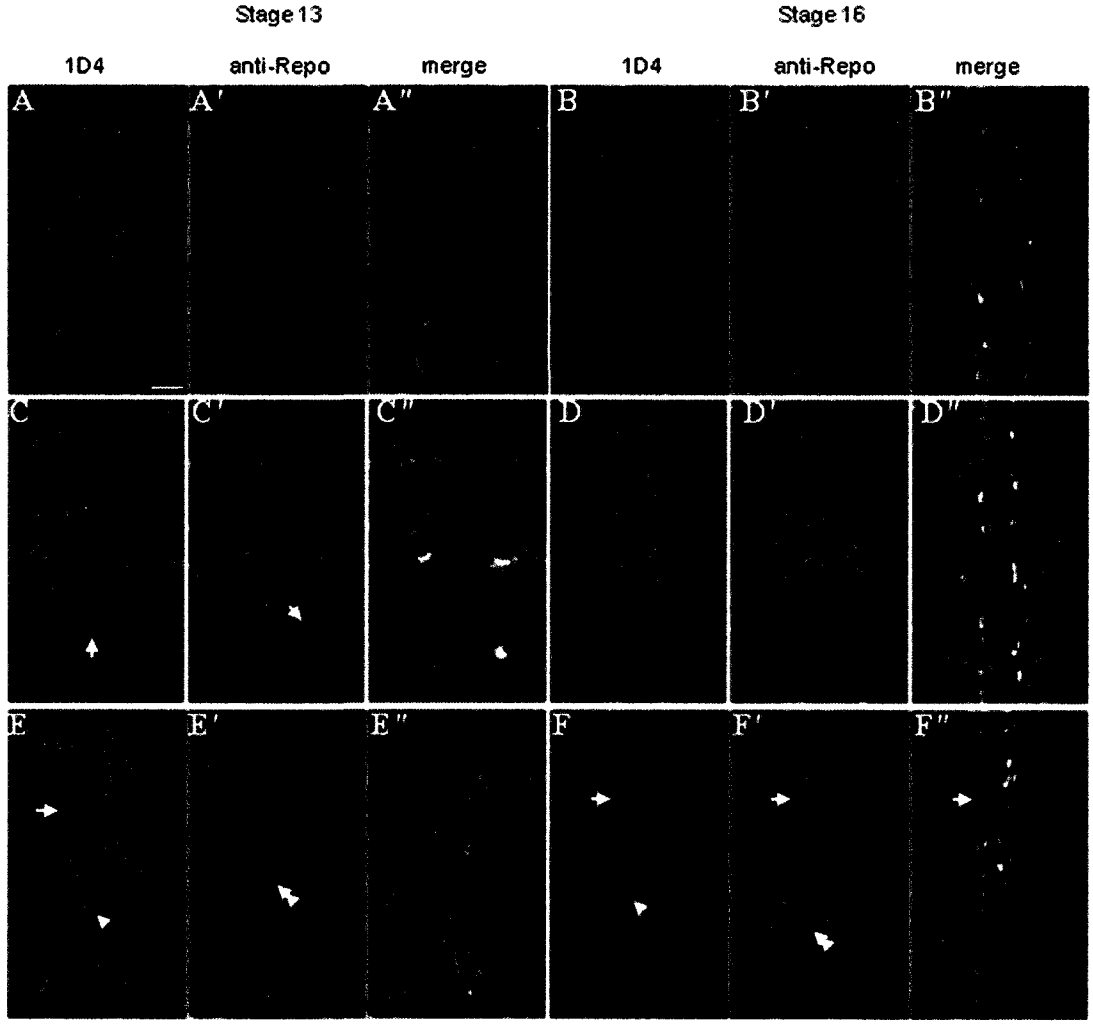

Stage 13
Stage 16
1D4
anti-Repo
merge
1D4
anti-Repo
merge
A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''
E
E'
E''
F
F'
F''

Figure 28. CNS phenotypes after *DATR-X* RNAi targeting to the MP2 neurons or LG. *DATR-X*-RNAi was directed to MP2 pioneer neurons using *15J2* (Hidalgo and Brand, 1997) or LG using *htl*-Gal4 (Shishido et al., 1997). Anterior is to the up for all. Scale bar, 10 μm. Embryos of stage 13 and 16 were doubly stained with longitudinal axon marker 1D4 anti-Fas II (red) (Hummel et al., 2000) and a glial marker rabbit anti-Repo (green) (Halter et al., 1995). Compared with wild type (A-B″), knocking-down *DATR-X* in MP2 neurons (C-D″) in very few embryos is associated with mild defects including axons crossing the midline (C, arrow) and glial mis-positioning (C′, arrow; Table 6). (E-F″) Targeting *DATR-X* RNAi to LG caused: 1) breaks in LG line (F′, arrow) or longitudinal pioneer axons (E, arrow); 2) misrouting of axons across the midline (E and F, arrowheads); 3) migration of LG to the midline (E′ and F′, double arrowheads). Lack of longitudinal defasciculation correlates with glial gap (F-F″, arrows)

Table 6. Quantification of longitudinal axon phenotypes in targeted *DATR-X* mutant embryos

		Penetrance			expressivity		
Genotype	Stage	broken	midline crossing	n	broken	midline crossing	n
w^{1118}	13	0	0	72	0	0	708
	16	0	0	78	0	0	757
15J2/+	13	1.4%	1.4%	70	0.2%	0.2%	611
	16	0	0	79	0	0	780
htl-Gal4/+	13	1.6%	1.6%	62	0.2%	0.2%	612
	16	1.2%	0	82	0.1%	0	809
15J2/UAS-*DATR-X* RNAi							
	13	4.3%	2.9%	70	0.5%	0.3%	659
	16	2.9%	2.9%	68	0.3%	0.3%	654
htl-Gal4/UAS-*DATR-X* RNAi							
	13	24.7%	23.5%	81	3.8%	3.4%	793
	16	18.4%	19.7%	76	2.1%	2.3%	746

Longitudinal axons were stained with 1D4 anti Fas II. The samples were counted as "broken" when the breakage was found along the longitudinal axons, and as "midline crossings" when these axons misrouted across the midline.

Penetrance is indicated as the percentage of embryos showing phenotype out of the total number of embryos counted. Expressivity is indicated as the percentage of abnormal segments out of the total number of segments examined.

Table 7. Quantification of longitudinal glial positioning in targeted *DATR-X* mutant embryos

		Penetrance			expressivity		
Genotype	Stage	gap	cross	n	gap	cross	n
w^{1118}	13	0	0	72	0	0	708
	16	0	0	78	0	0	757
15J2/+	13	1.4%	0	70	0.2%	0	611
	16	0	0	79	0	0	780
htl-Gal4/−	13	1.6%	1.6%	62	0.2%	0.2%	612
	16	0	1.2%	82	0	0.1%	809
15J2/UAS-*DATR-X* RNAi							
	13	1.4%	2.8%	70	0.2%	0.3%	659
	16	2.9%	2.9%	68	0.3%	0.3%	654
htl-Gal4/UAS-*DATR-X* RNAi							
	13	25.0%	25.0%	81	3.8%	5.3%	793
	16	19.7%	21.1%	76	3.1%	3.8%	746

Glia were stained with anti-Repo. "Gap" stands for the broken glia line and "cross" refers to the migration of glia towards the midline.

Penetrance is indicated as the percentage of embryos showing phenotype out of the total number of embryos counted. Expressivity is indicated as the percentage of abnormal segments out of the total number of segments examined.

To reveal the cellular function of *D*ATR-X in LG, *DATR-X* RNAi was targeted to these cells using the Gal4 driver *htl*-Gal4 whose expression is restricted to LG in the CNS (Shishido et al., 1997; Hidalgo et al., 2001). The positioning of glia and longitudinal axons (Fig. 28E-F″; Table 6 and 7) were monitored by staining these cells with anti-Repo (Halter et al., 1995) and 1D4 anti-Fas II. The effects of reducing *DATR-X* specifically in LG (Fig. 28) phenocopied those observed in homozygous *DATR-X* excision mutant embryos (Fig. 21) and those associated with pan-glial *DATR-X* RNAi directed by *gcm*-Gal4 (Fig. 25): gaps were found in the LG rows (Fig. 28F′, arrow) and some LG migrated inappropriately to the midline (Fig. 28, E′ and F′, double arrowheads). These defects are all within the spectrum of phenotypic abnormalities observed in *DATR-X* mutations affecting glial biology, revealing that *DATR-X* is required in LG for the positioning of these cells (Fig. 22 and 25).

Since LG play an essential role in longitudinal axon formation, the influences of LG-specific *DATR-X* knocking-down upon this process were also examined. Expectedly, there were breaks in the longitudinal axon bundles (Fig. 28E, arrow), misrouting of axons across the midline (Fig. 28F, arrowhead) and fusion of the axons into a single fascicle (Fig. 28E, arrowhead). Glial gaps sometimes coincided with a lack of separation of the three longitudinal bundles (Fig. 28F-F″), consistent with their known role in this process (Hidalgo et al., 1995; Hidalgo and Booth, 2000). Therefore, *DATR-X* in LG is required for the formation of the longitudinal axons.

