Phenotypic analysis of Pontin and Reptin mutations in the embryonic *Drosophila* nervous system

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Raunvísindadeild
Háskóli Íslands
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Svipgerðagreining Pontin og Reptin stökkbrigða
í taugakerfi *Drosophila* fóstra

Heiður Grétarsdóttir

16 eininga ritgerð sem er hluti af
Baccalaureus Scientiarum gráðu í Lífefnafræði

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Raunvísindadeild
Verkfræði- og náttúruvísindasvið
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I hereby declare that this thesis is based on my own observations, is written by me and has neither in part nor as whole been submitted for a higher degree.

Hér með lýsi ég því yfir að ritgerð þessi er samin af mér og hún hefur hvorki að hluta til né í heild verið lögð fram áður til hærri prófgráðu.

Reykjavík, maí 2013

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Útdráttur

Pontin og Reptin eru tvö nátengd protein sem hafa hlutverki að gegna í ýmsum frumutengdum ferlum. Bæði próteinin tilheyra AAA+ fjölskyldu ATPasa og hafa hliðstæður í gersveppi, plöntum og dýrum. Þau eru tengd mörgum fjölpróteina complexum, þar sem þau eru talin starfa aðallega sem siðvörslu- eða tengi-prótein. Í þessu verkefni eru hlutverk þessarra áhugaverðu próteina í þroskun taugakerfisins skoðuð með því að nota *Drosophila melanogaster* sem tilraunalífveru. Mótefnalitanir voru framkvæmdar til þess að sjá áhrif *pontin* og *reptin* stökkbreytinga á mismunandi hluta fóstur-taugakerfisins. Í þessarri skýrslu fer ég yfir fyrri rannsóknir á Pontin og Reptin, og lýsi niðurstöðum úr núverandi rannsókn.

Abstract

Pontin and Reptin are two closely related proteins involved in various cellular mechanisms. Both are members of the AAA+ family of ATPases and have orthologs in yeast, plants and animals. They are associated with several multiprotein complexes in which they are currently thought to act mainly as chaperones or adaptor proteins. In this project, the role of these intriguing proteins in the development of the nervous system was examined using *Drosophila melanogaster* as a model organism. Immunolabeling was employed to visualize the effect of *pontin* and *reptin* mutations on different components of the embryonic nervous system. In this report I briefly review previous studies of Pontin and Reptin, and describe the results of the current study.

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Abbreviations

AAA+	ATPases associated with diverse cellular activities
Anti-HRP	anti-horseradish peroxidase
Arm	Armadillo
Arp5	Actin-related protein 5
ATM	Ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bp	base pair(s)
BRM	Brahma
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CREBBP	cAMP-response element-binding protein
cryo-EM	cryo-electron microscopy
DKC1	TERC binding protein dyskerin
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
E-boxes	enhancer box sequences
ELAV	Embryonic lethal, abnormal vision
F2	second filial
ftz	*fushi tarazu*
GFP	Green fluorescent protein
HAT	Histone acetyltransferase
HSP90	Heat shock protein 90
lik	*liebeskummer*
mAb	monoclonal antibody
MAP1B	Microtubule-associated protein 1B
MG	midline glia
MP1	midline precursors
mTORC1	mammalian target of rapamycin
NBs	neuroblasts
NMD	nonsense-mediated mRNA decay

No.	number
NuA4	nucleosomal acetyltransferase of H4
PAF	Polymerase II associated factor
PBS	phosphate-buffered saline
PBT	PBS with Triton-X-100
PcG	Polycomb group
PIH1	Protein interacting with HSP90
PIH1D1	PIH1 domain-containing 1
PIKK	Phosphoinositide three-kinase-related kinase
PNS	peripheral nervous system
PRC1	Protein regulator of cytokinesis 1
pre-rRNA	pre-ribosomal RNA
RNA	ribonucleic acid
RNAP II	RNA polymerase II
RNP	ribonucleoprotein
RPAP3	RNA Polymerase II Associated Protein 3
rRNA	ribosomal RNA
RSB	reticulocyte standard buffer
RT	reverse transcriptase
Sb	stubble
SDS	sodium dodecyl sulfate
Ser	serrate
snoRNAs	small nucleolar RNAs
snoRNPs	small nucleolar ribonucleoproteins
snRNA	small nuclear RNA
SRCAP	SNF2-related CREBBP activator protein
ssDNA	single-stranded DNA
SWR1	SWI/SNF2-related
TAH1	TPR-containing protein associated with HSP90
TCF	T-cell factor
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TIP60	Tat-interactive protein 60 kDa

TOR	Target of rapamycin
tRNA	transfer RNA
TRs	telomeric retrotransposons
TrxG	Trithorax group
TTT	Tel2-Tti1-Tti2
UAS	upstream activating element
VUM	ventral unpaired median
WT	wild type
β-gal	β-galactosidase

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1 Introduction

1.1 Pontin and Reptin

1.1.1 General

Pontin (also known as RUVBL1 in human and Rvb1 in yeast) and Reptin (also known as RUVBL2 or Rvb2) are two closely related proteins which have been gaining attention since their discovery in the late 1990s. Both proteins are members of the AAA+ (adenosine triphosphatase (ATPase) associated with diverse cellular activities) family of ATPases and have been conserved from yeast to human (Diop et al., 2008). Both proteins are expressed in the nervous system of *Xenopus* (Etard et al., 2000), mouse embryos (Chauvet et al., 2005) and *Drosophila* (Chintapalli et al., 2007), although their exact roles there have not yet been determined. The proteins have been found to form part of various multiprotein complexes, and are sometimes found in lower quantities than other components of the complexes, which is one of the reasons it has been proposed that they act mainly as chaperones or adaptor proteins (Nano & Houry, 2013). They are usually found together in the same complexes but can have opposing effects within them especially in mechanisms of transcription control (Rosenbaum et al., 2013). They form part of several complexes involved in transcriptional regulation, such as the INO80, SCRAP, TIP60 and URI1 complexes in animals (Gallant, 2007) and have recently been shown to regulate all members of the PIKK family (Izumi et al., 2010). Both proteins also associate with HSP90 through the R2TP complex and have roles in snoRNP biogenesis (Jha et al., 2008). The *Drosophila* Pontin and Reptin proteins are 73 and 77% identical to their human ortholgues and 41% identical to each other (Bauer et al., 2000). They have mainly been studied in the context of signaling activity of oncogenic factors such as β-catenin and c-Myc and their roles as antagonistic mediators of *Drosophila* Hox gene transcription (Diop et al., 2008). Pontin and Reptin have non-redundant functions in all systems analyzed so far leading to a lethal phenotype if either gene is mutated (Diop et al., 2008; Gallant, 2007).

1.1.2 Expression in the nervous system

Little is known about the roles of Pontin and Reptin in the nervous system. In *Xenopus* both proteins were found to be expressed in neural crest cells, especially in the ones that later go on to form the adrenal medulla (Etard et al., 2000). In mouse embryos, the two proteins were found to be expressed in the neuroepithelium of the neural tube. Whole-mount *in situ* hybridization on dissected spinal cords of mouse embryos showed the expression of both proteins in motoneurons from cranial and spinal regions and Reptin expression extended dorsally in other neuronal cell types from thoracic and lumbar regions of the spinal cord (Chauvet et al., 2005). Research has also shown that Reptin is expressed during *in vitro* neural differentiation of human embryonic stem cells (Barthelery et al., 2009). Here, the embryonic nervous system of *Drosophila pontin* and *reptin* mutants, was analyzed by performing immunofluorescent stainings on the published null alleles $pont^{5.1}$ and $rept^{35}$ (Bauer et al., 2000), as well as several transposable element insertions in the 5' region of the two genes (see table 2.1.1). The aim was to find out whether loss of either gene caused any visible nervous system phenotype in the embryo and to find out whether the insertions affected *pontin* or *reptin.*

1.1.3 Structure

The crystal structure of human Pontin has been resolved with 2.2 Å resolution. The protein assembles into hexamers, forming a ring with an 18 Å wide central channel, wide enough to fit ssDNA (*single-stranded DNA)* but too small for dsDNA *(double-stranded DNA) (Gallant, 2007)*. The Pontin monomer contains three domains. Domains I and III function in ATP binding, and domain I also mediates ATP hydrolysis (Rosenbaum et al., 2013). Domain II is located between the conserved Walker A and Walker B sequence motifs in domain I, which are well known for their roles in ATP binding and hydrolysis (Gallant, 2007). Domain II has been found to bind *in vitro* to ssDNA, dsDNA and RNA (Chiraniya A, 2013; Gallant, 2007). The role of this 170 amino acid insertion still remains unclear although studies suggest that it serves in the regulation of ATPase and helicase activities of domains I and III (Gallant, 2007; Rosenbaum et al., 2013).

The crystal structure of Reptin has not yet been published, but the protein has been found to form a ~400 kDa homo-oligomer under certain conditions (Puri et al., 2007). X-ray and cryo-electron microscopy (*cryo-EM*) analyses have shown that together, Pontin and Reptin form a dodecameric complex. (Gorynia et al., 2008; Torreira et al., 2008) The

complex is known to consists of two hexameric rings, but it remains uncertain whether they are homo- or hetero-oligomeric, or if both types exist. cryoEM analysis has revealed a structure where the two rings display distinct conformations (Torreira et al., 2008), but different structures have also been published (Gribun et al., 2008; Puri et al., 2007). The dodecamer contains equimolar amounts of Pontin and Reptin and has a 26 Å wide central channel, wide enough to fit dsDNA (Gallant, 2007). The two hexameric rings interact through the ATPase-insert domains of each ring.

1.1.4 Chromatin remodeling

The information needed for the development and function of all living organisms is encoded in the DNA. In the nucleus, DNA and a variety of proteins, mainly histones, associate to form chromatin. Histones are alkaline proteins that function in the packaging of DNA into nucleosomes (the repeating units of eukaryotic chromatin), and play an important role in the regulation of gene expression. Five histone variants have been identified in canonical nucleosomes: H1 (or H5), H2A, H2B, H3 and H4. Approximately 147 base pairs of DNA are coiled around a histone octamer composed of the core histones H2A, H2B, H3 and H4. One or more histone variants (e.g. H2A.Z) can be found in non-canonical nucleosomes where they replace the canonical histones. In order for mechanisms such as DNA transcription, replication and repair to take place, the machinery related to these processes must be able to access the tightly packed DNA (Prescott et al., 2005; Rosenbaum et al., 2013). Chromatin remodeling complexes are therefore essential for the modulation of these processes, since they control the access of the DNA repair and transcriptional machinery by changing nucleosome positioning along the DNA (Griffiths, 2012).

The INO80 complex

Pontin and Reptin have been found to associate with several chromatin remodeling complexes. The INO80 complex is involved in the control of expression of a large number of genes. It is thought to function by mobilizing nucleosomes and altering accessibility of the transcription machinery to the underlying DNA. The complex has been well studied in yeast, where it is recruited to promoters, and functions in either activation or repression of a multitude of genes (Gallant, 2007). Yeast INO80 shares the same core subunits as the human version of the complex although both have distinct additional subunits. The subunits they share are: Pontin, Reptin, ACT1, IES2, IES6, ARP4, ARP5, ARP8 and the

INO80 helicase (Nano & Houry, 2013). The complex uses energy from ATP hydrolysis for its remodeling functions. By studying the INO80 complex in yeast it has been determined that Pontin and Reptin are not essential for the recruitment of the INO80 helicase to promoters or for the ATPase activity of the complex, but without them the INO80 complex loses the Actin-related protein 5 (Arp5), needed for its chromatin remodeling activities (Jonsson et al., 2004). Pontin and Reptin, might therefore act as assembly factors by directing the incorporation of Arp5 in the complex. Similar roles of the proteins have been discovered in the SWR1 complex, discussed in the following section (Conaway & Conaway, 2009).

The SRCAP/SWR1 complex

Many of the subunits in the INO80 complex, specifically ACT1, ARP4, Pontin and Reptin, are also found in the SNF2-related CREBBP activator protein (SRCAP) in vertebrates, and the SWI/SNF2-related (SWR1) complex in *S. cerevisiae*. In addition, the SWR1 complex shows further similarity to the INO80 complex, as the ATPase domain of the SWR1 protein (structural component of the complex which exchanges the histone variant H2A.Z for chromatin-bound histone H2A) has been shown to bind Pontin, Reptin, ARP6, SWC2, SWC3 and SWC6. Thus, it is not unlikely that Pontin and Reptin function in the assembly of the of the SWR1 complex, similar to the INO80 complex (Nano & Houry, 2013; Wu et al., 2005). Both SRCAP and the SWR1 complex catalyze ATP-dependent exchange of histone H2A/H2B dimers containing the histone variant H2A.Z into nucleosomes (Jha et al., 2008). Without the H2A.Z variant, heterochromatin spreads into the euchromatin where it silences the expression of local genes (Gallant, 2007). In *Drosophila,* the functional homolog of SRCAP is called DOM, and it is encoded by the *domino* gene (Eissenberg et al., 2005).

TIP60 complex

Another complex containing Pontin and Reptin that functions in transcriptional control is the TIP60 complex. In contrast to the ATPase mediated remodeling of the INO80 and SCRAP complexes, TIP60 is a histone acetyltransferase (HAT) remodeler. The complex shares many subunits with the SWR1 complex mentioned previously, and the Nucleosomal Acetyltransferase of H4 (NuA4) complex. One of its functions is to allow DNA transcription to take place by converting chromatin into euchromatin by histone acetylation. TIP60 HAT is also needed for ataxia telangiectasia mutated (ATM) protein

kinase activity in response to DNA damage. ATM and ATR protein kinases phosphorylate the histone variant H2AX when DNA is damaged and the phospho-H2AX then acts by recruiting other proteins to the DNA damage site for amplification of the damage signal or repair of the damage. Human Pontin and TIP60 are needed for the removal of phospho-H2AX in cells after DNA damage, and the HAT activity of TIP60 has been shown to require Pontin. Remodeling of phospho-H2AX-containing nucleosomes at DNA damage sites seems to require TIP60 *in vivo*. In *Drosophila*, remodeling of nucleosomes containing phospho-H2AV (the fly equivalent of phospho-H2AX) has also been shown to require TIP60 (Jha et al., 2008; Nano & Houry, 2013). In addition to its histone acetylation and DNA damage repair, TIP60 also plays an important role in apoptosis where it is required for the acetylation of tumor suppressor p53, which after acetylation can bind to promoters of proapoptotic genes (Nano & Houry, 2013; Sykes et al., 2006).

URI1 complex

Pontin and Reptin are known to form part of the the URI1 (also known as RMP) complex. URI1 is a scaffolding protein involved in cellular response to extracellular nutrient levels by mediating the repression of TOR (target of rapamycin)-repressed genes. It also associates with RNA polymerase II (RNAP II) and with components of the Polymerase II Associated Factor (PAF) complex in human cells. Additionally, increased number of double-strand breaks in the nematode *Caenorhabditis elegans* as a result of URI1 loss demonstrates that URI1 is involved in maintaining genomic integrity. The exact roles of Pontin and Reptin in the complex have not yet been identified (Gallant, 2007).

Chromatin decondensation and the Mitotic spindle

Yet another role of Pontin and Reptin in chromatin remodeling has been demonstrated in human HeLa cells and *Xenopus laevis* egg extracts, where both proteins are needed for chromatin decondensation at the end of mitosis (Rosenbaum et al., 2013). Reducing Pontin has also been shown to result in defects in spindle assembly in *Drosophila* S2 cells and in various mammalian cell lines. Human Pontin has been found in the the nucleus of U937 cells, and has been found to colocalize with tubulin at the centrosome and at the mitotic spindle. In addition, Pontin was shown to have an agonistic effect on tubulin assembly *in vitro* (Gartner et al., 2003).

1.1.5 Independent and antagonistic roles of Pontin and Reptin in transcriptional regulation

Pontin and Reptin have various roles in transcriptional regulation. Although they often function together, they have been found to have antagonistic effects in mechanisms such as the Wingless (Wg)/Wnt pathway and in the regulation of the metastasis suppressor KAI1 (Nano & Houry, 2013).

The Wnt signaling pathway is an intracellular transduction pathway necessary in various developmental processes. The β-catenin protein plays a major role in the pathway where it enters the nucleus of Wnt-stimulated cells, and then forms a complex with a member of the T-cell factor (TCF) family, which then stimulates the expression of specific target genes. Studies using *Drosophila melanogaster* as a model have demonstrated that Pontin and Reptin work antagonistically in the pathway where they interact with β-catenin or Armadillo (Arm), the *Drosophila* β-catenin homologue. Pontin was found to increase the transcriptional activation of target genes whereas Reptin was found to repress the β-catenin/TCF transactivation complex, thus decreasing the transcriptional activation of target genes (Bauer et al., 2000). Liebeskummer (*lik*) is a lethal mutation isolated in zebrafish causing cardiac hyperplasia. *lik* encodes Reptin, and the mutation enhances the ATPase activity of Reptin complexes. Reptin, Pontin and β-catenin seem to be involved in zebrafish heart and gut development. Reducing Pontin also results in cardiac hyperplasia indicating that Pontin and Reptin act as antagonistic regulators of embryonic heart and gut development, at least in part through the Wnt pathway previously described (Rottbauer et al., 2002).

KAI1 is a metastasis suppressor that promotes cell adhesion which inhibits metastasis (Nano & Houry, 2013). It is a member of the tetraspanin protein family and is down-regulated during tumor progression in various human cancers (Wood et al., 2000). Metastatic and non-metastatic cells recruit different co-factors to the KAI1 promoter. In non-metastatic cells Pontin and TIP60 bind to the KAI1 promoter region and acetylate histones H3 and H4 whereas in metastatic cells, Reptin and β-catenin are recruited to the promoter. Reptin and β-catenin are needed for the down-regulation of the KAI1 gene whereas TIP60 but not Pontin is needed for KAI1 expression, although inhibition of Pontin does reduce histon acetylation and target gene expression (Gallant, 2007).

Pontin and Reptin also interact with the transcription factor c-Myc which is involved in oncogenic transformation, apoptosis and cell proliferation (Nano & Houry, 2013). The

c-Myc protein is a oncogenic transcription factor which activates the expression of various genes by binding to Enhancer Box sequences (E-boxes) and recruiting HATs (McMahon et al., 2000; Perini et al., 2005). When overexpressed, the c-myc gene can cause unregulated expression of many genes. Some of these genes are involved in cell proliferation and when unregulated, cause the formation of cancer. Abnormal c-myc expression is found in approximately 80% of breast cancers, 70% of colon cancer, 90% of gynecological cancers, 50% of hepatocellular carcinomas and a variety of hematological tumors (Gardner et al., 2002). Pontin and Reptin complex with c-Myc *in vivo*, and mutation of the Walker B domain in Pontin has been shown to dominantly inhibit c-Myc oncogenic activity without inhibiting normal cell growth, indicating that the Pontin is essential for c-Myc oncogenic transformation (Wood et al., 2000). It has been demonstrated that the interactions between Pontin/Reptin and c-Myc are conserved in *Drosophila* where they also act as essential cofactors of the transcription factor. The two proteins can form a ternary complex with dMyc (the *Drosophila* c-Myc homologue) in *Drosophila* S2 cells. dMyc and Pontin show a strong genetic interaction and the two can interact in the absence of bound Reptin. dMyc:Pontin complexes regulate expression of genes such as *mfas* and seem to be essential for the regulation of growth and proliferation *in vivo*. Less is known about the function of Reptin in this context, and it remains uncertain whether it acts as an antagonist in a similar manner as with β-catenin (Bellosta et al., 2005).

1.1.6 PIKK signaling

The phosphoinositide three-kinase-related kinase (PIKK) family includes protein kinases with roles in the regulation of DNA damage response, nonsense-mediated mRNA decay (NMD) and nutrient-dependent signaling. ATM, ATR and DNA-PKCs are the main kinases responsible for signaling of DNA damage. They phosphorylate proteins involved in regulation of cell cycle progression, DNA repair, apoptosis, and cellular senescence. SMG1, TRRAP, and mTOR are three additional PIKKs found in human cells. SMG1 regulates NMD, TRRAP regulates transcription and TOR regulates nutrient-dependent signaling as mentioned previously (Lovejoy & Cortez, 2009). Pontin and Reptin associate with, and regulate all PIKK members. Knockdown of human Pontin and Reptin has been shown to decrease both mRNA and protein levels of ATM, ATR, DNA-PKcs, TRRAP and mTOR. It also impairs PIKK-mediated signaling by decreasing phosphorylation of

direct downstream effectors of ATM, ATR, mTOR and SMG-1. It seems as if the control of PIKK abundance relies on the ATPase activity of both Pontin and Reptin since the wild type proteins were able to rescue the decrease in abundance whereas ATPase-deficient mutants did not. The two proteins also promote the formation of mRNA surveillance complexes during NMD by associating with SMG-1 and messenger ribonucleoproteins in the cytoplasm. The process has been found to be dependent on the ATPase activity of Pontin (Izumi et al., 2010). mTORC1 (mammalian target of rapamycin complex 1) is a member of the PIKK family consisting of Raptor, mLST8 and the previously mentioned mTOR. The complex plays an important role in the regulation of metabolism of glucose and glutamine. These primary carbon sources supply carbons to the TCA cycle to produce ATP and give positive feedback to mTORC1 by regulating the assembly and lysosomal localization of the complex. Pontin and Reptin have important functions in the regulation of mTORC1 and interact with the Tel2-Tti1-Tti2 (TTT) complex which regulates the assembly of PIKK-containing complexes. The ATPase activity of both Pontin and Reptin is required for the formation of the TTT-Pontin/Reptin complex. The assembly of the complex is also disrupted during depletion of glucose/glutamine. Energetic stress thus inhibits the activity of the complex which is required for mTORC1 activation. Knockdown of either *pontin* or *reptin* disrupts lysosomal localization of mTOR. The effects of glucose/glutamine starvation are similar. The TTT-Pontin/Reptin complex is also needed for mTORC1 dimerization. Thus, the repression of mTORC1 involves both the mislocalization and the reduced dimerization of the complex. mTORC1 is hyperactive in several cancers and the expression of many TTT-Pontin/Reptin complex genes have been found to be significantly higher in breast and colorectal carcinomas in comparison to non-carcinogenic control tissues. It is likely that metabolic processes that are necessary for tumor cell growth are dependent upon elevated expression of the TTT-Pontin/Reptin complex. Human Pontin and Reptin are also involved in assembly and stabilization of PIKKs through the R2TP complex which both proteins form part of and will be described in the following section (Nano & Houry, 2013).

1.1.7 snoRNP biogenesis

Heat shock protein 90 (HSP90) is required for the activation of a number of signaling proteins in the eukaryotic cell. It is a molecular chaperone that acts by ATPase-coupled conformational changes and interactions with various co-chaperone proteins (Pearl &

Prodromou, 2006). Both Pontin and Reptin associate with HSP90 in the R2TP complex. The R2TP-HSP90 complex is involved in RNAP II assembly together with Prefoldin-like complex. In addition to Pontin and Reptin the R2TP complex in *S. cerevisiae* consists of the two HSP90 interactors TPR-containing protein associated with HSP90 (TAH1) and Protein interacting with HSP90 (PIH1), hence the name R2TP (Kakihara & Houry, 2012). The complex is highly conserved in eukaryotes, and both Pontin and Reptin form part of the human R2TP complex, although TAH1 and PIH1 are replaced by RNA polymerase II associated protein 3 (RPAP3) and PIH1 domain-containing 1 (PIH1D1) (Nano & Houry, 2013).

Small nucleolar ribonucleoproteins (snoRNPs) are complexes involved in the modification of RNA, mainly ribosomal RNA (rRNA), transfer RNA (tRNA) and small nuclear RNA (snRNA). Box C/D small nucleolar RNAs (snoRNAs) guide the ribose 2′-O methylation of pre-RNA whereas box H/ACA snoRNAs function as sequence-specific guides to pseudo-uridylation of pre-ribosomal RNA (pre-rRNA) (McKeegan et al., 2009; Nano & Houry, 2013). The R2TP complex is required for box C/D snoRNP biogenesis in yeast and mammalian cells (Kakihara & Houry, 2012). Pontin and Reptin interact with all four core proteins bound by box C/D snoRNAs: 15.5K, NOP56, NOP58, and the methyltransferase fibrillarin. Pontin and Reptin seem to connect 15.5K to both NOP56 and NOP58. The interaction of the Pontin/Reptin complex with 15.5K has been shown to be increased by the presence of ATP, which might act by changing the conformation of the complex so it is recognized by 15.5K (McKeegan et al., 2009). The R2TP complex is required for the assembly of the snoRNP complex, and the absence of Pontin and Reptin leads to changes in localization of the snoRNP core proteins in yeast and mammalian cells (McKeegan et al., 2009; Nano & Houry, 2013). Pontin and Reptin are also involved in biogenesis of Box H/ACA type of snoRNPs that include telomerase (Rosenbaum et al., 2013). During chromosome replication, the leading DNA strand is replicated to the end whereas the lagging strand loses sequences at the end of the strand. To prevent continuous loss of DNA sequences every time the DNA is replicated, multiple copies of a noncoding sequence, called telomeres, are added to the ends of chromosomes. After replication in eukaryotic germ cells, stem cells, and most cancer cells, the length of telomeres is restored by the enzyme telomerase which adds the noncoding repeats to the 3' ends of the DNA strands (Griffiths, 2012). Telomerase is synthesized by nearly all eukaryotic organisms (Greider & Blackburn, 1996) but it is not present in *D. melanogaster*, were telomeres are

maintained by telomeric retrotransposons (TRs) instead. (Villasante et al., 2007). The telomease enzyme is a ribonucleoprotein (RNP) complex that consists of the catalytic subunit telomerase reverse transcriptase (TERT), telomerase RNA component (TERC), and the TERC binding protein dyskerin (DKC1). Pontin and Reptin have been demonstrated to form part of immature telomerase complexes and are required for the assembly and biogenesis of telomerase complexes by maintaining the telomerase RNA stability (Nano & Houry, 2013; Venteicher et al., 2008). Both proteins associate with DKC1 and TERT at the endogenous level in human cells and they are essential for the telomerase activity and TERC accumulation (Venteicher et al., 2008). The decrease of the proteins leads to loss of TERC and DKC1 from the complex indicating that DKC1 may act by connecting the two proteins with TERC. ATPase mutant of *pontin* did not rescue the loss suggesting that the proteins act through a mechanism that requires ATPase activity. Pontin has been shown to interact directly with the reverse transcriptase (RT) domain of TERT by recruiting Reptin and bridging its interaction with the complex (Nano & Houry, 2013; Venteicher et al., 2008). Together, Pontin and Reptin form a cell-cycle regulated complex with TERT that peaks during each S phase, and it seem as though the two proteins then help bring together TERT, DKC1 and TERC to form the mature telomerase complex (Venteicher et al., 2008). Reptin has been shown to interact with the catalytic subunit of *S. cerevisiae* telomerase, Est2, *in vivo*. The Reptin-Est2 association takes place whether or not telomerase is recruited to telomeric ends. Mutant *reptin* alleles that display slight, stable telomere shortening have been isolated. It can be speculated that Reptin acts, by optimizing telomerase recruitment, possibly by changing the conformation of the telomeric ends (Grandin & Charbonneau, 2011).

1.1.8 *Drosophila* Hox gene transcription

Absence of Pontin and Reptin in *Drosophila* causes death in larval stages, indicating that the proteins have crucial and non-redundant roles during early development (Bauer et al., 2000). They have been found to act as antagonistic mediators in the transcription of the Hox genes, which further confirms their essential roles in development. The Hox genes in *Drosophila* control the identity of segments and their associated appendages in the fly (Griffiths, 2012). Polycomb group (PcG) proteins are a group of protein that silence Hox genes by chromatin remodeling whereas Trithorax group (TrxG) protein act in the opposite way to maintain gene activity (Ringrose et al., 2003). Pontin and Reptin function

with PcG and TrxG proteins and have been found to be essential for maintaining Hox gene expression states. Pontin is thought to act as a co-activator and Reptin as a co-repressor in the maintenance of Hox gene transcription through cooperation with PcG and TrxG proteins. Reptin has been shown to be a component of the Protein regulator of cytokinesis 1 (PRC1) PcG complex whereas Pontin co-purifies with the Brahma (BRM) complex. BRM belongs to the TrxG group of proteins which might explain the opposite effects of Pontin and Reptin on Hox gene expression (Diop et al., 2008).

1.2 *Drosophila melanogaster*

1.2.1 General

One of the key factors in the rapid development of genetics during the last century has been the use of model organisms. There are various reasons accounting for the popularity of *Drosophila melanogaster*, more commonly known as the fruit fly, in genetic research. The fly has a rapid life cycle, reaching sexual maturity in 10 days under standard laboratory conditions. This, and the large number of eggs produced (up to a 100 eggs per day), makes the process of creating new strains and maintaining them relatively easy compared to other animals. Culturing flies does not require a large amount of space and food, making it an economical *in vivo model* as well. Another great advantage of the species is the availability of sophisticated genetic tools, such as balancer chromosomes, P-elements and the GAL4/UAS system, which have been developed for *Drosophila* research (Stocker & Gallant, 2008). Last but not least it is estimated that 75% of human disease genes have functional homologs in the fly genome (Pandey & Nichols, 2011), making it an important animal model for therapeutic research.

1.2.2 Embryonic nervous system

Many *Drosophila* genes are essential and most mutations of them lethal, making it difficult to study their effects. As mentioned previously, mutations causing deficiency for Pontin and Reptin cause death in larval stages. Creating genetic mosaics is one way of circumventing the problem but another option is focusing on the effect of the mutations in the embryonic stages, as will be done here.

Two cell types, neuronal and glial, are needed in any complex nervous system. In mammals, glial cells are over 50% of the neural cells, whereas in *Drosophila* they make up only 10%. Glial cells are necessary in the nervous system of both vertebrates and

invertebrates. They play a crucial role in maintaining the correct environment around neurons so that they can function normally, perceiving and integrating information (Stork et al., 2008).

The embryonic development in *Drosophila* occurs in 22 hours at 25ºC and is divided into 17 stages shown in Table 1. Before gastrulation mesectodermal cells are situated on either side of the mesoderm. During stages 5-8 the mesectodermal cells are brought together by the invagination of the mesoderm. In stage 9 these midline progenitor cells go on to form a single cell row, the mesectoderm, which extends along the ventral surface of the embryo. During stages 9-12 all the midline cells except neuroblasts (NBs) divide once to form either midline glia (MG), midline precursors (MP1), or ventral unpaired median (VUM) neurons. The NBs are cells that originate in the so called neurogenic region or neuroectoderm, located next to the mesectoderm. Approximately 4 hours into embryogenesis, in embryonic stage 9, NBs delaminate from the ventral ectoderm, and their segregation continues for about 3 hours. During stage 10, whilst NBs keep segregating, some of them start stem cell-like division to give rise to ganglion mother cells. During stages 11-13 NBs keep dividing generating ganglion mother cells which then divide once to generate 2 neurons and/or glial cells. During stage 12 the stomatogastric nervous system start to form with the appearance of three invaginations at the upper part of the developing foregut. Axons start growing in the ventral nerve cord which separates completely from the epidermis during this stage. The cells of the three foregut invaginations becoming distributed within the clypeolabrum during stage 13. The ventral nerve cord and the supraoesophageal ganglion organize into neuromeres, and fiber connectives and commissures linking the neuromers form. Muscle cells and progenitors of sensory cells also become apparent. Cytodifferentiation (i.e. outgrowth of axonal processes) begins in sensory organs during stage 14. Condensation of the ventral nerve cord begins in stage 15 and it continues to shorten during stage 16. The sensory organs become visible during stage 16 and during stage 17, the last stage of embryogenesis and

Table 1. *Drosophila* embryonic stages*

Stage	Minutes after fertilization
1	0-15
2	15-70
3	70-90
4	90-130
5	130-180
6	180-195
7	195-200
8	200-230
9	230-260
10	260-320
11	320-440
12	440-580
13	560-620
14	620-680
15	680-800
16	800-900
17	Last until hatching
Hatch	21-22 hours

**(Weigmann et al., 2003)*

the embryo completes its organogenesis (Weigmann et al., 2003). Here, nervous system defects of embryos in stages 14-17, were analyzed using the BP-102 and 22C10 antibodies.