3.8. The N-terminus of *D*ATR-X is sufficient for its translocation from the cytoplasm to the nucleus

Sequence analysis suggested that *D*ATR-X belongs to the SWI/SNF chromatin remodeling protein family (Fig. 13; Sun et al., 2006). A prerequisite for *D*ATR-X to participate in any nuclear events would be that *D*ATR-X has a nuclear sublocalization. Consistent with this, our immunostaining localization studies have shown that *D*ATR-X is a nuclear protein *in vivo* (Fig. 19). In order to identify the region responsible for the nuclear localization of *D*ATR-X, expression constructs carrying HA-tagged full length or three truncated versions of *D*ATR-X were generated spanning the entire protein (T1-T3, corresponding to 1-449, 450-920 and 921-1311 amino acid residues respectively). These constructs were transfected into *Drosophila* S2 cells and their subcellular localization examined by immunostaining with anti-HA, anti *D*ATR-X and the nuclear marker 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI). Consistently, over-expressed *D*ATR-X-HA (Fig. 29D) as well as the endogenous *D*ATR-X (Fig. 29B, D, F, H and J) is confined to S2 cell nuclei. Anti-HA immunoreactivity for T1-HA was also confined to nuclei (Fig. 29E) while T2-HA and T3-HA were only present in the cytoplasm (Fig. 29, G and I, respectively). Together, these results show a sufficiency of the N-terminus of *D*ATR-X for its nuclear localization, consistent with the involvement of both DEXDc/SNF_N and Helicase C domains in nuclear cellular events (Eisen et al., 1995; Jankowsky and Jankowsky, 2000).

To more clearly define the region of the N-terminus sufficient for *D*ATR-X nuclear translocation, an NLS in *D*ATR-X was predicted against NLSdb using PredictNLS (http://cubic.bioc.columbia.edu/db/NLSdb/, Nair et al., 2003) and a peptide

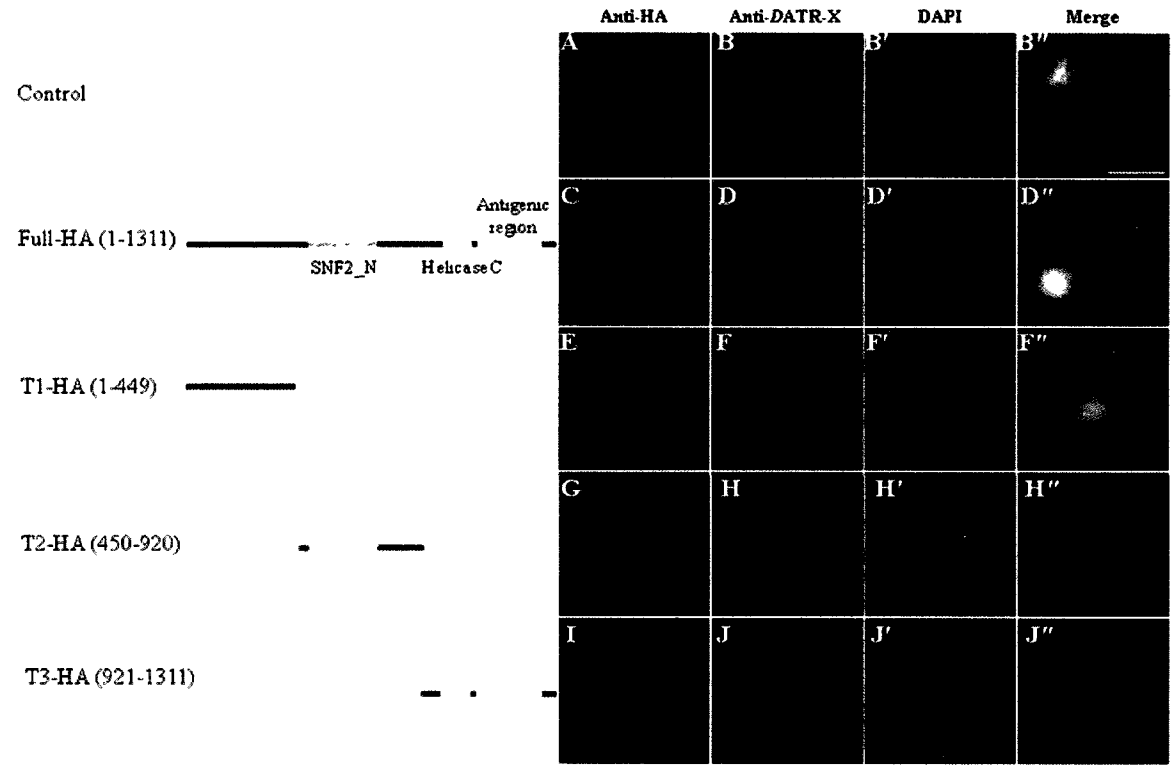


Figure 29. The N-terminus of *D*ATR-X is sufficient for its nuclear-localization. *Drosophila* S2 cells were transiently transfected with plasmids carrying various lengths of *D*ATR-X tagged with HA epitope at the C-terminus. Nuclear localization was assessed by staining with anti-HA (A, C, E, G and I), anti-*D*ATR-X (B, D, F, H and J) and DAPI (B′, D′, F′, H′ and J′). Anti-HA immunoreactivity showed the nuclear localization of endogenous *D*ATR-X (B-B″) as well as the HA-tagged full length *D*ATR-X transgene (C-D″). T1 was found in the nuclei of the transfected S2 cells (E-F″). T3 contains the region anti-*D*ATR-X was raised against but fails to travel to the nucleus by itself (I-J″). T2 doesn't have either the region anti-*D*ATR-X recognizes (H-H″) or the N-terminus for nuclear targeting (G) and therefore remains cytoplasmic. An empty pPAC vector was used as a control. Scale bar, 10 μm.

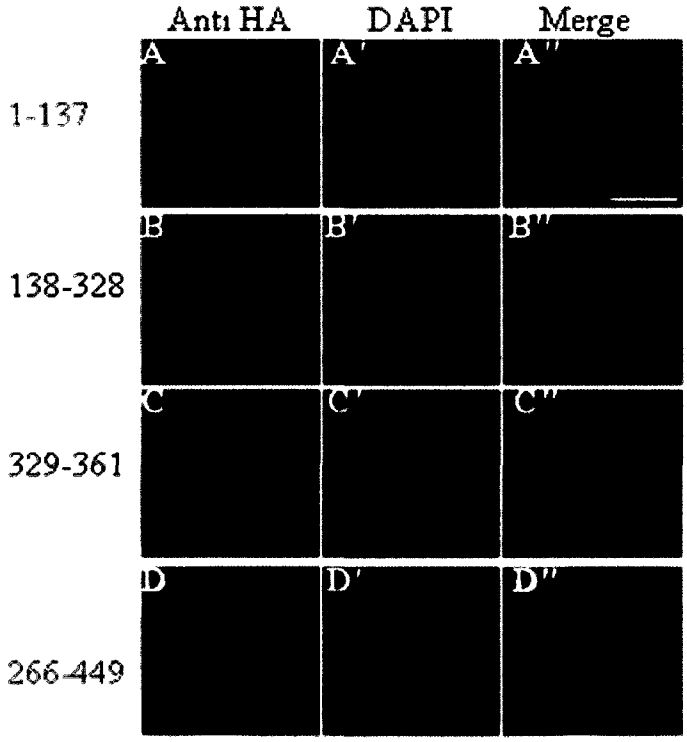


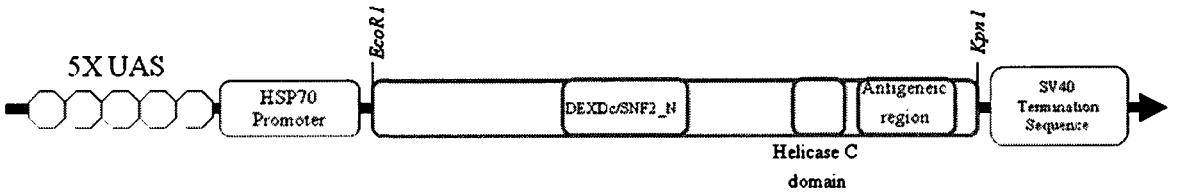
Figure 30. Refined mapping of the N-terminus sufficient for *D*ATR-X nuclear translocation. *Drosophila* S2 cells were transiently transfected with plasmids carrying C-terminal HA-tagged smaller truncations spanning the first 449 amino acid residues of *D*ATR-X. Nuclear localization was assessed by staining with anti-HA (A, B, C and D) and DAPI (A′, B′, C′ and D′). A peptide of 33 amino acid residues (329-361) in *D*ATR-X was predicted using PredictNLS (http://cubic.bioc.columbia.edu/db/NLSdb/, Nair et al., 2003). However, the predicted NLS alone was not detected in the nucleus (C-C″). The other smaller *D*ATR-X truncations, 1-137, 138-328 and 266-449 were found in the nuclei (A-A″, B-B″ and D-D″). Scale bar, 10 μm.

of 33 amino acid residues (329-361) was identified. However, a fragment containing the predicted NLS alone was detected cytoplasmically, indicating that it is unable to travel to nuclei by itself (Fig. 30C). Other smaller truncations spanning the first 449 amino acids were also made. The first two segments, 1-137 and 138-328, both were detected in the nuclei (Fig. 30, A and B). A construct which carries the sequence starting from the second ATG of *D*ATR-X (266 amino acid residue) was also created. It was observed that this region went to nuclei as well (Fig. 30D), revealing the plausible requirement of this part for the short isoform of *D*ATR-X to be present in the nuclei. Therefore, the region of 362-449 and 266-329 could target to the nucleus. Together, these results raise the possibility that there are multiple NLS sites in the N-terminus of *D*ATR-X consistent with an important nuclear role. Alternatively, all regions, excluding 329-361, may be chaperoned to the nucleus through multiple binding sites.