2 Materials and Methods

2.1 Materials

2.1.1 Fly stocks and maintenance

1. The stocks were crossed and kept on instant medium in plastic vials that were closed with either cotton or a sponge.

2. The flies were anesthetized with CO_2 gas before inspection and handling.

Table 2. *Drosophila melanogaster* stocks

No.	Stock	Abbreviation of stock*	Description	Origin/Reference **
1.	w^{1118}		white$^-$, otherwise wild type X chromosome	(Hazelrigg et al., 1984)
2.	$y^1 w^{67c23}$; $P\{EPgy2\}rept^{EY12756}$ /TM3, Sb^1 Ser^1		P-element insertion by *reptin*	Bloomington stock Centre #21384
3.	w^{1118}; $PBac\{WH\}rept^{f01801}$ /TM6B, Tb^1		piggyBac transposon insertion by *reptin*	Bloomington stock Centre #18476
4.	$rept^{D35}$/TM6		*reptin* P-element activity mutagen loss of function allele	(Bauer et al., 2000)
5.	FRT 82B $pont^{5.1}$ /TM3		*pontin* P-element activity mutagen, loss of function allele	(Bauer et al., 2000)

(Continued)

6.	w^{1118}; *P{EP}G5116*	*P{EP}G5116*	P-element insertion by *pontin*, fertile, viable	Bloomington stock Centre #30115
7.	*w[*]; Sb[1]/TM3, P{w[+mC]=ActGFP} JMR2,Ser[1]*	$\frac{Sb}{TM3^{Act\ GFP}, Ser}$	GFP balancer chromosome III	Bloomington stock Centre #4534
8.	*TM3, P{ry[+t7.2]=ftz-lacZ.ry[+]}TM3, Sb[1] ry[*]/Dr[Mio]*	$\frac{Dr^{Mio}}{TM3^{bb}, Sb}$	Blue balancer chromosome III	Bloomington stock Centre #3218
9.	$y^1 w^{67c23}$; *P{EPgy2} rept*^{EY12756}*/ TM3, P{w[+mC]=ActGFP} JMR2,Ser[1]*	$\frac{P\{EPgy2\}rept}{TM3^{Act\ GFP}, Ser}$	Balanced *reptin* mutation	Obtained by crossing No. 2 with No. 7
10.	w^{1118}; *PBac{WH} rept*^{f01801}*/TM3, P{w[+mC]=ActGFP} JMR2,Ser[1]*	$\frac{PBac\{WH\}rept}{TM3^{Act\ GFP}, Ser}$	Balanced *reptin* mutation	Obtained by crossing No. 3 with No. 7
11.	*rept*^{D35}*/TM3, P{w[+mC] =ActGFP} JMR2,Ser[1]*	$\frac{rept^{D35}}{TM3^{Act\ GFP}, Ser}$	Balanced *reptin* mutation	Obtained by crossing No. 4 with No. 7
12.	*FRT 82B pont*$^{5.1}$*/ TM3, P{w[+mC] =ActGFP}JMR2,Ser[1]*	$\frac{pont5.1}{TM3^{Act\ GFP}, Ser}$	Balanced *pontin* mutation	Obtained by crossing No. 5 with No. 7
13.	$y^1 w^{67c23}$; *P{EPgy2} rept*^{EY12756}*/P{ry[+t7.2] =ftz-lacZ.ry[+]}TM3, Sb[1] ry[*]*	$\frac{P\{EPgy2\}rept}{TM3^{bb}, Sb}$	Balanced *reptin* mutation	Obtained by crossing No. 2 with No. 8
14.	w^{1118}; *PBac{WH}rept*^{f01801} */P{ry[+t7.2]=ftz-lacZ.ry[+]}TM3, Sb[1]*	$\frac{PBac\{WH\}rept}{TM3^{bb}, Sb}$	Balanced *reptin* mutation	Obtained by crossing No. 3 with no. 8

(*Continued*)

15.	*$rept^{D35}$ P{ry[+t7.2]=ftz-lacZ.ry[+]}TM3, Sb[1]*	*reptD35 / TM3bb, Sb*	Balanced *reptin* mutation	Obtained by crossing No. 4 with No. 8
16.	*FRT 82B $pont^{5.1}$ / P{ry[+t7.2]=ftz-lacZ.ry[+]}TM3, Sb[1]*	*pont5.1 / $TM3^{bb}$, Sb*	Balanced *pontin* mutation	Obtained by crossing No. 5 with No. 8

*Further information about how the crossed strains were obtained is found in section 2.2.1.
** # designates Bloomington stock number

2.1.2 Collection and dechorionization of embryos

1. A vented 100-mL plastic beaker and a lid were used as a collection cage. The collection plate (see Subheading 2.1.2, No. 2) was placed into the collection cage by attaching it to the lid of the beaker with tape.

2. The collection plate consists of a 60 mm Petri dish filled two thirds with agar. Yeast paste was smeared on a part of the agar surface before use. The agar was prepared by mixing 9 g of Bacto™ Agar with c.a. 400 ml of deionized water. The mixture was then autoclaved, and 4 ml of acetic acid (puriss., 99-100%) added before pouring the liquid into Petri dishes. The plates were stored at 4°C before use.

3. The dechorionization was performed using retention-tubes. The tubes were prepared by cutting the closed end of falcon tubes and attaching either a stainless steel wire mesh or a Nitex® mesh to it. 5% sodium hypochlorite was used for the dechorionization.

2.1.3 Embryo fixation

1. Formaldehyde fixative: 4% formaldehyde (p.a. grade 37,5% stock solution) in phosphate-buffered saline (PBS).

2. Other chemicals used: hexane, methanol, 96% ethanol.

3. Fixation was performed on a rocking plate.

2.1.4 Immunolabeling

1. PBS with Triton-X-100 (PBT): 1X PBS (130 mM NaCl, 7 mM $NaHPO_4$, 3 mM NaH_2PO_4, pH 7,4) with Triton-X-100 (0.3%); that is 10 mL PBS with 30 µL Triton-X-100.

2. Rehydration: 50% ethanol in PBT.

3. Blocking solution: 10% goat serum in PBT.

4. Antibody solutions: primary and secondary antibodies were diluted in blocking solution; the strength depends on the antibody used (see Table 3 and Table 4).

5. Rehydration, washing and incubation took place on a rocking plate.

Table 3. Primary antibodies

Name	Source species	Dilution	Origin
Anti-CNS axons (BP102)	Mouse	1:100	Developmental Studies Hybridoma Bank
Anti-futch (22C10)	Mouse	1:100	Developmental Studies Hybridoma Bank
Anti-β-Galactosidase (40-1a-s)	Mouse	1:100	Developmental Studies Hybridoma Bank
Anti-GFP	Rabbit	1:750	invitrogen™
Anti-HRP blue*	Goat	1:500	Developmental Studies Hybridoma Bank

*Conjugate: Alexa Fluor® 647

Table 4. Secondary antibodies

Reactivity	Source species	Conjugate	Dilution	Origin
Anti-rabbit (IgG(H+L))	Goat	Alexa Fluor® 546	1:1000	invitrogen™

(*Continued*)

Anti-mouse (IgG1(γ1))	Goat	Alexa Fluor® 488, Alexa Fluor® 546	1:1000	invitrogen™
Anti-mouse (IgG2a(γ2a))	Goat	Alexa Fluor® 488	1:1000	invitrogen™

2.1.5 Mounting, embedding and observation

1. Mounting medium: *VECTASHIELD*®
2. Embedding was performed on a slide with spacers and cover slip.
3. The embryos were observed in a confocal laser scanning microscopy.

2.2 Methods

2.2.1 Rebalancing

Balancer chromosomes

Balancer chromosomes are important tools in *Drosophila* research. They are products of multiple chromosomal inversions and suppress recombination between homologous chromosomes during meiosis (Stocker & Gallant, 2008). They carry recessive mutations that cause lethality or sterility in homozygotes. In addition, they carry dominant markers affecting adult and occasionally larval morphology, which enables the distinction between mutant homo- or heterozygotes. Balancer chromosomes thus enable maintaining mutant stocks as balanced heterozygotes (Casso et al., 2000). Distinct balancers have been developed for the four chromosome pairs of the species (Stocker & Gallant, 2008).

Here, one *Drosophila* strain carrying a mutant allele of *pontin* (No. 3, Table 2) and three stocks carrying mutations of *reptin* (No. 4-6, Table 2) were rebalanced against two different third chromosomal balancer stocks (No. 7 and 8, Table 2) which enable the selection of homozygous mutant embryos. The genetic marker of the "$TM3^{bb}$, *Sb*" balancer used is a transgenic insertion of the *lacZ* gene, driven by the *fushi tarazu* (*ftz*) promoter. The *lacZ* gene from *Escherichia coli* encodes the β-galactosidase (β-gal) protein which can be stained using the artificial substrate X-gal that turns blue when it is cleaved by β-gal (hence the name *blue balancer),* or alternatively, β-gal can be detected by antibody staining. The *ftz* promoter causes the β-gal protein to be expressed in a seven-stripe pattern in embryos undergoing germ-band elongation (Dearolf et al., 1989). The genetic marker of

the "*Sb*/*TM3*$^{act,\ GFP}$, *Ser*" balancer used is the GFP (green fluorescent protein) gene, cloned from *Aequorea victoria* (jellyfish), driven by the Actin A5C promoter. The marker displays fluorescence when exposed to light in the blue to ultraviolet range (Tsien, 1998). *T* in "*TM3*^{bb}, *Sb*" stands for the third chromosome, M for "multiply inverted" and the number 3 distinguishes from other balancers of the same chromosome (Stocker & Gallant, 2008). The *bb* superscript stands for *blue balancer* and refers to the use of the *ftz-lacZ* genetic marker. *Sb* (stubble) is a dominant mutation and the phenotypic marker of the balancer. It is easily recognized in a microscope, as the flies containing the balancer have short and stubble bristles. The *act,GFP* superscript in "*TM3*^{act,GFP}, *Ser*" indicates the use of the *act-GFP* marker. *Ser* (serrate) is the phenotypic marker of the balancer and is recognized by the serrated wings of the flies. It is worth mentioning that in Table 2 the stocks containing the balancers are listed, not only the balancers themselves. The *blue balancer* stock, listed as "*Dr*$^{Mio/}$*TM3*^{bb}, *Sb*" in the abbreviation column, carries a dominant *Drop* eye mutation that severely disrupts eye development (Tearle et al., 1994) and makes the flies carrying the mutation easily recognizable by their eye shape which resembles an inverted drop. The *GFP balancer* stock, listed as "*Sb*/*TM3*^{act,GFP}, *Ser*", carries the previously mentioned *Sb* mutation.

P-element

The P-element was discovered by accident in the 1960s, when different wild-type *Drosophila* strains were crossed. The offspring of these crosses often turned out to have increased mutation rates. The wild-type strains were classified either as P- or M-cytotype. (Hummel & Klambt, 2008) When females of M-cytotype were crossed with males of P-cytotype the offspring displayed several defects such as sterility, diverse mutations, chromosome breakage, and male recombination. (Bachmann & Knust, 2008) This effect, known as hybrid dysgenesis, was not nearly as severe when females of P-cytotype were crossed with males of M-cytotype, and not present in offspring from pure crosses of P- or M-cytotypes. This effect is explained by the presence of the so called P-element in the P-cytotype flies. The functional P-element is a transposable element consisting of 2907 base pairs (bp), carrying 31 bp inverted terminal repeats and 11 bp inverted subterminal repeats of regulatory sequences. It encodes a functional transposase enabling it to move inside a genome by conducting a GTP dependent cut and paste reaction. The transposase activity only requires part of the P-element termini. The 31 bp inverted terminal repeat, the

transposase binding site, and the 11 bp inverted subterminal repeats are required for the transposition. The insertion of a P-element into a chromosome generates an 8 bp duplication at the target site. Subsequent excision of the P-element normally results in a 32 bp sequence of the 8 bp repeats being left behind. The P-element is used to genetically modify flies by inserting it into a vector and replacing the P transposase with a gene of interest before germline transformation. The autonomous movement of the P-element can be inhibited by transferring the transposase gene to a helper-plasmid vector which is unable to integrate into the genome but carries the transposase. A wide variety of P-element vectors now exist and can be used for numerous research purposes. The P-element activity mutagens used in this study are listed in table 2. The rebalancing procedure is is listed below.

1. Around 20 virgins were collected from stocks 2-5 in Table 2. One half of the virgins were crossed with 3 males from balancer stock No. 7 in Table 2 and the other half was crossed with 3 males from balancer stock No. 8 in Table 2.

2. The flies crossed were removed from the vials before eclosure of the offspring. Virgins and males that carried only the correct balancer phenotype (*Sb* or *Ser* depending on the balancer), were collected from the vials and crossed with each other in new vials. This selection must occur before the eclosure of the F2 (second filial) generation in order to be sure that the flies have the "correct" genotype.

3. The progeny from these crosses should all have the same genotype; that is the respective *pontin* or *reptin* mutation against one of the two balancer chromosomes.

4. Around 20 virgins were collected from strains 2-5 in table 2. One half of the virgins were crossed with 3 males from balancer strain no. 7 in table 2, and the other half was crossed was crossed with 3 males from balancer strain no. 8 in table 2.

5. The flies crossed were removed from the vials before eclosure of the offspring. Virgins and males that carried only the correct balancer phenotype (*Sb* or *Ser* depending on the balancer), were collected from the vials and crossed with each other in new vials. This selection must occur before the eclosure of the F2 (second filial) generation in order to be sure that the flies have the "correct" genotype.

6. The progeny from these crosses should all have the same genotype; that is the respective *pontin* or *reptin* mutation against one of the two balancer chromosomes.

Example of cross:

$$\frac{rept^{D35}}{TM6} \times \frac{Sb}{TM3^{Act\ GFP}, Ser}$$

The offspring will have one of the following genotypes:

a. $\frac{rept^{D35}}{TM6}$ b. $\frac{Sb}{TM3^{Act\ GFP}, Ser}$ c. $\frac{rept^{D35}}{TM3^{Act\ GFP}, Ser}$ d. $\frac{Sb}{TM6}$

e. $\frac{rept^{D35}}{Sb}$ f. $\frac{TM6}{TM3^{Act\ GFP}, Ser}$

The double balancer (f.) is not viable, so the only offspring with the *Ser* phenotype but without the *Sb* phenotype will be of genotype c. (*reptin* mutation against the balancer).

2.2.2 Collection and dechorionization of embryos

1. 15-30 flies, thereof 2/3 females, were put in a collection cage with a collection plate attached. The flies need 2-3 days to accustom to the cages before the egg production reaches its best. The collection plates were changed regularly during this period.

2. After 2-3 days egg collection begins. The collection plates were usually changed twice a day, once in the early morning and then again at around 5 PM. The flies lay most of their eggs early and changing the plates at 5 PM excludes unwanted eggs, that have not reached stage 14-17, from the embryo collection the next day. Since the temperature of the culture room is 24ºC and not the standard temperature (25ºC) it might have taken little longer than the standard time to reach the embryonic stages shown in Table 1. The embryos were collected from the plates in the early morning or in some instances late at night, in order to fix the embryos when most of them were in or between stages 14-17.

3. A soft brush was used for gathering the embryos from the collection plate. Deionized water was poured on to the plate, and after gently sweeping the surface with the brush the water was filtered through the retention-tubes. The embryos were washed with deionized water and then soaked in 5% sodium hypochlorite for approximately 5 minutes. After that the embryos were washed again and then carefully moved with a brush from the retention-tube to a 1.5 ml eppendorf tube containing hexane.

2.2.3 Embryo fixation

1. Hexane was added or removed so that it reached the 500 µl mark on the eppendorf tube containing the embryos. 500 µl of 4% formaldehyde in PBS was added to the tube.

2. The embryos were fixed for 30 minutes on a rocking plate.

3. The lower phase was removed and 500 µl of methanol were added. The eppendorf tube was shaken vigorously for 1 minute.

4. The upper phase was removed and more methanol was added. After a light shake the embryos were left to sink to the bottom.

5. The supernatant was replaced with 96% ethanol and stored in a freezer until the immunolabeling was performed.

2.2.4 Immunolabeling

In this study, the effect of *Drosophila* Pontin and Reptin on neuronal morphology and axonal projections was visualized using an antibody against the *Drosophila* Futsch/22C10 protein, which is required for dendric and axonal development (Hummel et al., 2000). The antibody labels all peripheral nervous system (PNS) neurons as well as some central nervous system (CNS) neurons. The *futsch* gene encodes the Futsch/22C10 protein which is recognized by the mAb (monoclonal antibody) 22C10. The vertebrate microtubule-associated protein 1B (MAP1B) contains N- and C-terminal domains homologous to those of Futsch/22C10 (Hummel et al., 2000). Axons in the *Drosophila* CNS were visualized using the mAb BP102, which effectively stains the axons of the central nervous system whilst leaving the neuron cell bodies, the PNS, and other embryonic tissue

unstained. In addition, the embryos were stained with a fluorescently conjugated anti-horseradish peroxidase (Anti-HRP) antibody which labels the entire nervous system, albeit not as clearly as the other two antibodies. Antibodies for the genetic markers of the balancers were also used in order to distinguish the embryos homozygous for the the *pontin* and *reptin* mutations from the rest. In theory, ¼ of the embryos should be homozygous for the *reptin/pontin* mutations. These embryos do not contain the balancer chromosomes and should not be stained by the antibodies against their genetic markers. One of the strains being studied is viable and does not need to be balanced (No. 6, Table 2). Images displaying the embryos collected from the mutant stocks rebalanced with the *blue balancer* are not shown in the results, because the β-gal staining was ineffective. Therefore, it was not possible to distinguish the embryos homozygous for the *pontin* or *reptin* mutations from other embryos by using this balancer. The immunolabeling (or staining) procedure is listed below.

1. The embryos were rehydrated in 50% ethanol in PBT for 10 minutes*.
2. The supernatant was removed and the embryos were washed three times for 10 minutes in PBT*.
3. The embryos were blocked in 500 µl of 10% goat serum in PBT for 1 hour*.
4. The primary antibody was diluted in 500 µl of PBT-10% goat serum, the concentration depending on which antibody was used (see Table 3).
5. The solution was left for either three hours at room temperature or overnight at 4ºC*.
6. The supernatant was removed and the embryos were washed in PBT for 20 minutes three times*.
7. The secondary antibody was diluted in 500 µl of PBT-10% goat serum, the concentration depending on which antibody was used (see Table 4).
8. The solution was left rocking or for either three hours at room temperature or overnight at 4ºC*.

*Steps 1, 2,3,5,6 and 8 were performed on a rocking plate.

2.2.5 Mounting, embedding and observation

1. The supernatant was removed and two drops of mounting medium were added to the tube.

2. The embryos in the mounting solution were placed on a slide with spacers and a cover slip.

3. The embryos were imaged with a confocal laser scanning microscope.

2.2.6 Viability count

In order to find out whether the three transgenic insertions near *reptin* and *pontin* are viable against each other and against the published null alleles $pont^{5.1}$ and $rept^{D35}$ (Bauer et al., 2000), the rebalanced stocks (and the unbalanced *P{EP}G5116* stock) were crossed with each other, and offspring from the crosses were examined under a microscope.

Example of cross between *reptin* mutants:

$$\frac{rept^{D35}}{TM3^{bb}, Sb} \times \frac{P\{EPgy2\}rept}{TM3^{bb}, Sb}$$

The offspring will have one of the following genotypes:

a. $\frac{rept^{D35}}{P\{EPgy2\}rept}$ b. $\frac{P\{EPgy2\}rept}{TM3^{bb}, Sb}$ c. $\frac{rept^{D35}}{TM3^{bb}, Sb}$ d. $\frac{TM3^{bb}, Sb}{TM3^{bb}, Sb}$

a. If the offspring have this genotype they will have normal bristles and the homozygous mutations are viable.

b. This is the same genotype as the ones that were crossed and these flies will have the *Sb* phenotype.

c. This is the same genotype as the ones that were crossed and these flies will have the *Sb* phenotype.

d. These offspring are homozygous for the balancer and are therefore not viable.

The viable *P{EP}G5116* stock was crossed with the rebalanced *pont*$^{5.1}$ stock (No. 16 in table 2.1.1), and the offspring was examined under a microscope.

Cross between *pontin* mutants:

$$\frac{pont^{5.1}}{TM3^{bb}, Sb} \quad \times \quad P\{EP\}G5116$$

The offspring will have one of the following genotypes:

a. $\frac{pont^{5.1}}{P\{EP\}G5116}$ b. $\frac{P\{EP\}G5116}{TM3^{bb}, Sb}$

a. If the offspring have this genotype, the *pontin* mutations are viable against each other and the flies will have normal bristles.

b. Flies with this genotype have the *P{EP}G5116* allele against the balancer and therefore have the *Sb* phenotype.

3 Results

3.1 Mutant analysis

In this section the results from the viability count and the immunofluorescent stains are shown and analyzed. Immunofluorescent stains were performed on the published null alleles *pont*$^{5.1}$ and *rept*^{D35} (Bauer et al., 2000), as well as several transposable element insertions in the 5' region of the two genes (see Table 2), to find out whether loss of either gene caused any visible nervous system phenotype in the embryo and to find out whether the insertions affected *pontin* or *reptin*. If a homozygous transposable element insertion, which is lethal *in trans* to a null-allele, causes the same phenotype as the null-allele, it is likely to have caused a loss of function in the same gene. As described in the methods section, two types of stains were performed, one for the PNS and the other for CNS axons. The images that share the same initial number show the same embryo. The embryos shown did not display significant anti-GFP staining unless otherwise noted.

3.1.1 Viability

Table 5. Mutant crosses and viability results

Stocks crossed			***Sb*/All***	**%**	**Viability of homozygous mutations**
$\frac{rept^{D35}}{TM3^{bb}, Sb}$	×	$\frac{P\{EPgy2\}rept}{TM3^{bb}, Sb}$	100/100	100	Not viable
$\frac{rept^{D35}}{TM3^{bb}, Sb}$	×	$\frac{PBac\{WH\}rept}{TM3^{bb}, Sb}$	100/100	100	Not viable
$\frac{PBac\{WH\}rept}{TM3^{bb}, Sb}$	×	$\frac{P\{EPgy2\}rept}{TM3^{bb}, Sb}$	100/100	100	Not viable
$\frac{pont^{5.1}}{TM3^{bb}, Sb}$	×	*P{EP}G5116*	51/ 152	33.6	Inconclusive

*The quantity of flies with the *Sb* phenotype out of the number of flies inspected

All of the offspring from the *reptin* mutant crosses had the *Sb* phenotype. It can thus be concluded that none of the *reptin* alleles are viable against each other. The result of the *pontin* mutant crosses was inconclusive. If the mutant *pontin* alleles are viable against each other, half of the flies should have normal bristles. The result was that more than half of the flies had normal bristles. The F2 generation might have eclosed by the time the

counting was performed resulting in this inconsistency. However, the fact that more than half of the flies had normal bristles does indicate that the mutant *pontin* alleles, *P{EP}G5116* and $pont^{5.1}$, are viable against each other.

3.1.2 Analysis of peripheral nervous system phenotypes

Wild type control

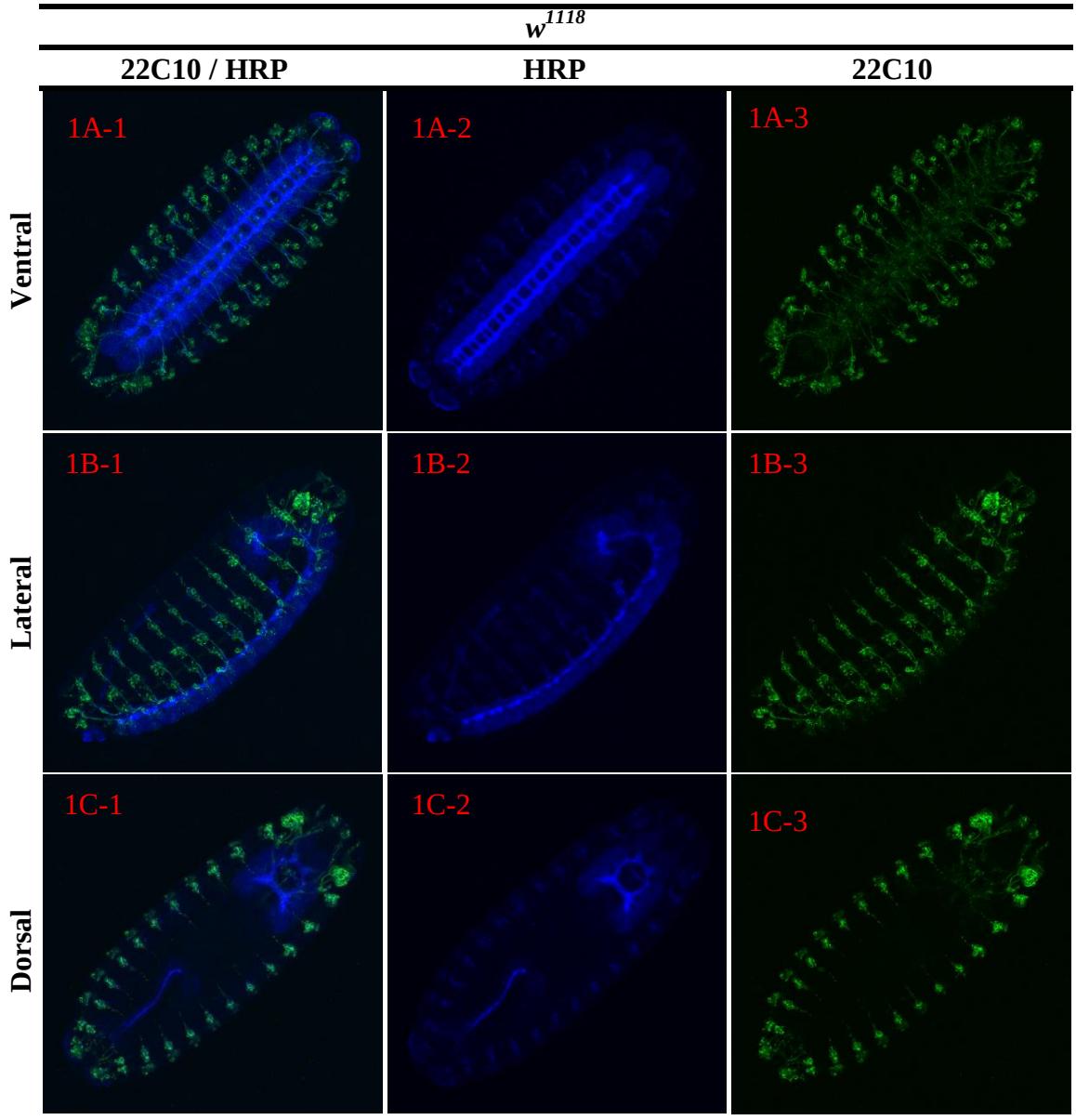


Figure 1. Futch (22C10) and HRP staining of w^{1118} embryos. The figure shows confocal images of wild type embryos stained with the 22C10 antibody (green) to label PNS neurons (and some CNS neurons) and anti-HRP (blue) to label all neurons.

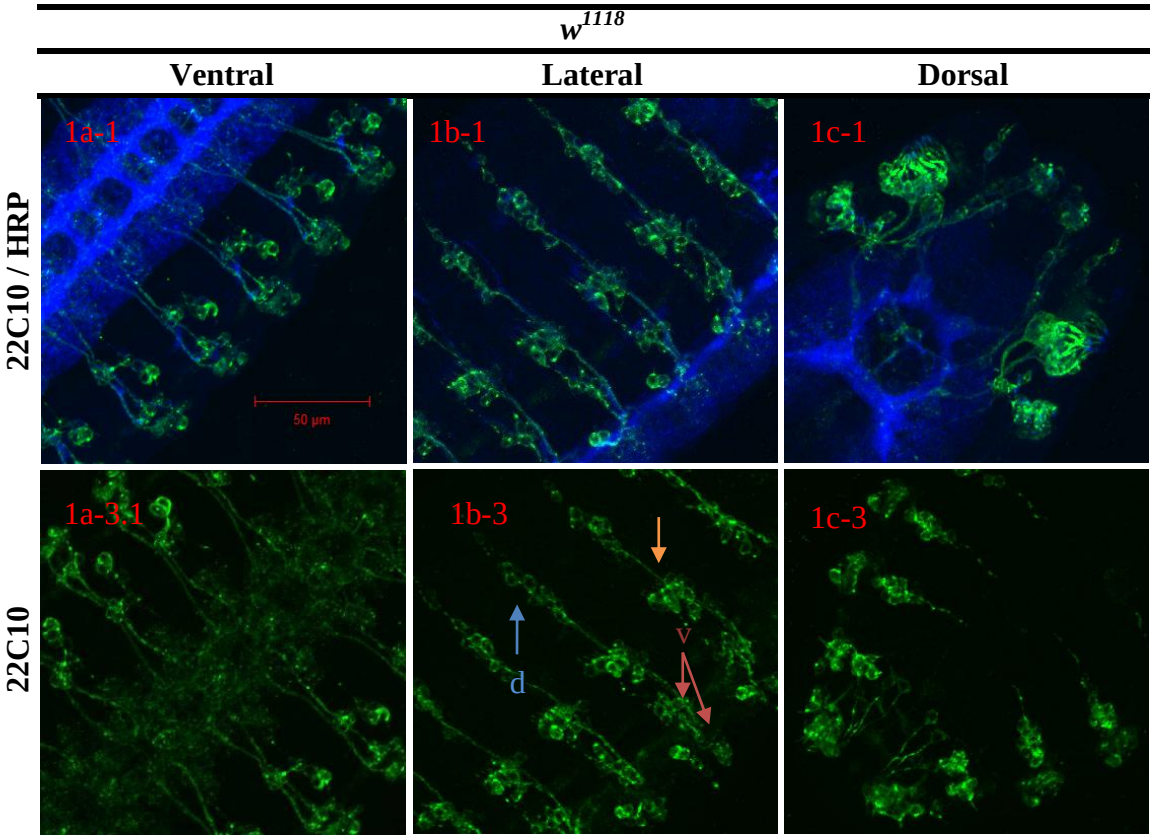
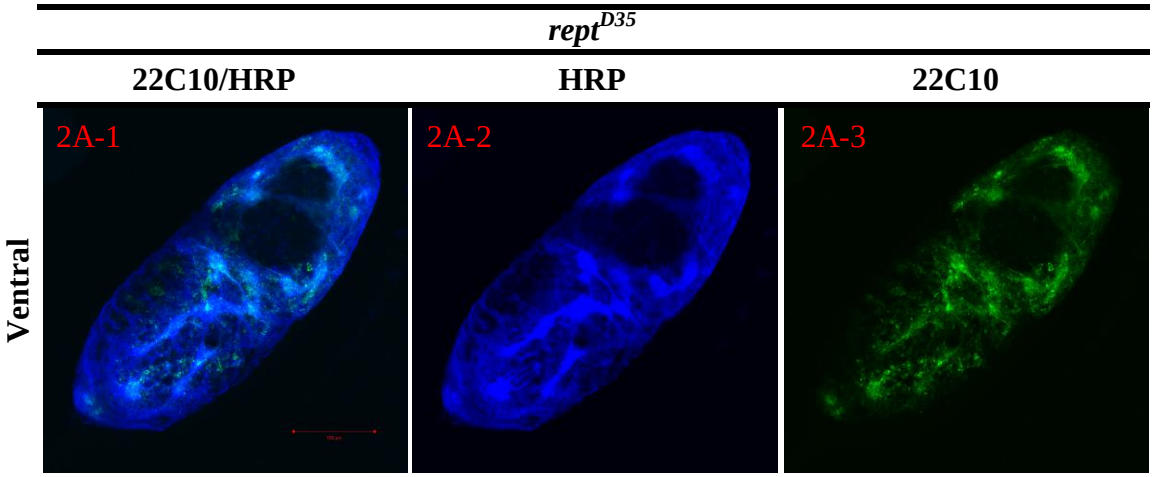


Figure 2. Detailed view of Futch (22C10) and HRP staining of w^{1118} embryos. The arrows in image 1b-3 show the chordotonial neuron clusters with the blue arrow indicating the dorsal cluster (d), the orange arrow the later cluster (l) and the red arrows the ventral clusters (v). The names if the images in Figure 2 are the same as in the original images in Figure 1., except a lower case letter is used instead of uppercase. If there are more than one detailed view of an image another number is added to the end. For example, image 1a-3.2 is the second detailed view of image 1A-3. The same applies to all of the following images.

$rept^{D35}$ null allele



(*Continued*)

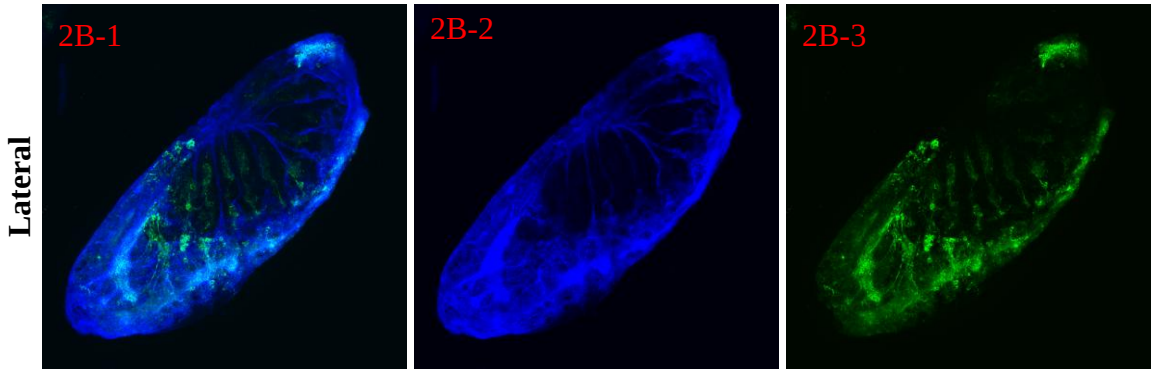


Figure 3. Futch (22C10) and HRP staining of an embryo likely to be homozygous for the *rept*^{D35} mutation. The embryo shows severe defective morphology and staining pattern. The axonal projections are distorted and closer to each other than in *w*^{1118} embryos. The embryo shows strong HRP staining (image 2B-2) of what resembles trachea. HRP does not normally stain trachea, but trachea staining can occur with antibodies that are not trachea specific.

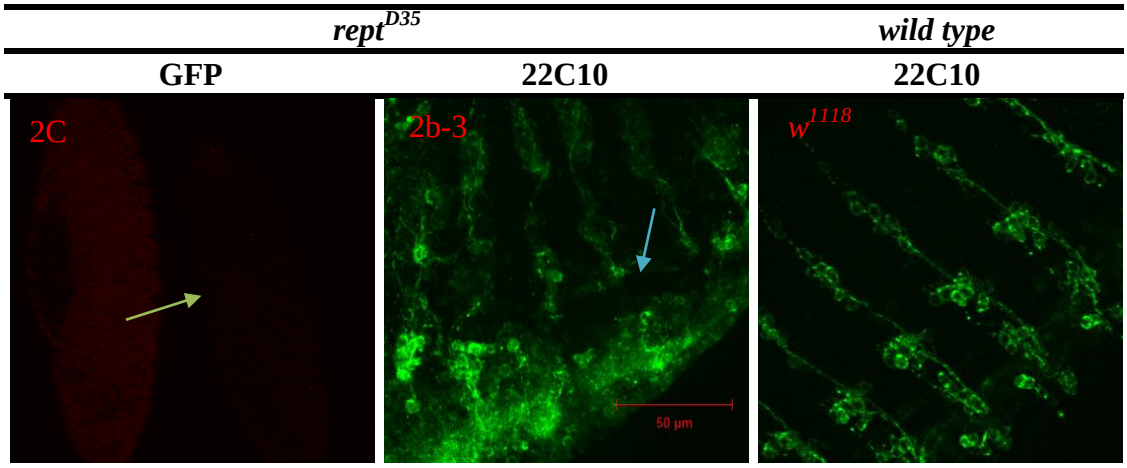


Figure 4. Detailed view of Futch (22C10) and HRP staining of an embryo likely to be homozygous for the *rept*^{D35} mutation as well as GFP staining and wild type (*w*^{1118}) comparison. There is almost no visible GFP staining of the embryo (indicated with the green arrow in image 2C). There seems to be a disruption between the lateral and ventral chordotonial neuron clusters (blue arrow in image 2b-3) in the *rept*^{D35} mutant.