3.9. Phenotypes of *DATR-X* over-expression in LG phenocopy those of RNAi

An important functional role for the Helicase/adenosine triphosphatase (ATPase) domain in vertebrate ATR-X has been suggested (Xue et al., 2003; Tang et al, 2004). In order to evaluate whether the Helicase conserved C domain in *D*ATR-X contributes to its normal function, a truncated form of the protein was generated. The C-terminus, including the Helicase C region, was deleted from *D*ATR-X to determine if this truncated version could interfere with endogenous *D*ATR-X. Toward this end, a transgenic fly was generated that contained UAS construct encoding truncated *D*ATR-X without the

A



B

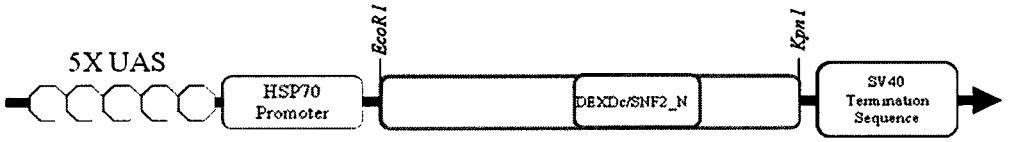


Figure 31. The tagged full length and truncated *D*ATR-X constructs. Both full length and truncated DATR-X were tagged with HA at the C-terminus and subcloned into the pUAST vector (Brand and Perrimon, 1993). The transgenic flies carrying these constructs were generated. (A) UAS-*DATR-X-HA* full length construct. (B) UAS-*DATR-X*$^{\Delta HelC}$*-HA*. The C-terminus containing Helicase C domain and the antigenic region for anti-*D*ATR-X were removed in the truncated *D*ATR-X construct.

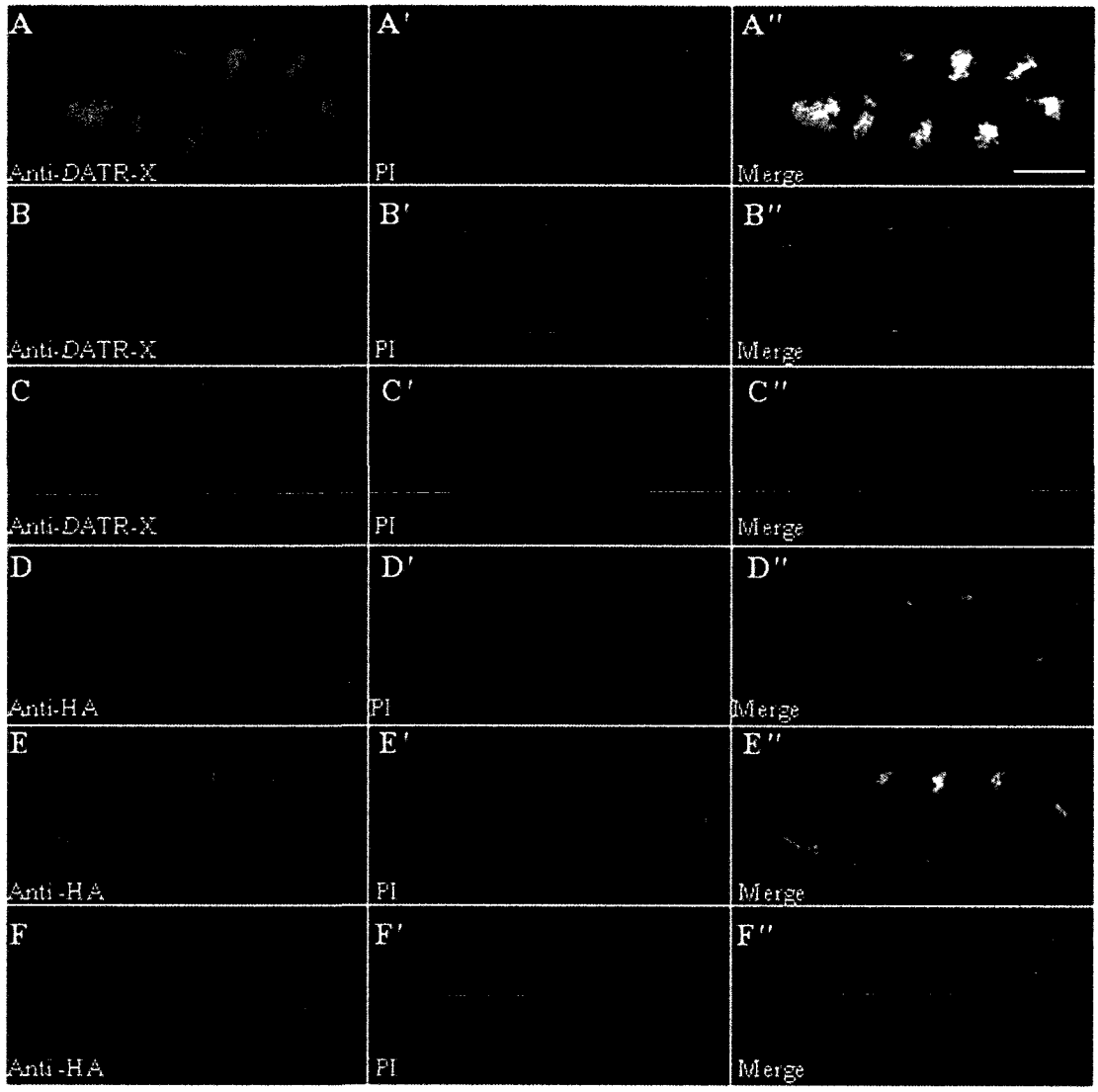

A
Anti-DATR-X
A'
PI
A''
Merge
B
Anti-DATR-X
B'
PI
B''
Merge
C
Anti-DATR-X
C'
PI
C''
Merge
D
Anti-HA
D'
PI
D''
Merge
E
Anti-HA
E'
PI
E''
Merge
F
Anti-HA
F'
PI
F''
Merge

Figure 32. The truncated *DATR-X* transgene can be delivered to the nucleus. Both full length and truncated (without Helicase C domain containing C-terminal region) forms of *D*ATR-X were C-terminally tagged with HA. Embryos of stage 10-11 were stained with rabbit anti-*D*ATR-X (green) or rabbit anti-HA (green). The nuclei were marked with propidium iodide (PI) (red). Images in the last column are the merge of those in the first two columns. Anterior is left and dorsal is up for all. Scale bar, 100 μm. Genotype: *prd*-gal4/UAS-*DATR-X-HA* (A-A″ and D-D″); *prd*-gal4/UAS-*DATR-X*$^{\Delta HelC}$-*HA* (B-B″ and E-E″); *prd*-gal4/UAS-*jing* (C-C″ and F-F″). *paired* (*prd*)-*Gal4* allows the expression of UAS transgene in a characteristic pattern of seven stripes. Over-expression of full-length *DATR-X-HA* was verified by the staining from both anti-*D*ATR-X (A) and anti-HA (D). Only anti-HA was detected for the over-expression of *DATR-X*$^{\Delta HelC}$ due to its lacking the recognizable epitope for the anti-*D*ATR-X antibody (B and E). UAS-*jing* was used as a negative control (C and F). Overlapped staining of PI and anti-HA or anti-*D*ATR-X confirmed the nuclear localization of both full-length and truncated forms of *D*ATR-X (A″, D″ and E″).

Helicase conserved C domain and subsequent carboxyl tail region and designated as *D*ATR-X$^{\Delta HelC}$ (Fig. 31B).

Using the *paired* (*prd*)-Gal4/UAS system (Brand and Perrimon, 1993), the characteristic pair-rule pattern of seven stripes was observed in *prd*-Gal4/UAS-*DATR-X-HA* embryos after staining with anti-HA and anti-*D*ATR-X (Fig. 32, A and D). Next *D*ATR-X$^{\Delta HelC}$ protein was tested to be induced by Gal4 and reach the nucleus upon targeted over-expression in *Drosophila* embryos. *D*ATR-X$^{\Delta HelC}$ still contains the N-terminus which is sufficient for its translocation from the cytoplasm to the nuclei (Fig. 29 and 31B), as was confirmed with *in vivo* double staining of *prd*-Gal4/UAS-*DATR-X$^{\Delta HelC}$–HA* carrying embryos with anti-HA and propidium iodide (Fig. 32, E and E″). The antigenic region for anti-*D*ATR-X is localized at the C-terminus which was removed together with the Helicase C domain in the transgenic construct UAS-*DATR-X$^{\Delta HelC}$–HA* (Fig. 31B), therefore, the characteristic stripes were not found for the immunoreactivity against *D*ATR-X in *prd*-Gal4/UAS-*DATR-X$^{\Delta HelC}$–HA* embryos (Fig. 32B). No staining for HA or stripes were found in *prd*-Gal4/UAS-*jing* which was used as a negative control (Fig. 32, C and F; Sedaghat et al., 2002). These results served four purposes: 1) they verified the specificity of the anti-*D*ATR-X polyclonal antibody; 2) they provided *in vivo* evidence that the N-terminus of *D*ATR-X is sufficient for it to travel to the nucleus; 3) both the full-length and the truncated versions of the *D*ATR-X constructs were indeed over-expressed in the desired specific cell types/tissues and 4) nuclear sublocalization of *D*ATR-X$^{\Delta HelC}$ is not disturbed and therefore, any associated phenotypes can thus be assumed to reveal the functional significance of the absence of the Helicase C domain.

Next, over-expression assays were used to evaluate whether the Helicase C containing region contributed to the function of *D*ATR-X. Over-expression of the full length form of *DATR-X* causes apoptosis in developing eyes, wings and neurons (Lee et al., 2007; Hong et al., 2009) and fewer neurons in mushroom body (Nicolaï et al., 2003), comparable to the neural tube defects associated with the over-expression of ATR-X in transgenic mice (Bérubé et al., 2002). We have also previously shown that over-expression of the wild type form of *Drosophila DATR-X* is sufficient to generate CNS neuronal and glial defects (Fig. 20; Sun et al., 2006). Consistently, after full-length *DATR-X-HA* was over-expressed specifically in LG using *htl*-Gal4, gaps were found along the Repo-positive LG line (Fig. 33B, arrow) and some migrated to the midline (Fig. 33E, arrowhead), reminiscent of the repulsive defects in glial-specific *DATR-X* RNAi mutants (Fig. 28E-F″). Thus, the sensitivity of *Drosophila* LG to elevated *DATR-X* levels could be used to test whether the Helicase C domain is essential for high levels of *DATR-X* to disturb the longitudinal glial biology

The *D*ATR-X$^{\Delta HelC}$ protein was over-expressed in LG using the *htl*-Gal4 driver (Shishido et al., 1997) and its capacity to affect LG positioning was determined using anti-Repo (Halter et al., 1995). It was observed that removing the Helicase C domain at the *D*ATR-X C-terminal in LG was sufficient to disturb longitudinal glial organization including repulsion from the CNS midline (Fig. 33, C and F), but not as frequently as over-expression of full-length *DATR-X* (Table 8). Similar results were obtained with two independent transgenic UAS-*DATR-X*$^{\Delta HelC}$*-HA* fly lines confirming that the phenotype was due to the *D*ATR-X truncation. These data show that a lack of Helicase C domain suppresses the LG positioning defects associated with full-length *DATR-X* over-

expression. However, this information is inadequate to clarify the importance of the Helicase C domain for full length *D*ATR-X function in LG. More studies are required to delineate the precise role of this domain for *D*ATR-X function. Nevertheless, the transgenic tools generated here could be used in further studies to evaluate the functional involvement of the Helicase C domain in the identified cells/tissues expressing *D*ATR-X.

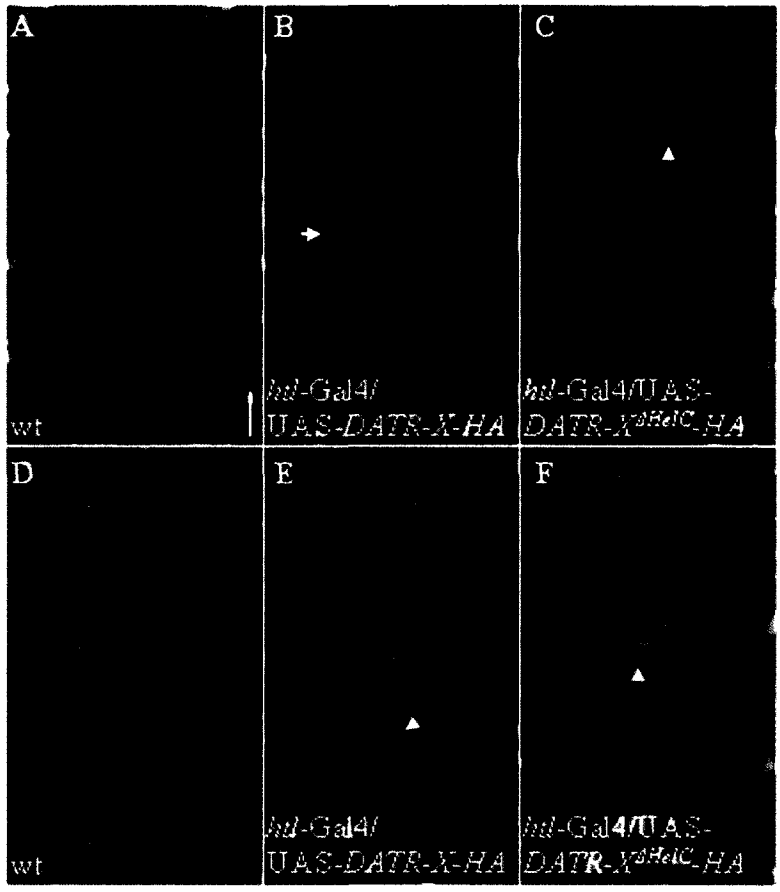


Figure 33. Deletion of the *D*ATR-X Helicase conserved C domain does not alter *DATR-X* over-expression effects in LG. Full length or truncated forms of *DATR-X* were directed to the LG by the driver *heartless* (*htl*)-Gal4. Embryos of stage 13 (A-C) and 16 (B-D) were stained with anti-Repo (Halter et al., 1995). Anterior is to the up for all. Scale bar, 10 μm. After over-expression of full length *DATR-X* in LG, some of these cells were missing (B, arrow) and some migrated to the midline (E, arrowhead). In the embryos carrying over-expressed *DATR-X*$^{\Delta HelC}$, some LG were found positioned in the middle (C and F, arrowheads).

Table 8. Quantification of longitudinal glial positioning in embryos over-expressing wild-type and truncated *DATR-X*

		Penetrance			expressivity		
Genotype	Stage	gap	cross	n	gap	cross	n
w^{1118}	13	0	0	72	0	0	708
	16	0	0	78	0	0	757
UAS-*DATR-X-HA*/+	13	0	0	65	0	0	572
	16	0	0	62	0	0	552
UAS-*DATR-X*$^{\Delta HelC}$-*HA*/+							
	13	0	0	59	0	0	582
	16	0	0	61	0	0	598
htl-Gal4/UAS-*DATR-X-HA*							
	13	37.8%	42.2%	90	6.1%	12.5%	800
	16	30.7%	37.5%	88	6.5%	13.4%	848
htl-Gal4/UAS-*DATR-X*$^{\Delta HelC}$-*HA*							
	13	22.0%	30.5%	82	2.3%	3.2%	789
	16	22.4%	25.9%	85	2.6%	3.0%	724

Glia were stained with anti-Repo. "Gap" stands for the broken glia line and "cross" refers to the migration of glia towards the midline.

Penetrance is indicated as the percentage of embryos showing phenotype out of the total number of embryos counted. Expressivity is indicated as the percentage of abnormal segments out of the total number of segments examined.

DISCUSSION

In order to determine if *D*ATR-X function is required for embryonic CNS development, cellular and molecular assays were carried out using *Drosophila melanogaster* as a model system. The genetic basis for these assays was facilitated by using *Drosophila* research tools to create specific genetic backgrounds allowing for the comparison of potential neuronal and glial roles for *D*ATR-X. These studies were aided by the wealth of information regarding the roles of specific neuronal and glial lineages during axon guidance (Hiramoto and Hiromi, 2006; Keleman et al., 2005; Garbe and Bashaw, 2007; Kinrade and Hidalgo, 2004; Kinrade et al., 2001; Bonkowsky et al., 1999; Brankatschk and Dickson, 2006). Furthermore, the conserved genetic patterning mechanism of the *Drosophila* ventral nerve cord and brain (Lichtneckert and Reichert, 2005) allow for a comparison of the role of ATR-X during vertebrate embryogenesis. These studies will contribute to our knowledge of how transcription factors affect axon guidance (Butler and Tear, 2007).

*D*ATR-X has a very similar gene structure but is smaller than its vertebrate counterparts due to the absence of an amino-terminally located zinc finger domain. Nevertheless, N-terminal sequences of *D*ATR-X were found to be sufficient for its nuclear localization, *in vitro*. *D*ATR-X was widely expressed throughout embryogenesis and was enriched in the developed CNS. Similarly, vertebrate embryonic ATR-X is expressed in the CNS including the spinal cord (Cardoso et al., 1998).

The studies described in this thesis are the first to demonstrate the requirement of *D*ATR-X for embryonic CNS axon initial extension and maturation, and longitudinal glial survival and positioning. The availability of *DATR-X* strains and the creation of *gain-of-DATR-X* and targeted RNA interference (RNAi) against *DATR-X* (Sun et al.,

2006) allowed for the specific analysis of *D*ATR-X function in the neurons and glia that are instrumental in the formation of the major CNS axon pathways. The similarities in axon guidance phenotypes associated with targeted mis-expression of *DATR-X* and/or *jing* to neurons or glia suggested that *D*ATR-X and *jing* may have similar functions. We also identified the CNS cell subtypes expressing *D*ATR-X and carried forward the analysis of *D*ATR-X function in specific CNS cell lineages. Furthermore, it was demonstrated that the removal of the Helicase C domain containing C-terminal region can suppress the LG positioning defects associated with full-length *DATR-X* over-expression. Together, these studies establish *D*ATR-X as an important player during the formation of the longitudinal axon scaffold and for glial survival and positioning in the developing *Drosophila* embryonic CNS.

4.1. A conserved role for ATR-X in the embryonic CNS

Mutations in the human ATR-X gene are associated with several X-linked mental retardation syndromes which are characterized by cognitive delay, facial dysmorphism, microcephaly, skeletal and genital abnormalities and neonatal hypotonia (Gibbons et al., 1995a; Gibbons et al., 1995b; Gibbons and Higgs, 2000; Villard et al., 1996a; Villard et al., 1996b). A total of 87% of mental retardation (MR) genes have a fruit fly homolog and 76% have at least one functional ortholog revealing a remarkable conservation between humans and *Drosophila melanogaster* (Inlow and Restifo, 2004). Some orthologs of human MR genes have cellular phenotypes involving neurons, glia and neural precursor cells and arise from defects in proliferation, migration and process

extension or arborization (Inlow and Restifo, 2004). For example, targeted mutation of ATR-X to the early forebrain in mice leads to cortical progenitor cell death and reduced forebrain size (Bérubé et al., 2005). In addition, mutations in genes controlling the identity of forebrain neuronal precursors can result in holoprosencephaly where the brain hemispheres do not separate (Wallis and Muenke, 2000). An increased understanding of these from molecular and cellular bases for hereditary MR is critical for therapeutic drug discovery. *Drosophila* not only can be used in a systematic manner to study MR, but also can help to reveal molecular and cellular mechanisms of nervous system development relevant to MR.