P{EPgy2}rept insertion

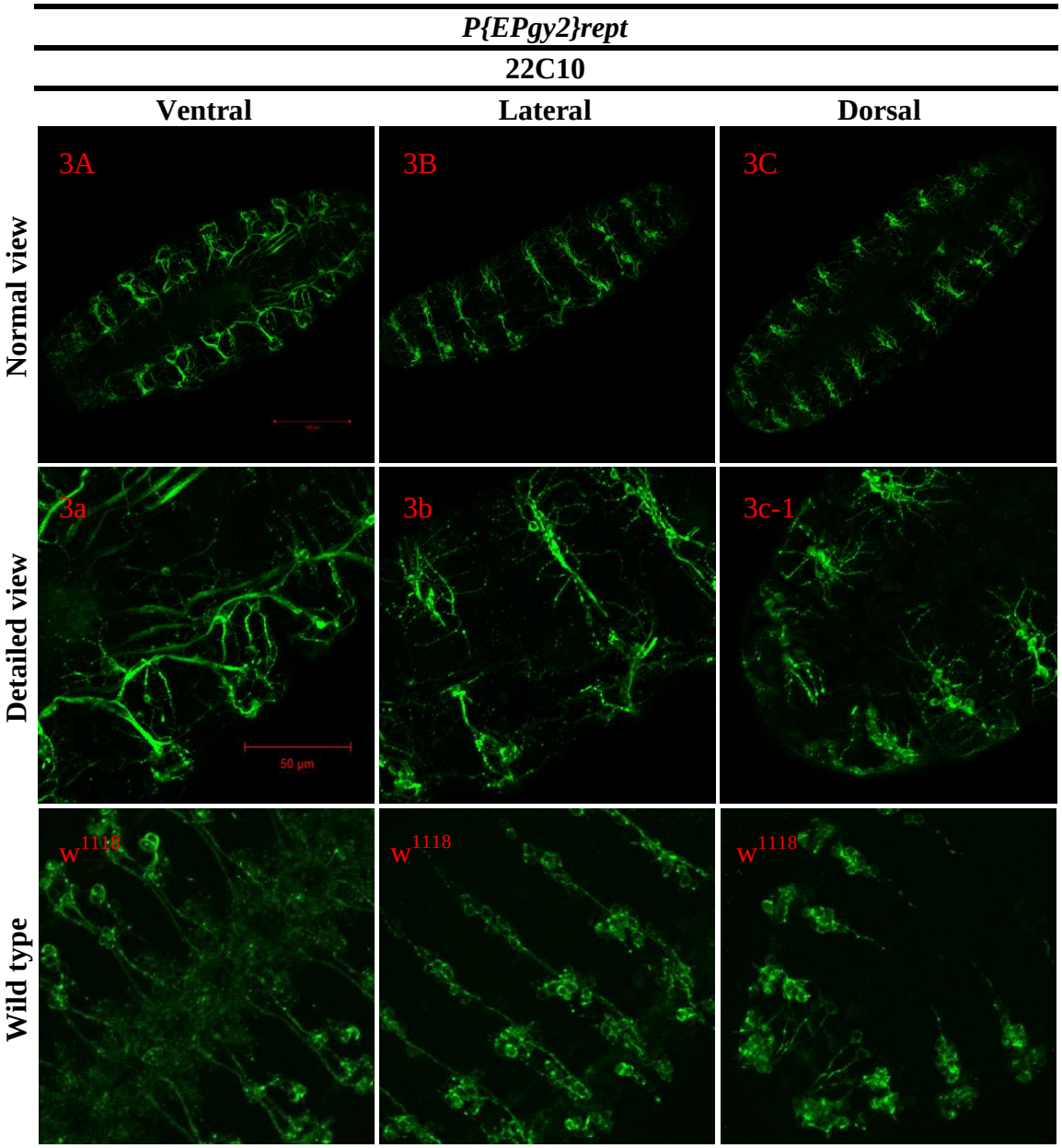


Figure 5. Futch (22C10) staining of an embryo likely to be homozygous for the *P{EPgy2}rept* insertion and wild type comparison. The embryo displays very weak staining of an abnormaly short ventral midline and the arrangement of the PNS neurons extending from the midline have a distinct morphology from all other embryos observed in this study. The dorsal and ventral parts of the axonal projections do not connect, as can be seen from the lateral view in image 3b. One explanation for this phenotype could be that the embryo is lacking all motoneurons and that only sensory neurons are seen in the image. The HRP antibody was not used.

P{EPgy2}rept

22C10

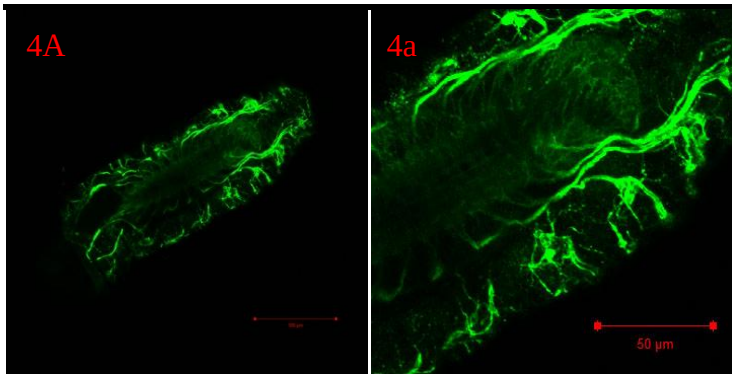


Figure 6. Futch (22C10) staining of an embryo likely to be homozygous for the *P{EPgy2}rept* insertion. The embryo shows a similar phenotype as the one shown in Figure 5. The HRP antibody was not used.

PBac{WH}rept insertion

PBac{WH}rept

22C10/HRP

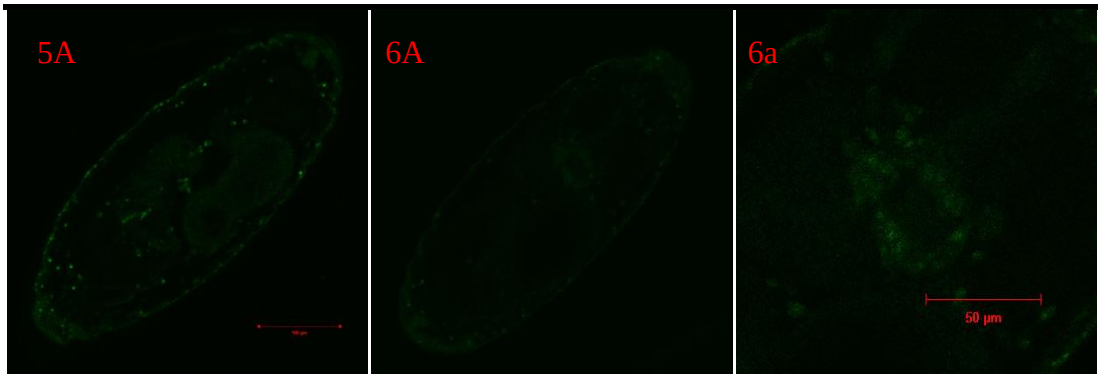


Figure 7. Futch (22C10) and HRP staining of embryos likely to be homozygous for the *PBac{WH}rept* insertion. Embryos 5 and 6 show little staining. No specific phenotype was observed other then staining of a circular area that was seen in a couple of embryos (image 6a). There was no HRP staining. Other similar embryos were observed. All images have been modified by increasing their brightness and contrast.

pont$^{5.1}$ null allele

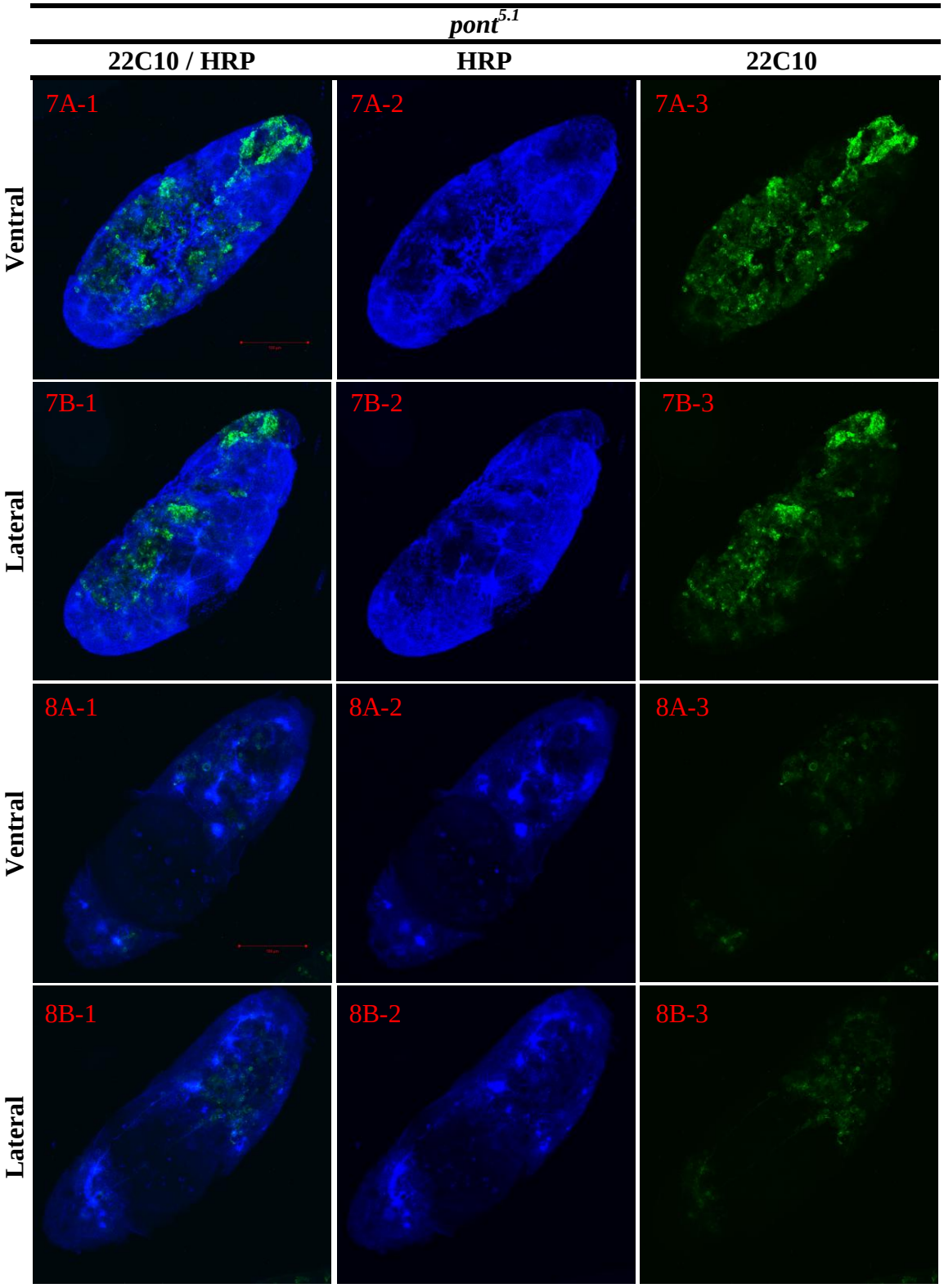


Figure 8. Futch (22C10) and HRP staining of embryos likely to be homozygous for the *pont*$^{5.1}$mutation. Both embryos have a severely distorted nervous system. No organized segments can be seen and both embryos have what seems to be a hollow

space in the lower ventral side. More embryos showed similar morphology, some of which displayed anti-GFP staining, indicating that this might be a balancer phenotype.

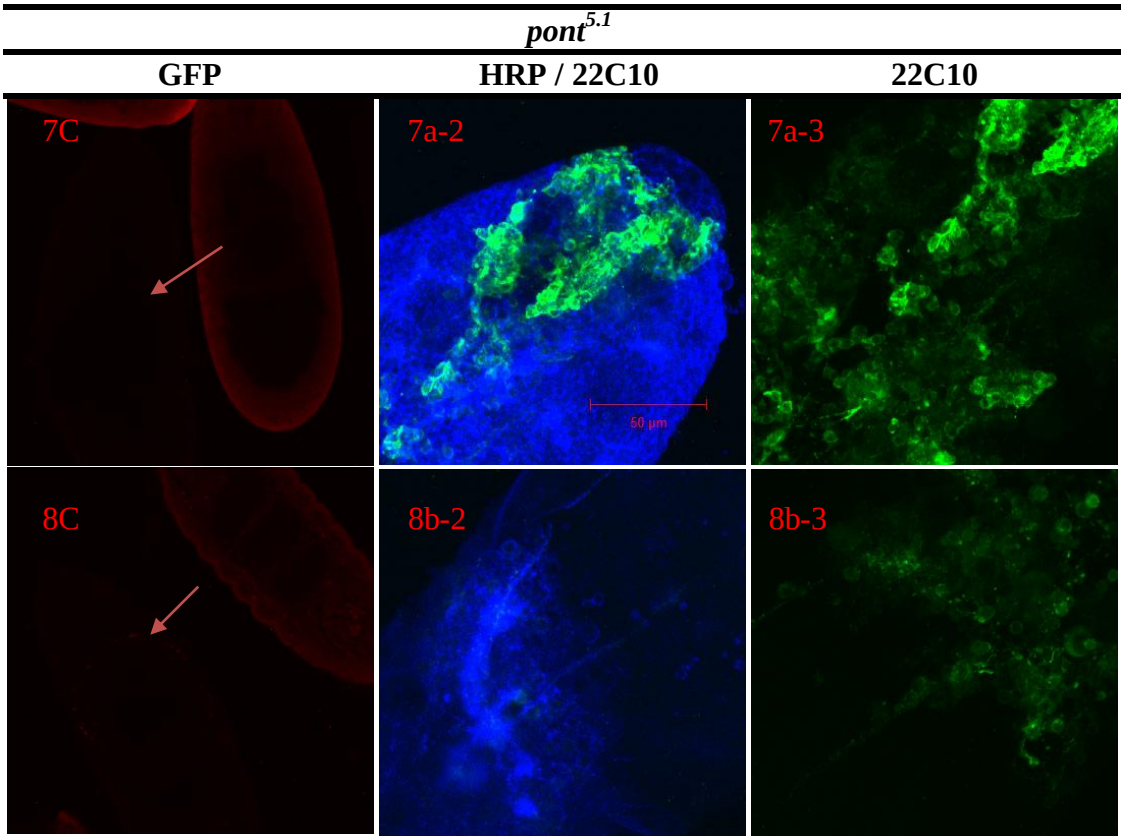
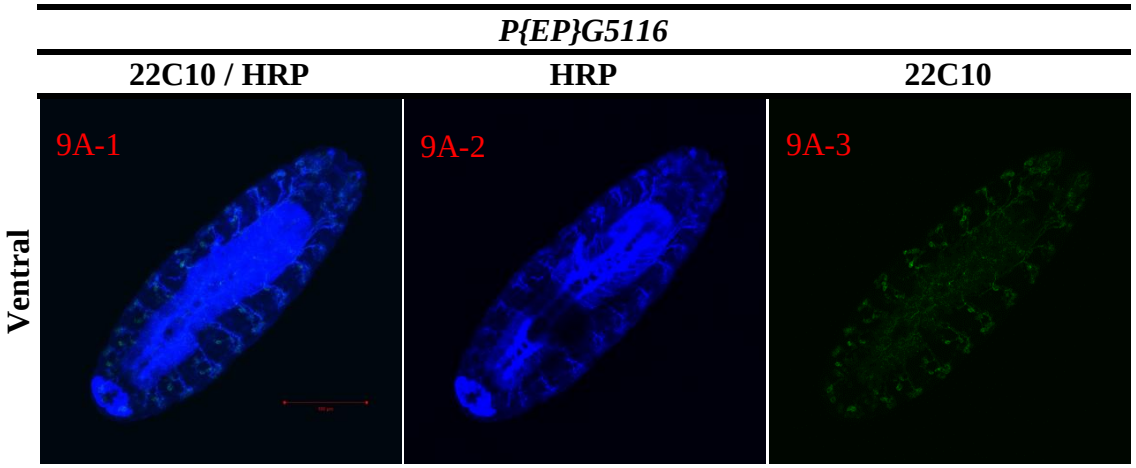


Figure 9. Detailed view of Futch (22C10) and HRP staining of embryos likely to be homozygous for the *pont*$^{5.1}$mutation as well as GFP staining. Both embryos lack GFP staining (images 7C and 8C). Either the *pont*$^{5.1}$mutation and the balancer have similar phenotypes or the GFP staining was ineffective on some of the balancer embryos.