ATR-X belongs to the SWI/SNF group of chromatin remodeling proteins which use the energy provided by ATP hydrolysis to disrupt histone-DNA associations and move nucleosomes to different positions (Kingston and Narlikar, 1999; Whitehouse et al., 1999). This chromatin modulation allows for the access of activators or repressors to their DNA binding sites in their target genes. The helicase C and SNF2N domains of ATR-X have been shown to have DNA translocase and nucleosome-remodeling activities (Tang et al., 2004; Xue et al., 2003). Accordingly, mutations in ATR-X have been mapped to the helicase C and SNF2N domains which show approximately 60% homology with those in *D*ATR-X and have been conserved from *C. elegans* to humans (Fig. 13). This conservation supports a conserved role for *Drosophila* ATR-X in chromatin remodeling (Fig. 13; Tang et al., 2004).

Vertebrate ATR-X has a C_2C_2 zinc finger motif in the amino terminus that is similar to a plant homeodomain (PHD) finger previously identified in proteins involved in chromatin-mediated transcriptional regulation (Gibbons et al., 1997; Aasland et al.,

1995). The structure of this domain has recently been solved and suggests a DNA-binding function for ATR-X (Argentaro et al., 2007). Interestingly, the zinc finger domain is missing in *D. melanogaster* and *C. elegans* ATR-X proteins, suggesting that these structures may have been acquired through evolution due to a necessity in vertebrate chromatin remodeling mechanisms. More than 80% of patients reported have been identified with mutations in the ATPase and the PHD zinc finger domains of ATR-X confirming that these are essential functional regions of the protein (Villard et al., 1996b and 1996c; Gibbons and Higgs, 2000).

Given the absence of the zinc finger domains in *D*ATR-X, invertebrate *D*ATR-X proteins may be complexed with a targeting protein containing DNA-binding motif in order to regulate gene expression at the proper regulatory sites. This may be one role of Jing since it has a very similar embryonic expression pattern as well as mutant and over-expression phenotypes as those of *D*ATR-X (Fig. 15, 17, 19, 20, 24 and 25; Sedaghat et al., 2002). Therefore, it seems that the ATPase domain of *D*ATR-X has been conserved through evolution and that the other regions of the protein may have evolved to suit the specific needs of the cell. Different mechanisms of ATR-X function and different binding partners across species may account for the divergence of sequence with respect to the amino terminal and Q-rich repeats while the main chromatin remodeling aspects of ATR-X remain similar.

4.2. Dosage effects of *DATR-X* and *jing*

The *Glass multimer reporter* (*GMR*)-Gal4 is a commonly used driver for the ectopic expression of transgene in the developing eye as the *GMR* promoter is induced in third-instar eye-imaginal cells (Moses and Rubin, 1991). The fly eye is a valid system for targeting genes that function in other tissues (Raymond et al., 2004). Consistently, mis-expression of *jing* in the eye in combination with other genes potentially involved in *jing* transcriptional regulation disrupt ommatidial formation, a consequence probably due to alterations in gene expression (Sun et al., 2006). The genetic relationship between *D*ATR-X and *jing* in embryonic neurons and glia shows that the function of *D*ATR-X and *jing* in adult neuronal cells is relevant to *jing* function in the embryonic CNS.

Over-expression of one copy of *D*ATR-X in the *Drosophila* adult eye has no visible effect on the eye (Sun et al., 2006). However, the eye defects appear after an increased amount of *D*ATR-X protein is produced by expression of two copies of *D*ATR-X in the eye (Lee et al., 2007), suggesting that the development of the eye is sensitive to the gene dosage of *D*ATR-X. Jing alone is not sufficient to result in such defects. However, the rough eye phenotypes are observed in the flies mis-expressing both *jing* and *DATR-X* in the eye (Sun et al., 2006), suggesting a possible genetic interactions between the genes.

Over-expression of *D*ATR-X in *Drosophila* embryonic neurons with *Elav*-Gal4 also showed a dosage –dependency (Fig. 20), similar to the sensitivity of the adult eye to the amount of *D*ATR-X. Expression of one copy of *D*ATR-X is already sufficient to result in subtle defects in the CNS axon scaffold (Fig. 20C), in contrast to the non-effect

on the eye morphology in the fly carrying one copy of over-expressed *DATR-X*. Given that *D*ATR-X has an active role in multiple cellular functions, different cells may have a different degree of sensitivity to the dysregulation of its function which is revealed as different levels of tolerance to *DATR-X* expression levels. Similar to *D*ATR-X, over-expressing one copy of *jing* also results in subtle changes in CNS axonal patterning, which is exacerbated in the flies carrying over-expressed two copies of either *jing* or *DATR-X*. Much more severe defects are obtained after *jing* and *DATR-X* are co-overexpressed pan-neuronally, indicating a genetic synergistic effect.

Consistently, the importance of ATR-X dosage in normal development and cortex organization has been revealed in transgenic mice over-expressing the ATR-X protein in which are found growth retardation, neural tube defects and a high incidence of embryonic death (Bérubé et al., 2002).

Over-expression of *DATR-X* in the mushroom body, which is the substructure in the *Drosophila* brain essential for memory (Heisenberg, 1998), also reduces neuron number but doesn't result in axonal growth and guidance defects (Nicolaï et al., 2003). It is not clear whether additional factors are required for over-expressed *D*ATR-X proteins to induce axonal defects in the mushroom body. Interestingly, *jing* was not recovered from the screen to identify genes that are involved in mushroom body development (Nicolaï et al., 2003). It would be interesting to examine whether co-overexpression of *jing* and *DATR-X* or increasing dosages of *DATR-X* can result in axonal defects in the mushroom body.

4.3. *DATR-X* RNAi induced mutant phenotypes resemble those in genetic mutants

dsRNA was first found to be a potent and specific inhibitor of gene activity in *C. elegans* (Fire, et al., 1998). The similar effects of dsRNA were also found in other organisms including *Drosophila* (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999), zebrafish (Li et al., 2000) and mice (Wianny and Zernicka-Goetz, 2000). Injection of dsRNA into organisms is capable of silencing expression of the specific gene (Fire, et al., 1998; Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999; Li et al., 2000; Wianny and Zernicka-Goetz, 2000). RNAi introduced by injected dsRNA has been shown to induce a phenotype as potent as loss-of-function mutations in *Drosophila* (Kennerdell and Carthew, 1998). A conventional RNAi based screen identified *DATR-X* as a gene required for *Drosophila* embryonic CNS development (Koizumi et al., 2007). However, the dsRNA effect through injection into preblastoderm embryos is systemic (Koizumi et al., 2007); therefore gene interference is not restricted to a specific cell type and genotype-phenotype analysis cannot be carried forward in specific places at specific times. In contrast, our results not only linked *Drosophila* CNS phenotypes to *DATR-X*, but also attribute them to specific cell types by using transgenic RNAi (Kennerdell and Carthew, 2000). The method was adapted from *C. elegans* (Tavernarakis et al., 2000) and takes advantage of transgenes carrying hairpin forms of dsRNA to generate biological effects in *Drosophila* similar to RNAi by injected dsRNA in a controlled temporal and spatial pattern (Kennerdell and Carthew, 2000). The CNS has also been tested not refractory to the transgenic RNAi in many studies (e.g., Dietzl et al., 2007).

It was found that a sequence shared between several closely related genes may interfere with several members of the gene family (Kennerdell and Carthew, 1998). In our studies, the region selected for *DATR-X* dsRNAi production was from non-conserved sequence. Pan-neural or pan-glial *DATR-X* RNAi phenotypes resemble those in loss-of-*DATR-X* mutants, but were shown generally in a lower frequency than loss-of-*DATR-X* mutants. These were consistent with other reported applications of RNAi in that the RNAi phenotypes sometimes corresponded to the null phenotypes for classical loss-of-function mutations, but more often resembled hypomorphic phenotypes (Dietzl et al., 2007). The phenotypes varied significantly even between individuals carrying *DATR-X* RNAi. Similar phenomena were also found in assays with conventional RNAi, which may be due to the variability in the injected dose of dsRNA (Kennerdell and Carthew, 1998; Koizumi et al., 2007). Some phenotypes may be less sensitive to the level of gene activity; or abundant mRNAs may be more sensitive to interference than mRNAs of lower abundance (Kennerdell and Carthew, 1998). For transgenic RNAi, the variability between individuals carrying *DATR-X* RNAi may also be due to the irregularity or levels of expression by the driver lines and the concentration dependence of RNAi (Yang et al., 2000; Sun et al., 2006; Dietzl et al., 2007). A method to enhance transgenic RNAi potency is through over-expression of the RNAi machinery, *dicer-2* (*Dcr-2*) (Lee et al., 2004); however, co-expression of UAS-*Dcr-2* is likely to enhance off-targeting effects as well (Dietzl et al., 2007).

4.4. Neuronal and glial functions of *D*ATR-X and *jing* dictate longitudinal axon tract formation

During the formation of axonal tracts in wild type embryos, the midline repulsive cue Slit signals to the Robo receptor on ipsilateral fascicles to not cross the midline (Kidd et al., 1999). Therefore, contralateral misprojections of axons may be caused by loss of Slit/Robo signalling. In *DATR-X* mutants, and neural or glial mutants, ipsilateral axons project contralaterally and cross the midline, suggestive of a breakdown in midline repulsive mechanisms. Axon repulsion is controlled by Slit-Robo signalling (Sánchez-Soriano et al., 2007). It has been shown that growth cones that express *robo* will not enter and cross the midline where *slit* is expressed at high levels; therefore, in *robo* mutants axons cross and re-cross the midline, whereas in *slit* mutants, axons that normally are kept away from the midline enter and stay in the midline (Kidd et al., 1999; Rajagopalan et al., 2000a, b). Therefore, the Slit-Robo repellent mechanisms might be affected in the *DATR-X* mutants. However, defects in longitudinal pioneers are not only caused by a loss of repulsion (Garbe and Bashaw, 2007; Hiramoto et al., 2000; Hiramoto and Hiromi, 2006). Robo also suppresses relocalized Netrin preventing the latter from attracting longitudinal axons to the midline as they cross segment boundaries (Hiramoto and Hiromi, 2006). A failure to maintain this suppressed responsiveness, if occurred in *DATR-X* mutants, would also lead longitudinal axons to enter the midline.

In the wild type *Drosophila* embryonic CNS, longitudinal glia (LG) occupy characteristic positions and do not cross the midline (Ito et al., 1995; Beckervordersandforth, et al., 2008). This is controlled by multiple mechanisms at different developmental stages including response to repulsive and attractive molecules,

cell-cell contact, trophic support and axon contact (Kinrade et al., 2001). One can expect a disruption in any of these processes will perturb formation of the glial and axonal scaffolds. The expression of *DATR-X* and *jing* in LG correlates with the longitudinal glial phenotypes associated with mutations in these genes.

During stage 12, LG are kept away from the midline by a response of Robo present in these cells to Slit secreted from midline (Kinrade et al., 2001). However, the LG become irresponsive to the repulsion and mis-placed medially in *DATR-X* and *jing* mutants (Fig. 22; Sun et al., 2006). Similar phenotypes were found after *jing* and *DATR-X* are conditionally knocked down in all glial cells including LG, indicative of a breakdown in Robo-dependent repulsive mechanisms. However, the Robo protein was found still present in these embryos carrying reduced level of neuronal or glial *DATR-X* and *jing* (Sun et al., 2006), suggesting that *robo* expression may be independent from gene regulation controlled by *D*ATR-X and Jing. Alternatively, Jing and *D*ATR-X may regulate the expression of a factor that controls how Robo respond to the Slit signal. This is supported by the misrouting of axons across the midline in the presence of Robo occurs in *calmodulin* and *Son of sevenless* mutants where these proteins are required to process the Slit signal (Fritz and VanBerkum, 2000). It is also possible that *jing* and *DATR-X* regulate the expression of factors controlling glial and neuronal positioning in a Robo-independent fashion (Kinrade et al., 2001).

DATR-X and *jing* mutations affect more than Robo-mediated LG positioning. First, glial survival is not affected in *robo* mutant embryos whereas glial number is reduced in *DATR-X* and *jing* mutants, as well as in *DATR-X* and *jing* glial-specific mutants (Sun et al., 2006). It was shown that continuous axonal contact was still

maintained in embryos carrying reduced level of glial *DATR-X* and *jing* (Sun et al., 2006); therefore, the loss of CNS glia may reflect a breakdown in an intrinsic survival pathway mediated by *DATR-X* and *jing*. The expression of *DATR-X* and *jing* in glia is consistent with such a role. Furthermore, both *jing* and *DATR-X*/ATR-X have been implicated in cell survival processes in the CNS midline and tracheal cells and in cortical progenitors, respectively (Bérubé et al., 2005; Sedaghat et al., 2002; Sonnenfeld et al., 2004).

Second, in *robo* mutants only the innermost fascicle but not the outer two longitudinal fascicles are affected. The *robo* mutant phenotype can also result from the derepression of the responsiveness to Netrin (Hiramoto and Hiromi, 2006). However, in *DATR-X* and *jing* mutants or glial and neuronal mutants, the outer fascicles are fused, often broken and can be seen crossing the midline. These defects are similar to those after neural or glial ablation and after genetic loss of glia as in *gcm* mutants (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996; Hidalgo and Brand, 1997). These observations suggest that multiple biological processes require the proper function of these genes and are consistent with an important upstream role for *jing* and *D*ATR-X in glial and neuronal differentiation.

In summary, *D*ATR-X and *jing* genetically interact in adult neuronal cells, consistent with the specific neuronal and glial developmental defects which underlie the problems in axon guidance associated with mutations in *DATR-X* and *jing*.

4.5. Cellular function of *D*ATR-X

DATR-X is expressed throughout the entire embryonic stages and in both neurons and glia. Analysis of pan-neural and pan-glial expression of wild-type and RNAi transgenes revealed an axon guidance function for *D*ATR-X. However, *Elav*-gal4 has been recently reported not to be a true pan-neural driver since *Elav* expression was detected in both neurons and glia (Berger et al., 2007). Therefore a secondary effect from glia was not taken into account when the interpretation was sought for the phenotypic analysis of pan-neural over-expression of *DATR-X* (Sun et al., 2006). Furthermore, these studies did not address the role of *D*ATRX in specific neuronal or glial lineages known for their roles in guiding CNS axons. *D*ATR-X expression was found expressed in MP2 pioneer neurons and LG which both are important for *Drosophila* embryonic CNS axon formation (Goodman and Doe, 1993). *D*ATR-X expression is not restricted to MP2 neurons and LG; though, identifying its function in these specific cell types would no doubt enable us to better appreciate the role for *D*ATR-X in CNS axon formation. To define this more accurate cellular role for *D*ATR-X, *DATRX* expression levels were selectively altered in the MP2 neurons and LG using two previously characterized Gal4 drivers. Having confirmed the presence of nuclear *D*ATRX in the MP2 and LG lineages, *15J2*-Gal4 and *heartless* (*htl*)-Gal4 were used to selectively knockdown *DATRX*. Axon guidance phenotypes associated with altered *DATRX* expression were assessed using antibodies that label large groups of commissural and longitudinal axons and antibodies that identify the ipsilateral and contralateral projections of the MP2 and other pioneer neurons.

Previous cell ablation experiments showed that removal of MP2 neurons by targeting toxin to these cells with *15J2* affects the longitudinal pathways at stage 14 and results in occasional misrouting across the midline at stage 17 (Hidalgo and Brand, 1997). Also, targeting disruption of *Drosophila* Neuregulin Vein by RNAi in MP2 neurons using *15J2* is able to induce longitudinal glial apoptosis (Hidalgo et al., 2001), suggesting neuron-glial interaction can be disturbed through reduced protein levels in these cells. Therefore, axonal phenotypes associated with knocking down *DATR-X* specifically in MP2 neurons may result if *D*ATR-X plays a role in repulsive pathways. However, knocking-down *DATR-X* in these neurons was not sufficient to cause an obvious effect, suggesting that *D*ATR-X in MP2 neurons may not be definitely required with regard to axon formation and cell survival. It should be noted that *15J2* driven expression was first detectable at stage 12/1 (Hidalgo and Brand, 1997), while *D*ATR-X is already present in these neurons at stage 11 (Fig. 27). Therefore, the data here does not rule out that *D*ATR-X in these cells is involved in initial axon extension.

Knocking down *DATR-X* expression in all glia is associated with reduction in the number of glia and their mispositioning at the CNS midline (Sun et al., 2006). This was consistent with the abnormal glial biology observed in four different homozygous *DATR-X* mutant alleles, supporting a requirement of *D*ATR-X for glial survival and positioning. This role can be assigned at least in some part to LG since the embryos carrying longitudinal glial *DATR-X* RNAi phenocopied *DATR-X* mutants and pan-glial *DATR-X* RNAi (Sun et al., 2006). However, the *htl*-Gal4 driven expression is not found in longitudinal glioblasts and starts at stage 13 (Hidalgo et al., 2001). The effects assessed in our study therefore occur late in the lineage.

Since the longitudinal connectives are pioneered at the end of stage 12, *htl*-Gal4 would drive expression of *DATR-X* RNAi in the LG after the pioneer growth cones have started to grow out. At this time, longitudinal glioblast fate has been determined and LG have already established contact with the pioneer neuron cell bodies and axons. Knocking-down *DATR-X* specifically in LG leads to disorganization of LG, suggesting that *D*ATR-X in LG is required for these cells to be correctly positioned. However, LG already become dependent on neurons for their survival at stage 13 (Kinrade et al., 2001). Interestingly, defects in the longitudinal tracts were also found in these embryos carrying LG-specific *DATR-X* RNAi. This argues that the *D*ATR-X function in LG are necessary for longitudinal tract formation even after the axon scaffold has been initiated by the pioneer growth cones at stage 12 (Hidalgo and Booth, 2000). Therefore, *D*ATR-X function in LG might be necessary to maintain the axon scaffold established by the pioneer neurons. This may be supported by the similar defects in the longitudinal tracts after the LG were ablated at or after stage 13 (Hidalgo et al., 1995).

4.6. The N-terminus of *D*ATR-X is sufficient for its nuclear localization

*D*ATR-X contains SNF2 domain and Helicase conserved C-terminal domain (Helicase C) (Sun et al., 2006), which both are found in those proteins implicated in processes which occur in nuclei (Eisen et al., 1995; Caruthers and McKay, 2002). A prerequisite to enable the involvement of *D*ATR-X with these nuclear events would be that *D*ATR-X has a nuclear subcellular localization. For a protein such as ATR-X which is distributed at multiple places, the identification of the region required for its subcellular

localization could be indicative of its specific function or molecular interaction. The inter-relationship between these regions and other functional domains can be investigated only after they were identified (Bérubé et al., 2007). We found that the N-terminal region prior to the two conserved domains is sufficient for *D*ATR-X to be shipped into nuclei, consistent with the functions of both SNF2 and Helicase C domains being most likely involved with DNA-related events within nuclei. Interestingly, relative N-terminal region carrying nuclear localization signals in vertebrate ATR-X are also identified (Bérubé et al., 2007). This may provide another level of conservation for ATR-X proteins.