P{EP}G5116 insertion



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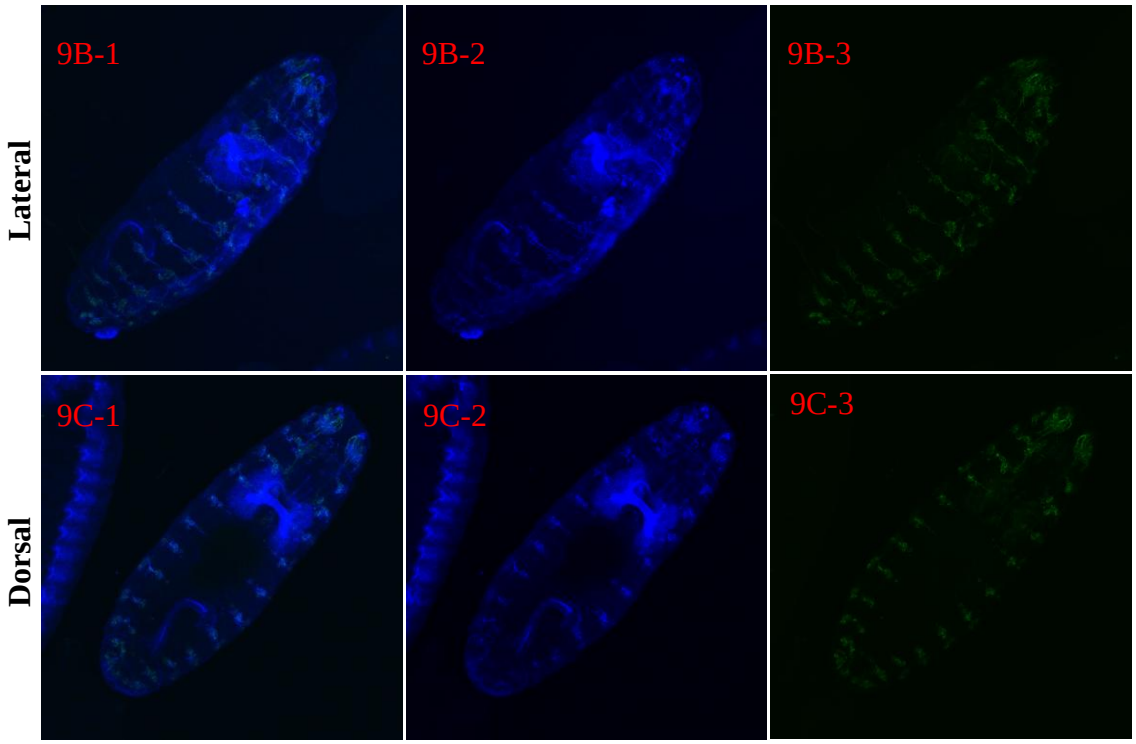


Figure 10. Futch (22C10) and HRP staining of an embryo homozygous for the *P{EP}G5116* mutation. The ventral midline of the embryo is defectious and the chordotonial neuron clusters are irregular. This embryo was the only one examined that showed abnormal morphology. Most of the embryos did not have any obvious abnormalties, which is consistent with the fact that the *P{EP}G5116* insertion is viable.

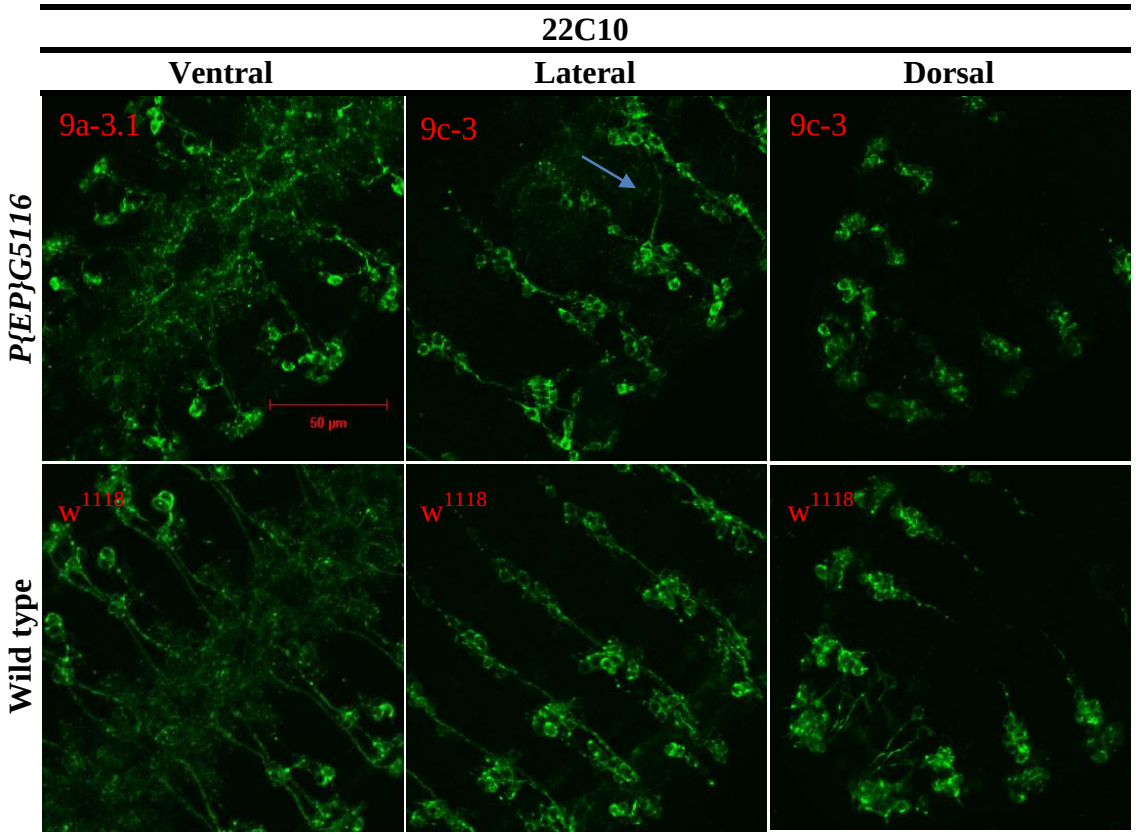


Figure 11. Detailed view of Futch (22C10) and HRP staining of an embryo homozygous for the *P{EP}G5116* insertion and wild type comparison. The midline defect of the embryo can be seen in image 9a-3.1. The embryo also seems to have an axon pathfinding defect. Image 9c-3 shows an axonal projection between two hemisegments (blue arrow). The *P{EP}G5116* mutant embryo shown is somewhat older than the wild type embryo used for comparison. The images have been modified by increasing their brightness and contrast.

3.1.3 Analysis of central nervous system phenotypes

Wild type control

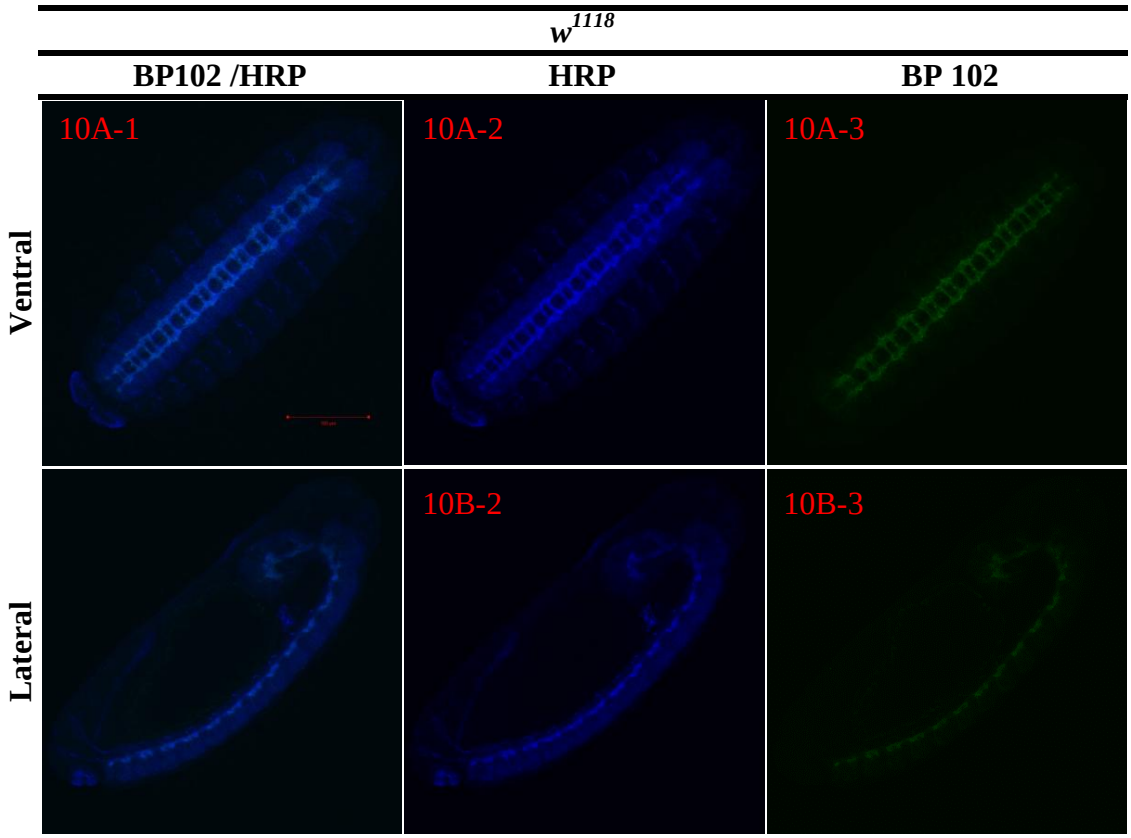


Figure 12. CNS axons (BP102) and HRP staining of w^{1118} embryos. The figure shows confocal images of wild type embryos stained with the BP102 antibody (green) to label CNS axons and anti-HRP (blue) to label all neurons.

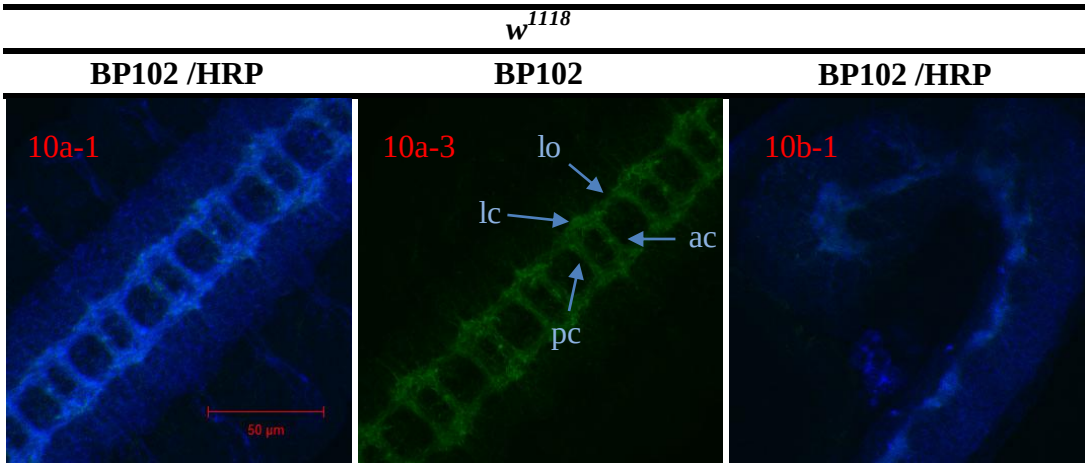


Figure 13. Detailed view of CNS axons (BP102) and HRP staining of w^{1118} embryos. The images display the CNS midline of a w^{1118} embryo. The arrows in image 10a-3 point out a longitudinal tract (lo), a longitudinal connectitive (lc), an anterior comissure (ac) and a posterior comissure (pc) of the midline.

P{EPgy2}rept insertion

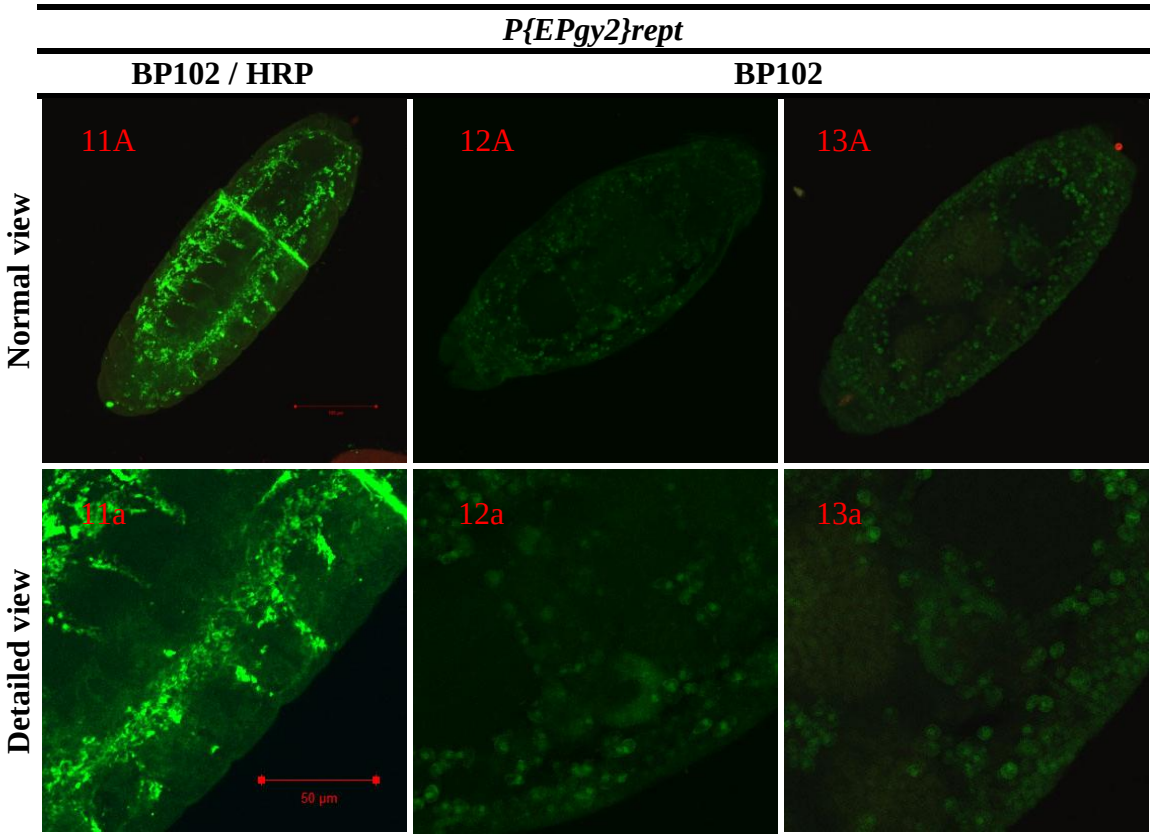
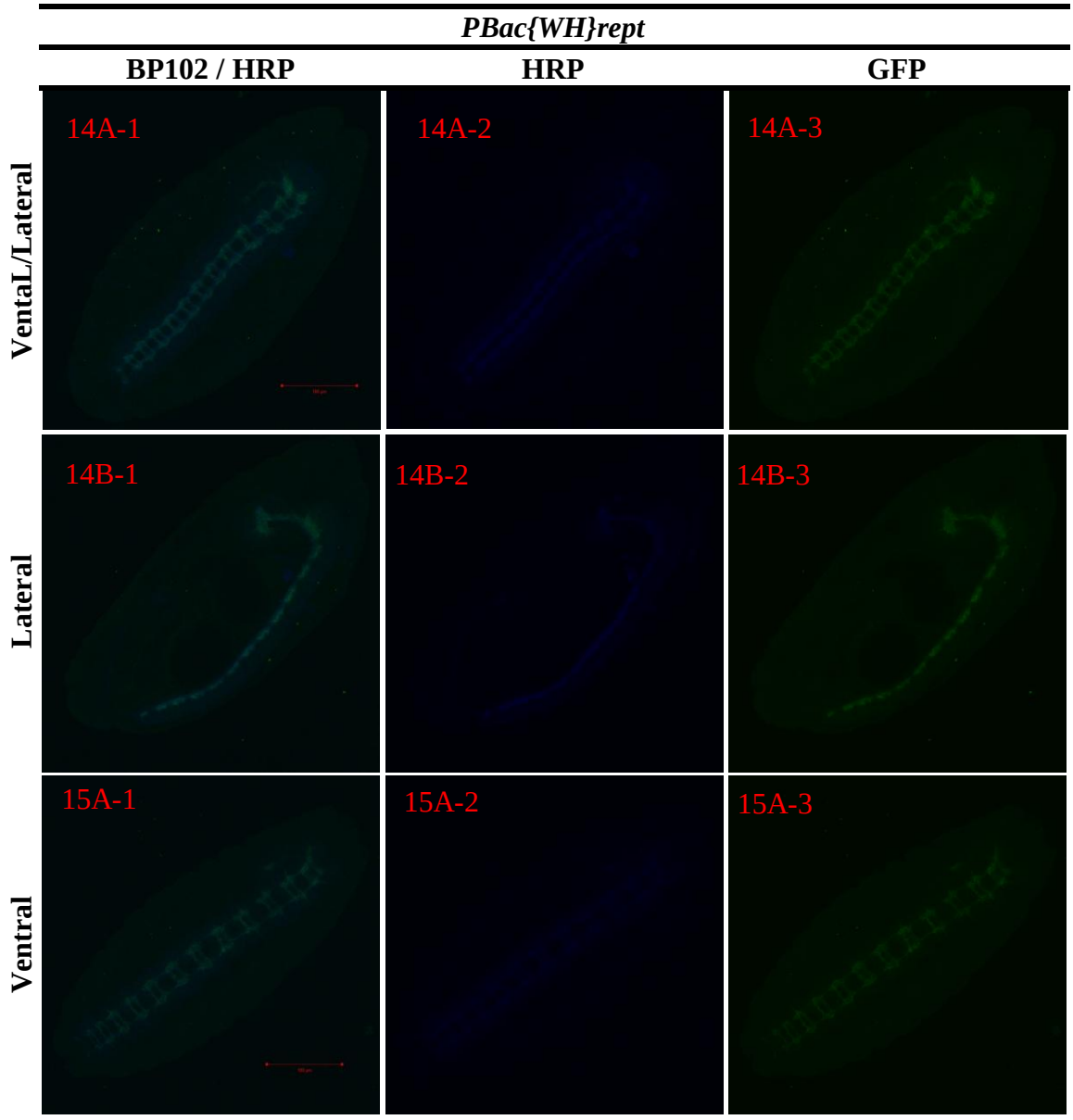


Figure 14. CNS axons (BP102) and HRP staining of embryos likely to be homozygous for the *P{EPgy2}rept* insertion. The embryo in image 11A has a very distorted ventral midline lacking the central comissures and did not show any HRP staining (image not shown). It does show some GFP staining so it could be a balancer phenotype. The embryos in images 12A and 13A are similar to 5A and 6A stained with the 22C10 antibody.

PBac{WH}rept insertion



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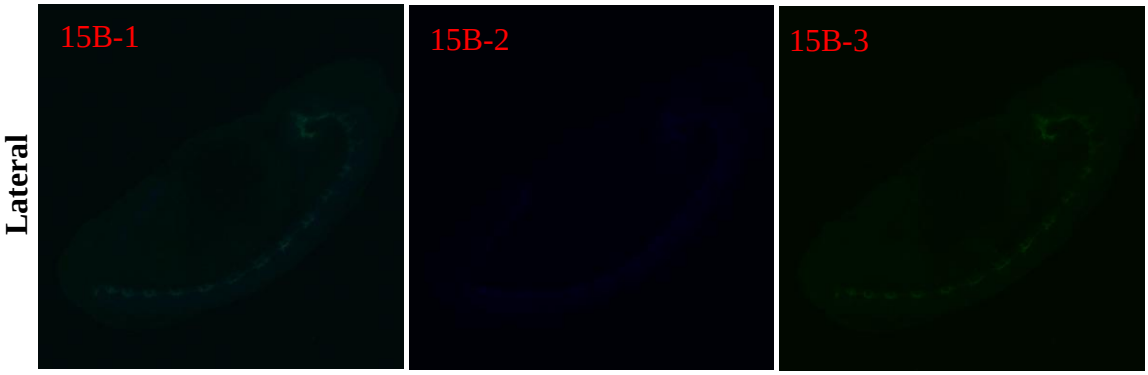


Figure 15. CNS axons (BP102) and HRP staining of embryos likely to be homozygous for the *PBac{WH}rept* insertion.

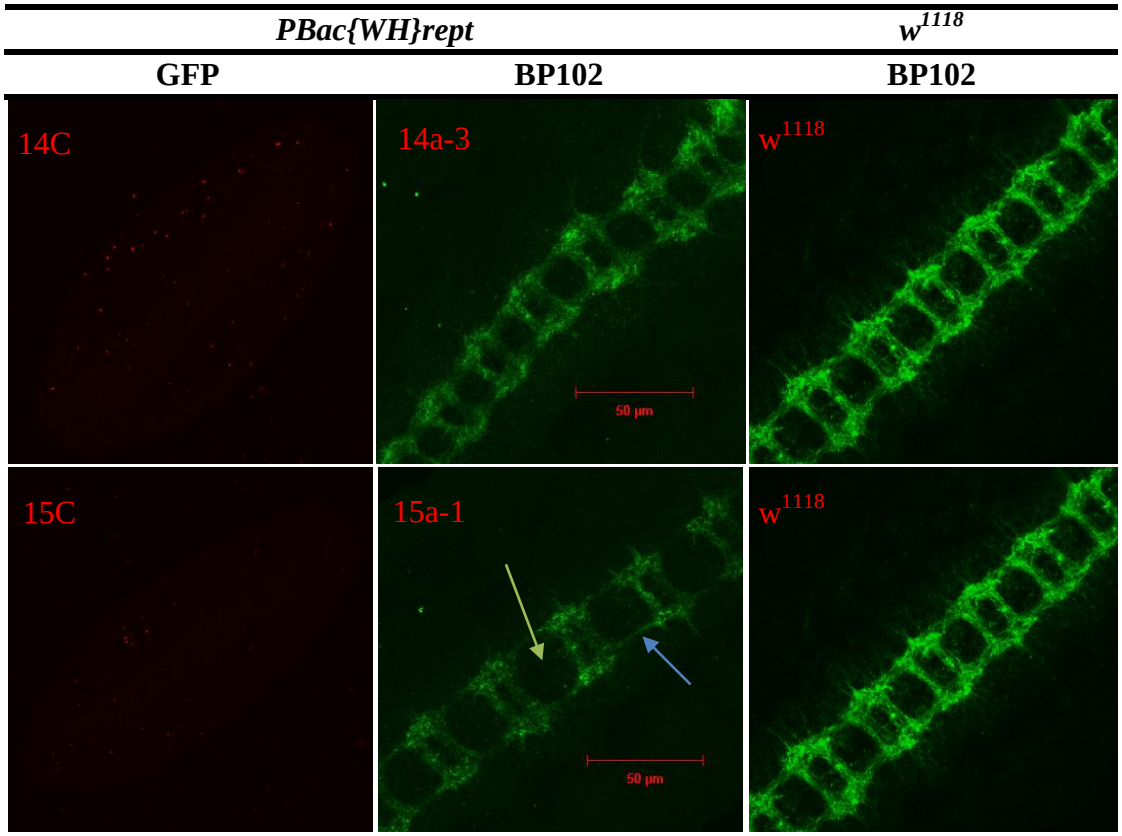


Figure 16. Detailed view of CNS axons (BP102), HRP and GFP staining of embryos likely to be homozygous for the *PBac{WH}rept* insertion. Comparison of image 15a-1 to the wild type image shows that the bilateral longitudinal tracts (blue arrow) are thinner in the embryo likely to carry the *PBac{WH}rept* mutation. There is also a wider space between the two commissural tracts (green arrow) in each segment in embryo 15. Images 14C and 15C show that there is almost no GFP-staining in the embryos. The images have been modified by increasing their brightness and contrast.

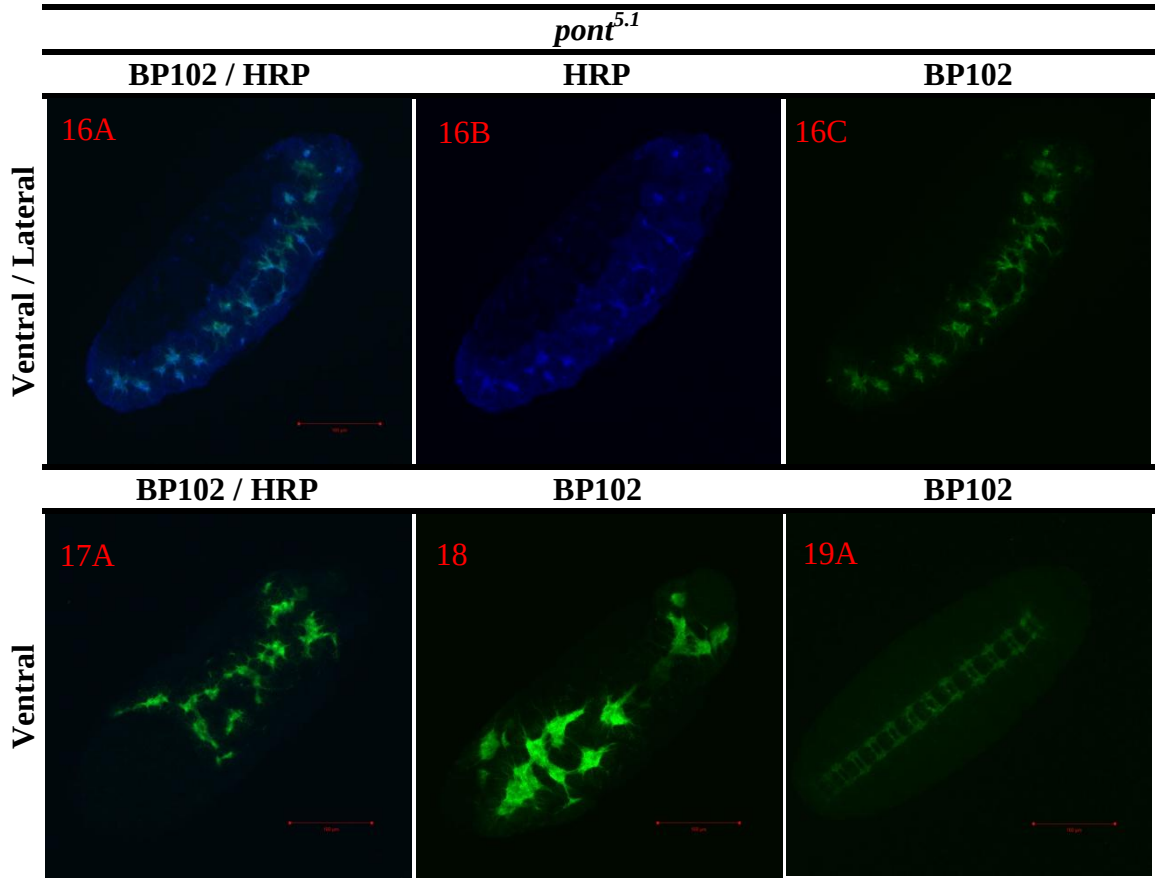
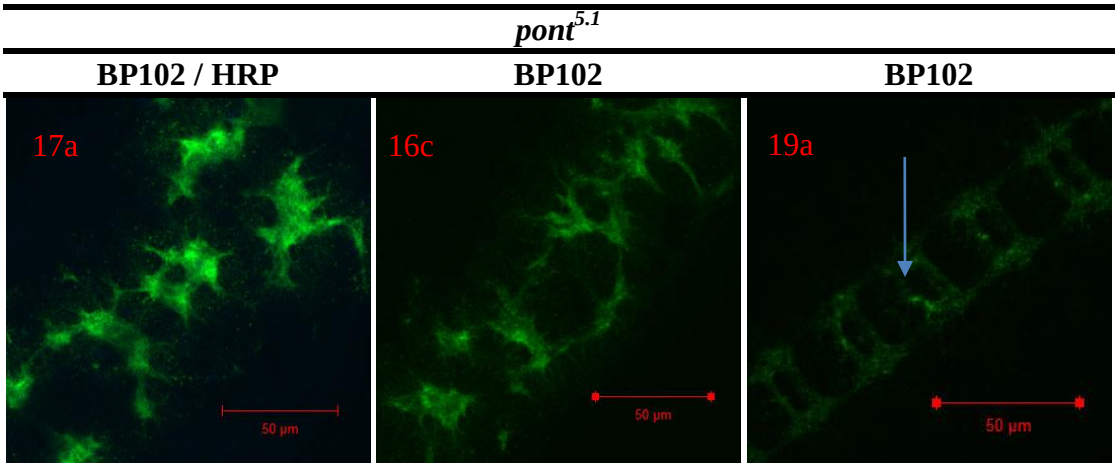


Figure 17. CNS axons (BP102) and HRP staining of embryos homozygous for the *pont*$^{5.1}$ mutation or the GFP balancer. Embryos 17 and 18 have the same phonotype and embryo 16 is very similar. GFP stained embryos with similar morphology have been seen in the immunolabeling of other stocks in this study (e.g. image 23), and the possibility that the embryos are homozygous for the balancer and not the *pont*$^{5.1}$ mutation must be taken into account.



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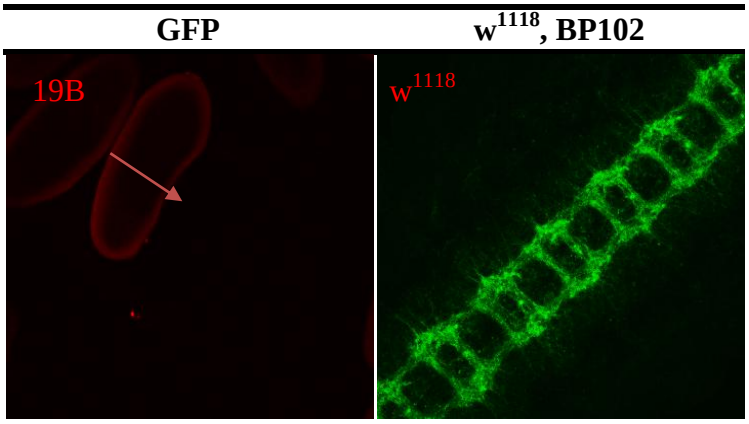
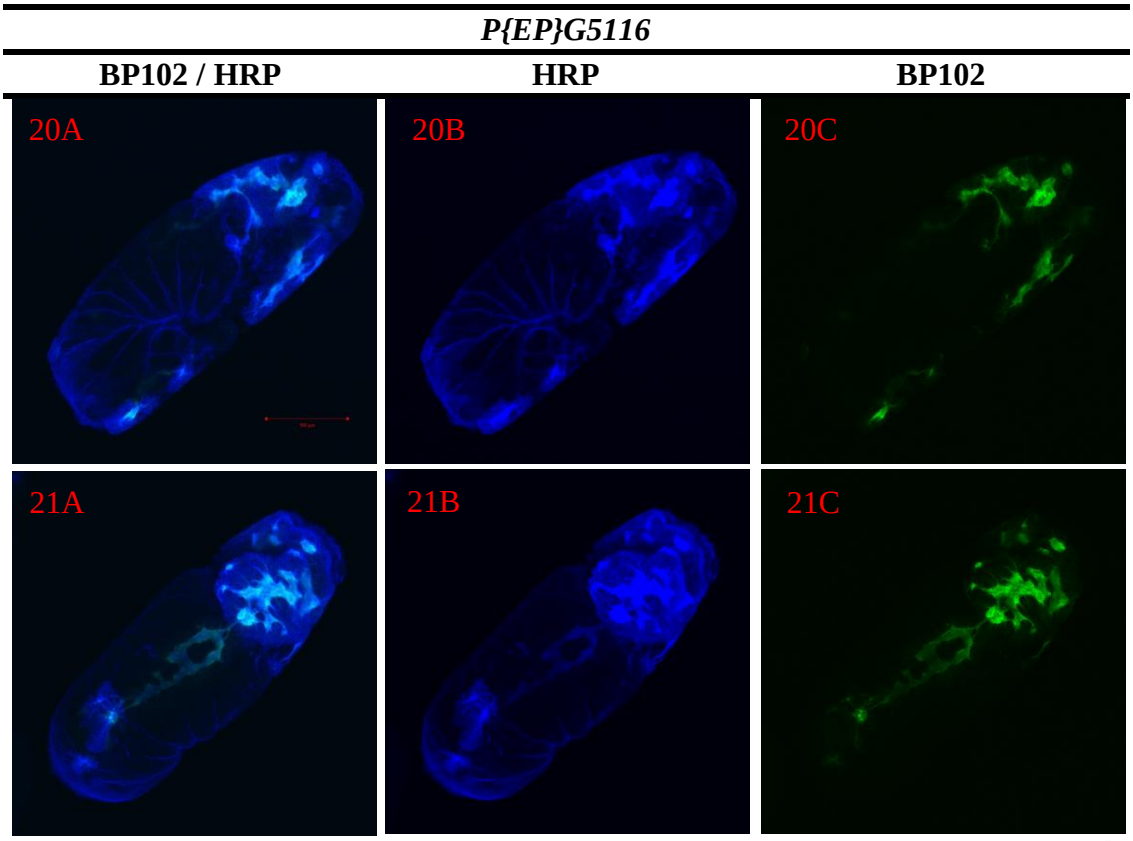


Figure 18. Detailed view CNS axons (BP102) and HRP staining of embryos homozygous for the *pont*$^{5.1}$ mutation or the GFP balancer. Embryo 19 is distinct from the others and shows some abnormalties in the posterior comissure of the midline (blue arrow in image 19a).