4.7. The functional role of Helicase C domain for *D*ATR-X LG function

The Helicase/ATPase domain shows a high occurrence of mutations in ATR-X patients and is highly conserved in evolution (Gibbons et al., 2008; Sun et al., 2006). Though this domain has been shown required for its chromatin remodeling activity (Xue et al., 2003; Tang et al., 2004), its contribution to ATR-X function in cellular processes is unknown. To address this issue, the Helicase C domain-containing region was removed from the C-terminus of *D*ATR-X and the truncated form was over-expressed specifically in LG. The deleted C-terminal region contains no other domain except a Helicase C domain defined by Prosite patterns (Hulo et al., 2004). This domain may be an integral part of helicase-related motifs to utilize the energy of ATP-hydrolysis for the remodeling of nucleic acid or protein-nucleic acid structure (Xue et al., 2003; Dürr et al., 2006). Therefore, if *D*ATR-X$^{\Delta HelC}$ were able to assemble into *D*ATR-X regulatory complexes at

target sites then endogenous associated activity may be reduced resulting in a mutant effect. This may indicate a contribution of the *D*ATR-X C-terminus in its regulatory activities. Indeed, high levels of *DATR-X*$^{\Delta HelC}$ specifically in LG resulted in a loss of LG repulsion and the presence of *DATR-X*$^{\Delta HelC}$ in nuclei supports the possibility that truncated *D*ATR-X is in the right location to form regulatory complexes. However, it is noted that this should be supported by assays to show that the Helicase/ATPase domain of *D*ATRX has ATP-dependent DNA translocase activity which can be attenuated or abolished by point mutations in Helicase domain (Xue et al., 2003; Tang et al., 2004).

Presently, we have shown that a subtype of glial cells in the *Drosophila* embryonic CNS were less tolerant to over-expressed full-length *D*ATR-X than a mutant form of *D*ATR-X without the C terminus. LG-specific over-expression of full-length *DATR-X* was associated with a higher penetrance and expressivity of repulsion defects and gaps than that of *DATR-X*$^{\Delta HelC}$. Similar phenotypes were also found in embryos carrying reduced *D*ATR-X in LG. This may be due to that the absence of the C terminus might have negative effects upon the stability of either the *D*ATR-X or the regulatory protein complex which contain *D*ATR-X (see discussion below), or both. Nevertheless, more studies are required to define the contribution of the Helicase/ATPase domain for *D*ATR-X to the repulsion of LG. For example, to generate point mutations in Helicase/ATPase domain and test whether over-expression of wild-type and point-mutated forms of *D*ATR-X are able to rescue *D*ATR-X loss-of-function in LG.

4.8. Are *D*ATR-X and Jing members of a protein complex?

Evidence is accumulating that chromatin accessibility plays a key role in the transcriptional regulation of cell-type-specific gene expression in the CNS (Hsieh et al., 2004; Huang et al., 1999). The region of highest sequence conservation correlated with ATPase domains between human and *Drosophila*, along with the similarities in the phenotype of *D*ATR-X and *jing* mutations and in their expression patterns raises the possibility that Jing is involved in the targeting of a chromatin remodeling complex containing *D*ATR-X to transcriptional target genes whose products are required for the response of longitudinal growth cones and glia to midline guidance cues.

Jing is a good candidate as a targeting protein since synergistic effects have been shown by knocking down Jing and *D*ATR-X in neurons (Sun et al., 2006). Both over-expression and loss-of-function *D*ATR-X mutants displayed disruptions in CNS axonal patterning. These results are consistent with *jing* gain and loss-of-function CNS phenotypes (Sedaghat et al., 2002). This non-linear dosage sensitivity is not in disagreement with the importance of a stoichiometric balance among protein components in a complex (Veitia et al., 2008). This can be possible if Jing and *D*ATR-X function in a form of protein complex. The integrity of the protein complex depends on each component, because LOF (or GOF) mutations in any one member may lead to destabilization of the whole complex. Therefore, the LOF and GOF phenotypes are not necessarily opposite in many cases. This has become more likely given some accumulated evidences. First, Jing and *D*ATR-X are both nuclear proteins (Fig. 19 and 29; Sedaghat et al., 2002). Their domain structures suggest that they are both involved with DNA related events, which usually require orchestration of many proteins (Näär et

al., 2001). Second, Jing has been found associated with Trachealess (Trh) / Tango (Tgo) to bind to the promoter region of the *fibroblast growth factor receptor homolog* (Sonnenfeld et al., submitted). Though this occurs in another distinctive biological context, it is likely Jing is associated with different activators/repressors in the CNS. The function and composition of transcriptional regulation complexes may be different in a tissue-specific and target gene-specific manner (Otte and Kwaks, 2003; Strutt and Paro, 1997). Third, the Zn finger domain present in the vertebrate ATR-X is missing in *D*ATR-X. Jing encodes a nuclear protein with putative DNA-binding and transcriptional regulatory domains (Sedaghat et al., 2002; Liu and Montell, 2001). The mouse homologue of *jing*, *adipocyte enhancer binding protein 2* (*AEBP2*), has strong expression in the brain and has been shown to function as a potential targeting protein (He et al., 1999; Kim et al., 2009). Furthermore, *D*ATR-X belongs to the SWI/SNF chromatin remodeling protein family whose functioning normally requires sequence-specific activators (Lusser and Kadonaga, 2003). It has been shown that ATR-X transcription repression is targeting-dependent because ATR-X by itself does not inhibit *luciferase* expression (Tang et al., 2004). ATR-X is recruited by different targeting proteins and directed to different places. For example, ATR-X is recruited by HP1 in most tissues and MeCP2 in postmitotic neurons to pericentrometic heterochromatin and recruited by Daxx to promyelocytic leukemia nuclear bodies (PML NBs) (McDowell et al., 1999; Kourmouli et al., 2005; Nan et al., 2007; Xue; 2003; Ishov et al., 2004; Tang et al., 2004).

There also exists the possibility that Jing and *D*ATR-X function in parallel pathways which converge on the same downstream process such as axon formation and glia behaviour (Sun et al., 2006). Testing the genetic relationship between *jing* and

DATR-X is difficult as maternal supplies rescue zygotic phenotypes. Co-immunoprecipitation did not show an ability for full-length *D*ATR-X to pull down truncated versions of Jing that collectively span the whole Jing protein in S2 cells (Fig. 26). These two versions of Jing were also tested by ChIP at the FGFR promoter and only Jing2, which contains the zinc finger (Fig. 26A), was capable of an association in the presence of Trh and Tgo (Sonnenfeld et al., submitted). Considering usually only those most robust interactions survive co-immunoprecipitation studies, the possibility has yet to be confirmed that Jing and *D*ATR-X belong to one protein complex. There might also exist other endogenous factors that are required to ensure proper conformation. It would not be a surprise to identify a variety of accessory proteins that interact through purified preparations and genetic procedures. For example, these may have been identified in the genetic screen for Jing coactivators (Table 1; Sun et al., 2006). A similar strategy can also be applied for *D*ATR-X since wild type and mutant forms of *D*ATR-X have a phenotype when over-expressed in the *Drosophila* embryonic CNS. The transgenic tools developed in this study can also be used to introduce mutant (or wild type) *D*ATR-X into different genetic background to search for those factors able to suppress or enhance the phenotype.

4.9. Targets of ATR-X proteins

Though it has long been suggested a role for ATR-X in gene repression, α-globin is still the only confirmed transcriptional target of regulation by human ATR-X (Steensma et al., 2005). Additional targets of ATR-X remain to be identified. Therefore,

it is not clear whether ATR-X proteins only play a role in gene repression or maybe also involved in gene activation. The dual roles for SWI2/SNF2 in both gene activation and gene repression have been demonstrated (Schulze and Wallrath, 2007). Considering that the function of SWI2/SNF2 is to alter nucleosome positions in the promoter region of genes, the shift can promote activation in some cases, whereas in other cases it may prevent gene activation by covering *trans*-acting factor binding sites (Schulze and Wallrath, 2007).

Jing encodes a nuclear protein with putative DNA-binding and transcriptional regulatory domains (Sedaghat et al., 2002; Liu and Montell, 2001). Further genetic studies suggested that *jing* may interact with fly Polycomb Group (PcG) protein complexes, which are required to maintain gene-expression patterns of important developmental regulators like the *hox* genes by repression (Culi et al., 2006; McClure and Schubiger, 2008). The mouse homologue of *jing*, *AEBP2*, which has strong expression in the brain, is a component of the EED-EZH2 PcG protein complex (He et al., 1999; Cao et al., 2002; Cao and Zhang, 2004). AEBP2 can interact with other components of PcG, including EED, EZH2, Su(z)12, and may function as a targeting protein (Cao and Zhang, 2004; Cardoso et al., 1998; Kim et al., 2009). Many of AEBP2's target loci are co-occupied by AEBP2 and Su(z)12 (Kim et al., 2009). A functional homology would be better demonstrated if AEBP2 can compensate for a mutation in the corresponding *Drosophila jing* gene. Interestingly, ATR-X has been shown interact with EZH2 *in vitro* (Cardoso et al., 1998). If further evidence can be obtained showing *D*ATR-X in association with PcG in a manner with or without the

involvement of Jing, a role in gene repression could be postulated for *D*ATR-X (Ringrose and Raro, 2004).