P{EP}G5116 insertion



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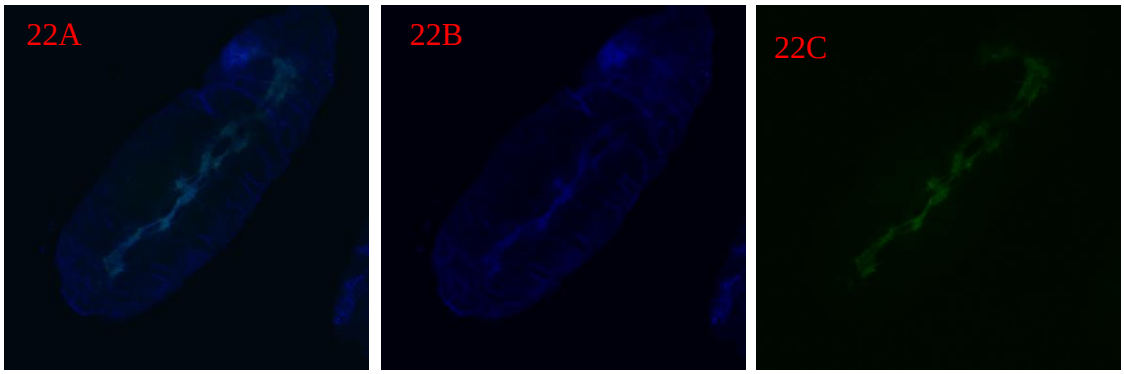

Figure 19. CNS axons (BP102) and HRP staining of embryos that are homozygous for the *P{EP}G5116* insertion or the balancer. The images were taken of embryos collected from a *P{EP}G5116* stock which by mistake there had been an attempt to rebalance. Therefore the images might show embryos that are homozygous for the balancer and not the *P{EP}G5116* insertion. No similarities were found in the 22C10 staining of *P{EP}G5116* mutant embryos.

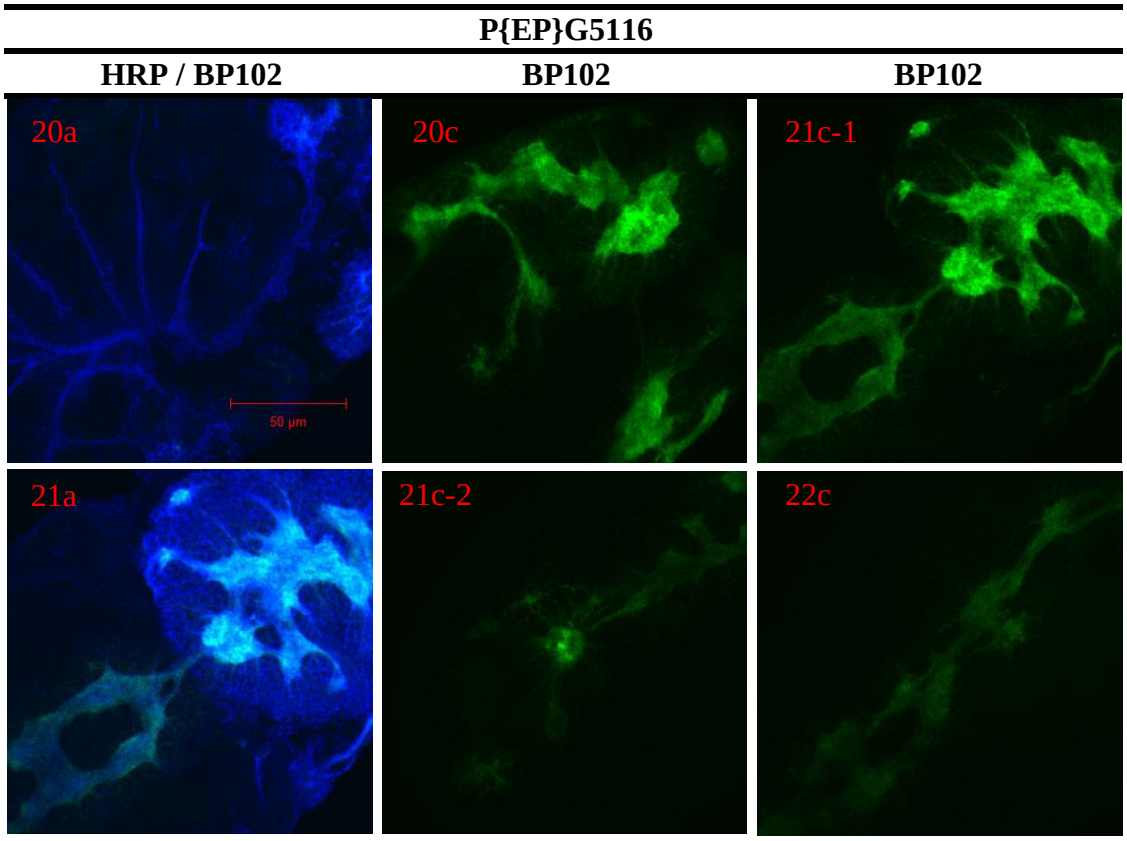

Figure 20. Detailed view of CNS axons (BP102) and HRP staining of embryos that are homozygous for the *P{EP}G5116* insertion or the balancer. Embryo 20 shows similar HRP staining to the embryo shown in image 2B-2 in Figure 2. The BP102 staining in image 20-C shows that the ventral midline is completely distorted and lacks the central commissures. Embryos 20 and 21 share the same phenotype, which is similar to those observed in embryos 16,17 and 18 in Figure 17 and in one of the GFP balancer embryos (24) shown in Figure 21 on next page.

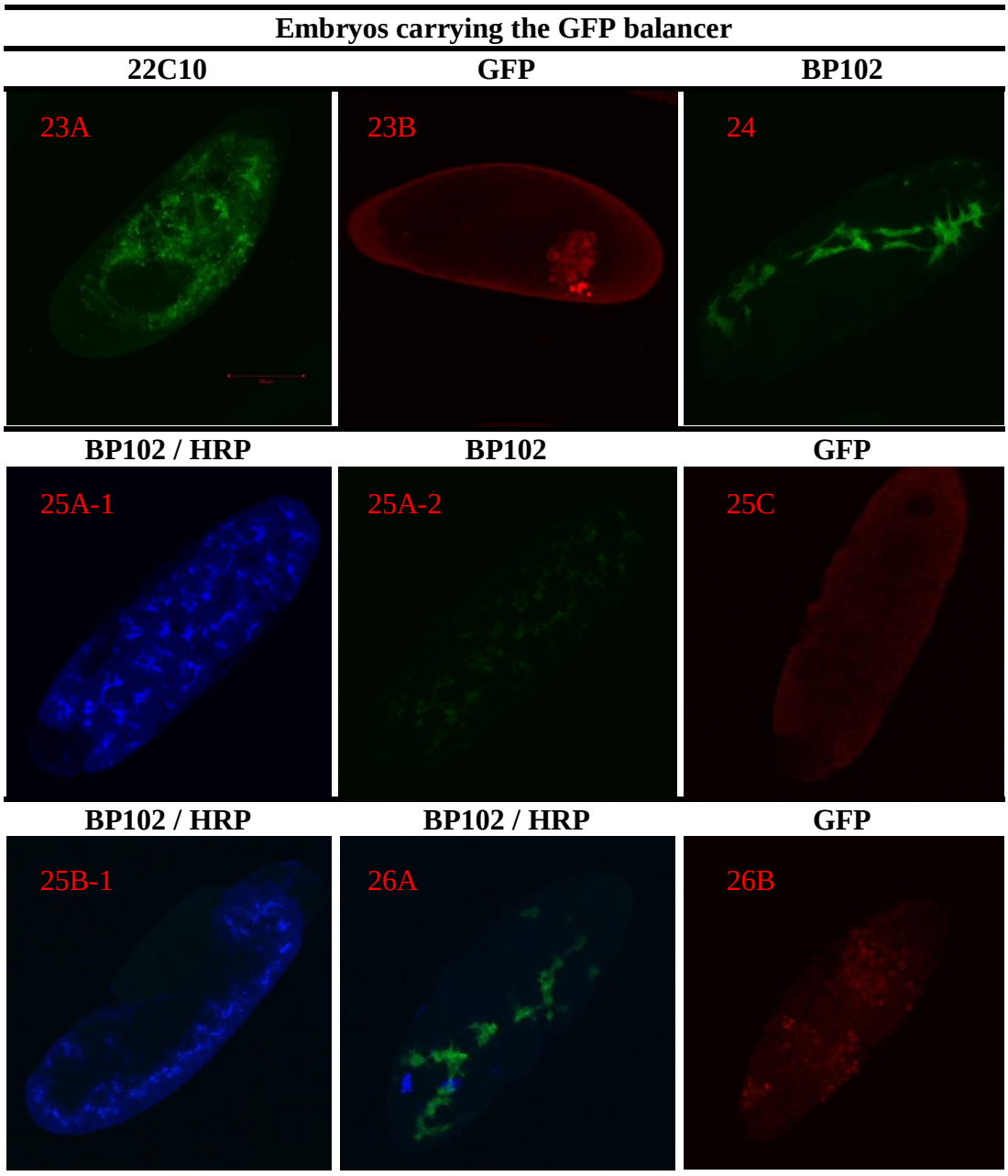

Figure 21. Embryos carrying the GFP balancer. The embryos all display strong GFP staining, and are thus likely to be homozygous for the GFP balancer. Images 23A and B were taken of an embryo from the rebalanced *pont*$^{5.1}$ stock (No. 12 in table 2). Image 24 was taken of an embryo from the rebalanced *rept*^{D35} stock (No.11 in table 2), and is the only image that was taken from the immunolabeling of that stock since no embryos in the right embryonic stages (14-17) which lacked GFP staining were observed. This embryo (24) displayed strong GFP staining (not shown) and is thus likely to be homozygous for the GFP balancer. The embryos in images 25 and 26 were collected from the rebalanced *P{EPgy2}rept* sock (No. 9 in table 2).

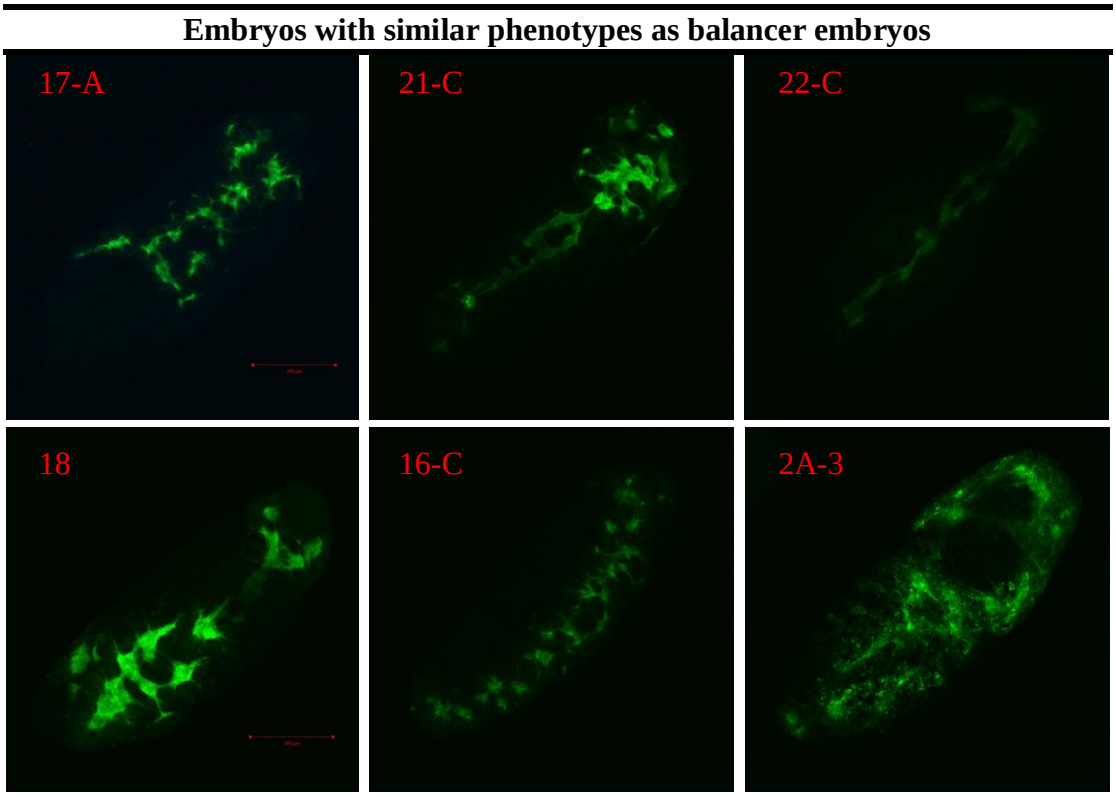

Figure 22. Embryos that do not display GFP staining but have phenotypes similar to the balancer embryos. The distorted PNS of the embryo shown in image 2A-3 resembles that of embryo 23A in Figure 21, and might therefore carry the balancer although no GFP staining was observed. Embryos 21 and 22 have similar phenotypes as embryo 24 in figure 21. Embryo 25 is very similar to 16 and embryo 26 has a phenotype resembling embryos 17 and 16. Either the GFP staining must have been ineffective in several cases, or the *pont*$^{5.1}$ mutation has similar phenotypes as the balancer.

4 Discussion

It is clear that the Pontin and Reptin proteins have roles in the developing Drosophila nervous system. The confocal microscopy imaging of embryos with *reptin* and *pontin* null alleles, and transgenic insertions near the genes revealed several abnormal phenotypes, indicating that the disruption of the *pontin* and *reptin* genes severely affects the developing nervous system. Analysis of the *reptin* null allele ($rept^{D35}$) using the Futch antibody (22C10) revealed a phenotype with defective morphology and distorted axonal projections that are closer to each other than in wild type embryos. Only one embryo from the $rept^{D35}$ mutant stock is shown since few embryos in the right embryonic stages that lacked GFP were observed, and none were seen in the anti-CNS (BP102) stain. Further investigation is therefore needed to confirm that this phenotype is indeed caused by the *reptin* null allele, and to see the effect of the allele on the CNS. Transgenic insertions near the *reptin* gene caused defects in most embryos. The *P{EPgy2}rept* insertion caused an intriguing phenotype when stained with the 22C10 antibody, which might be the result of motoneuron absence (see Figures 5 and 6). The BP102 stain showed two possible phenotypes associated with the *P{EPgy2}rept* insertion. The embryo shown in image 11A has a much distorted ventral midline lacking the central comissures, whilst most of the embryos lacking GFP staining have no apparent CNS (see Figure 14). The CNS absence coincides with the 22C10 staining where only PNS staining is visible. To confirm whether the *P{EPgy2}rept* insertion results in absence of motoneurons and/or the CNS, the immunolabeling could be repeated using the HRP antibody and an antibody specific for motoneurons. There was no PNS staining in embryos from the *PBac{WH}rept* insertion stock lacking GFP. There are two possible causes, one is that the embryos lack the PNS and the second possibility is that the staining was ineffective. The second option is more likely, since no HRP staining is observed in these embryos, whilst HRP staining is clearly visible in the HRP/BP102 images (Figure 15). In addition, the CNS stain revealed embryos with a slightly defective central midline. Figure 16 shows a possible *PBac{WH}rept* insertion phenotype where the bilateral longitudinal tracts are thinner than in wild type embryos. Analysis of the *pontin* null allele ($pont^{5.1}$) using the Futch antibody (22C10) revealed severely defective phenotypes in embryos lacking GFP. In addition to

the ones showed in Figure 9, several embryos displayed similar morphology, some of which had GFP staining. This indicates that the *pont*$^{5.1}$ mutation and the balancer have similar phenotypes, or that the GFP staining was ineffective on some of the embryos. The CNS staining also revealed defective phenotypes, some of which are similar to embryos carrying the balancer (see Figure 17). Only one embryo carrying the *P{EP}G5116* insertion displayed defects when stained with anti-Futch. The normal appearance of the rest of the embryos is consistent with the fact that the *P{EP}G5116* insertion is viable, and must therefore have somewhat of a functioning nervous system. It is harder to draw conclusions from the CNS stain, since the embryos used were collected from a stock which by mistake there had been an attempt to rebalance. Therefore the images might show embryos that are homozygous for the balancer. As can be seen from Figures 21 and 22, embryos homozygous for balancers are likely to display abnormal phenotypes, making it difficult to determine whether the phenotypes of the embryos are caused by the balancer or the *pontin/reptin* mutation. It would therefore be useful to test the antibodies on embryos known to be homozygous for the balancer, to determine if any of the phenotypes observed in this study can be found, compare them to the results of the study and to results from other studies using *TM3*^{actGFP}, *Ser* balancer.

The results from the viability count show that the mutant *reptin* alleles are not viable against each other whereas the mutant *pontin* alleles, *P{EP}G5116* and *pont*$^{5.1}$, seem to be viable against each other. To investigate further, offspring from the *pontin* mutant cross with normal bristles could be screened for abnormalities, phenotypic and behavioral, caused by carrying the two *pontin* mutant alleles, and compared to wild type flies and to flies that carry only one of the alleles. The crosses used for the viability count could also be analyzed by immunolabeling, as done with the individual mutant alleles here. In addition to the suggestions made here, it is clear that a variety of studies will be needed to clarify the roles of Pontin and Reptin in the nervous system where they are likely to function in several ways, as they do elsewhere.

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