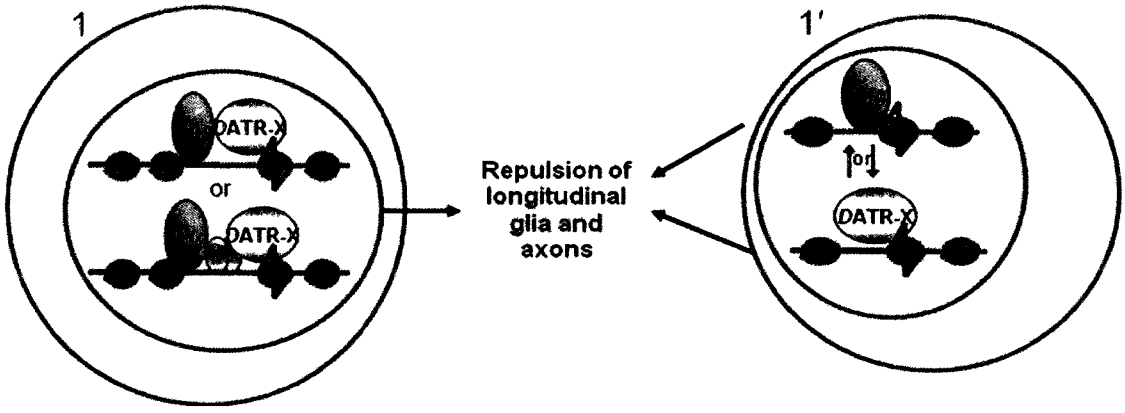
During embryogenesis, general heterochromatic silencing is initiated by proteins such as HP1 (Schulze and Wallrath, 2007). Heterochromatin is cytologically and molecularly different from euchromatin which encompasses gene-rich regions and is associated with transcriptional activity (Grewal and Elgin, 2002). In contrast, heterochromatin is gene-poor with transcriptional repression/silencing. ATR-X has been shown to be recruited by its interaction with HP1 to heterochromatin (McDowell et al., 1999; Lechner et al., 2005). In *Drosophila*, *D*ATR-X has also been shown to co-localize and interact with *Drosophila* HP1 (Bassett et al., 2008). Therefore, a role in heterochromatin formation for *D*ATR-X is also possible.

Therefore, *D*ATR-X mutant phenotypes may be associated with altered regulation of chromatin structure and/or transcription mechanisms that result in a deregulation of gene expression and abnormal expression of genes in the *Drosophila* embryonic CNS. This would be expected to result in defective CNS phenotypes shown as disrupted axonal scaffolds and neural and glial positioning (Fig. 34).

4.10. Multi-functionality of ATR-X proteins

The wide spectrum of phenotypes associated with ATR-X mutations suggests that ATR-X may be involved in many cellular and molecular processes. An association of ATR-X proteins with apoptosis has also been demonstrated.

A


1
DATR-X
or
DATR-X
Repulsion of longitudinal glia and axons
1'
or
DATR-X

B

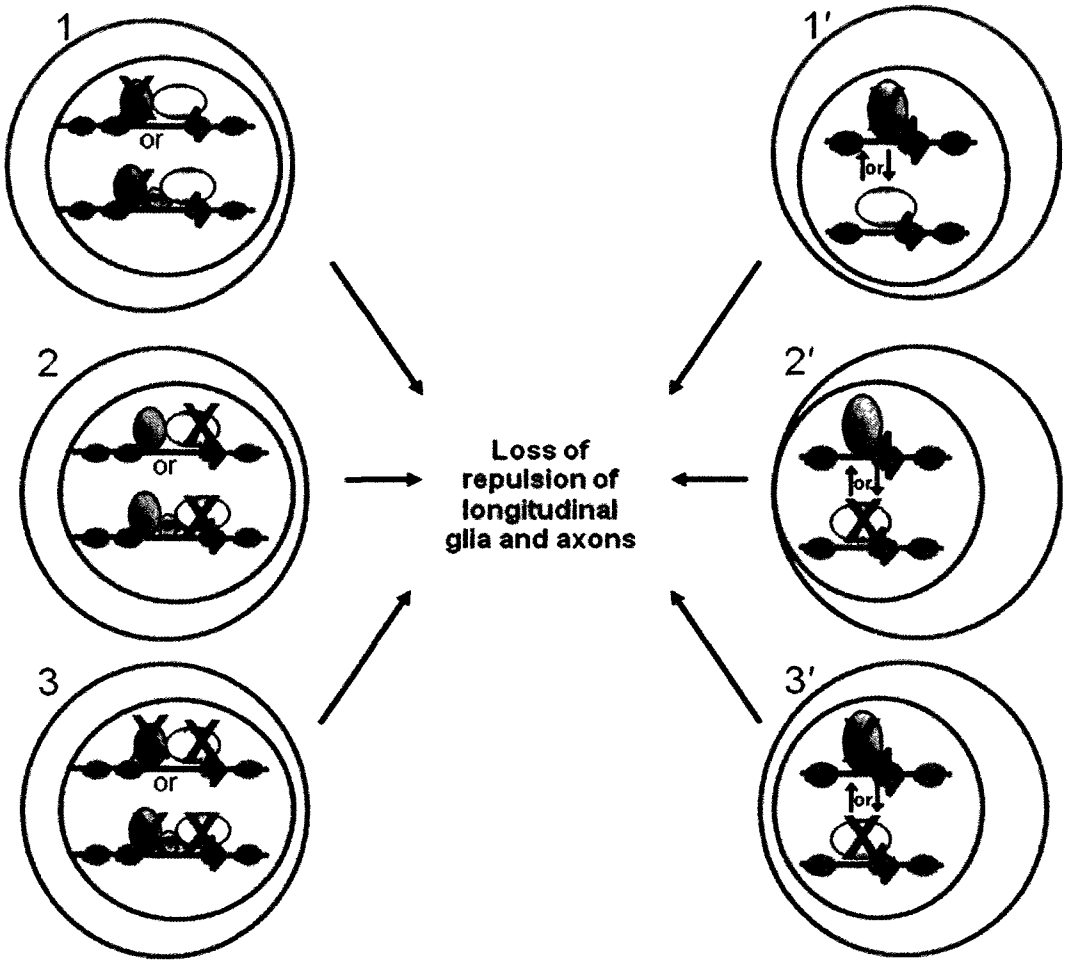

1
or
2
or
3
or
Loss of repulsion of longitudinal glia and axons
1'
or
2'
or
3'
or

Figure 34. Model of *D*ATR-X-Jing LG function. Based on the studies in this thesis, a model is postulated that downstream gene expression controlled by *D*ATR-X and Jing facilitates axon guidance and cell survival which ultimately allows for proper connectivity in the developing *Drosophila* CNS. (A) In wild-type embryos, *D*ATR-X and Jing ensure the repulsion of longitudinal glia and axons. (A1) They may function within a nuclear protein complex either in contact or not. (A1′) They may function in parallel. In this case, *D*ATR-X and Jing function may have different targets. When they have different targets, it is also possible that one functions upstream of the other (indicated by the arrows between the two genes). (B) When *D*ATR-X (B2 or B2′) or Jing (B1 or B1′) or together (B3 or B3′) are knocked-down, the repulsion of longitudinal glia and axons is lost.

Over-expression of *DATR-X* affected embryonic CNS axon patterning (this study), resulted in fewer neurons in the mushroom bodies (Nicolaï et al., 2003), and induced apoptosis in the developing imaginal discs (Lee et al., 2007), consistent with observations that ATR-X over-expression in mouse brain results in neural tube defects (Bérubé et al., 2002). Loss-of-*DATR-X* affects the development of *Drosophila* embryonic CNS neurons and glial survival (this study). Embryonic injection of *DATR-X* RNAi results in disruption of the ventral nerve cord and reduction of the intersegmental and segmental nerves (Koizumi et al., 2007). Consistently, conditional ablation of ATR-X in the mouse forebrain caused neural apoptosis and hypocellularity in the mutant brain (Bérubé et al., 2005; Seah et al., 2008). ATR-X is also important for the survival of interneurons of the retina (Medina et al., 2009). It was found that over-expression of *DATR-X* increases apoptosis in the developing eye, wings and neurons via the Jun-N-terminal kinase (JNK) pathway (Lee et al., 2007; Hong et al., 2009; Dhanasekaran and Reddy, 2008). ATR-X proteins are found in promyelocytic leukemia nuclear bodies (PML-NBs) (Ishov et al., 2005), which have been implicated in multiple cellular processes including apoptosis (Krieghoff-Henning and Hofmann, 2008). The localization of ATR-X to PML-NBs occurs by its interaction with Daxx, a multifunctional apoptosis-related protein (Ishov et al., 2005; Salomoni, P. and Khelifi, 2006). Despite this accumulated data, the precise roles for ATR-X proteins in apoptosis remain to be determined.

4.11. In summary, this thesis work through implementation of combined classic genetics and modern molecular biology and various techniques, has added plenty of content to portray *D*ATR-X function from the genetic perspective, cellular function, and

its molecular properties. The extent of conservation of the protein throughout evolution suggests that *D*ATR-X may serve a conserved role for its cellular function. And the conservations shown at various levels, from genetic property, molecular biology to cellular role, assure us to expect some conservation up to behavioural level. In order to fully understand the function of *D*ATR-X, diverse behavioural assays should be done at the identified cell level. These assays must have a genetic basis. All the functional defects in the nervous system explicated by behavioural analysis can be associated with specific CNS cell types. A collection of behavioural paradigms has been established (http://www.sdbonline.org/fly/aimain/6behavior.htm) to monitor a wide spectrum of fly behaviours and this no doubt will increase the power to decipher pathways and the cellular bases of ATR-X syndrome.

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