Transcriptional Regulation on Development of *Drosophila* 
Type II Neuroblast Lineages

by

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A Dissertation in Neuroscience and Physiology

Submitted in partial fulfillment of the requirements for the degree of Doctor of 
Philosophy in the College of Graduate Studies of State University of New York,
Upstate Medical University

Approved ____________________________
(Sponsor’s signature)

Date 12/16/21

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Contribution Statement

Abstract

Introduction

Results

Six4 is specifically expressed in Drosophila larval type II NB lineages

 Knockdown of Six4 results in supernumerary type II NB lineages

 Six4 in imINPs prevents dedifferentiation of imINPs to ectopic type II NBs

 Six4 has synergistic effect with Erm to prevent dedifferentiation of imINPs

 Overexpressing Six4 in imINPs substitutes Erm to prevent dedifferentiation of imINPs

 Six4 prevents dedifferentiation of imINPs likely by antagonizing PntP1 function

 Six4, Erm, and PntP1 likely form a trimeric complex to inhibit PntP1 function

 Endogenous Six4 suppresses Pros expression in newly born Ase imINPs

 Simultaneous reduction of Six4 and PntP1 results in depletion of mINPs but not an increase of type II NBs

Discussion

Expression of Six4 in cell types within type II NB lineages

Six4 prevents dedifferentiation of imINPs

Functional relationship between Six4 and Erm in preventing dedifferentiation of imINPs

Equilibrate the expression levels and activity of Six4 in type II NBs

A potential role of Six4 in preventing premature differentiation of INPs

Materials and Methods

Acknowledgements

Competing interests

Funding
CHAPTER IV. Mediator complex subunits Kohtalo and Skuld interact with the Ets protein Pointed P1 to prevent dedifferentiation of intermediate neural progenitors

Contribution Statement

Abstract

Introduction

Results

Skd and Kto act synergistically to prevent generation of supernumerary type II NBs.

Kto and Skd are ubiquitously expressed in cells of Drosophila central nervous system.

Skd and Kto are required to maintain self-renewal of type I NBs.

Supernumerary type II NBs resulting from Skd/Kto knockdown arise from dedifferentiation of imINPs due to loss of Erm expression.

Skd and Kto are essential cofactors of PntP1.

Six4 genetically interacts with mediator complex to prevent supernumerary type II NBs.

Discussion

Skd and Kto function as cofactors of PntP1 in both type II NBs and imINPs.

Erm mediates indirect interactions between Six4 and Skd or Kto in preventing dedifferentiation of imINPs.

Prevention of dedifferentiation of imINPs by the mediator complex and Six4.

Potential function of Skd and Kto in mediating the cell cycle exit in type I NBs versus type II NBs.

Materials and Methods

Acknowledgements

Competing interests

Funding

Supplementary Figures for CHAPTER IV

References

CHAPTER V. General Discussion

Type II NB lineage determination during Drosophila embryogenesis and post-embryogenesis

Determining functional specificity of the master regulator in type II NB lineage progression.

Regulations and contributions of intermediate neuronal progenitors during neurogenic process or disorders.
ACKNOWLEDGMENTS

At this moment when I finally accomplished this dissertation based on six-year research on neural stem cells, I actually recall the first moment I was interviewed by my mentor Dr. Sijun Zhu when he accepted me directly to his talented research group and reminded me science needs one’s whole dedication. And I believe I did it! I would like sincerely to thank Dr. Zhu for his generosity to offer me the chance to resume my career on science and for trusting me who muddled through undergraduate study and master program with a mediocre GPA and gapped for three years in non-academic field. During the past time of my study and research, Dr. Zhu provided me outstanding mentorship on training my scientific thinking, writing, experimental design and skills, offered me the best resource for research, and drove my scientific career beyond my limits at every crucial step.

I wish to thank my advisory committee, Drs. Pignoni, Matthews, Zuber, Amack and Lewis for their invaluable suggestions that always inspire me on my research.

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I would like to thank my parents who support me without any reservation. And finally, I dedicate the work herein to my wife, Lan Shi, who sacrifices her own career in our hometown, dedicates herself to keep our small family safe and warm, and encourages me at difficult times. Without her sacrifices behind me, I could not be able to focus on my work and achieve the stage now I am.

Thank you, Lan!
ABSTRACT

In order to promote neural stem cells (NSCs) to generate progeny cells functionally contributing to central nervous system (CNS), each step involved needs to be finely controlled. Self-renewing type II neuroblasts (NBs, the NSCs of *Drosophila*) and their derived self-renewing intermediate neural progenitors (INPs) drastically boost the output and diversity of neurons and glia compared to other types of NBs, such as type I NBs or type 0 NBs. Therefore, type II NBs and their derived lineages stand a unique position for investigating the fundamentals of neural stem cell biology and addressing neurogenetic disorders during development. Cell-type-specific transcription factors along with their cofactors play critical roles in determining the fate and homeostasis of type II NBs, immature INPs (imINPs) and mature INPs (mINPs). However, there are gaps in our understanding of the complete pathway or network for regulating type II NB lineage development, due to either unknown transcriptional regulators or unknown mechanisms for those known factors. In this dissertation, I dissect the underlying molecular mechanisms for maintaining the identity of type II NBs through the master transcriptional activator, Pointed P1 (PntP1) and its direct downstream target Tailless (Tll), a transcriptional repressor that suppresses type I NB marker Asense (Ase). I also identify the transcription factor Six4 and two novel cofactors of PntP1, Skuld (Skd) and Kohtalo (Kto), which function through interacting with PntP1 to regulate the homeostasis of INPs. I find that PntP1 needs Skd and Kto as cofactors to activate dFez transcriptional repressor earmuff (Erm) in imINPs, which in turn directly binds Six4 to decommission PntP1’s function and thus promote imINPs’ transition into fate-committed mINPs and thereby preventing imINPs’ dedifferentiation into NBs. I also show that Six4 prevents premature differentiation of imINPs to maintain the pool of mINPs by repressing cell cycle exit determinant Prospero (Pros). Taken together, I discovered novel transcriptional regulations involved in maintaining the identity of type II NBs and regulating the pool of INPs for
consistently boosting neurogenesis. This dissertation includes published and unpublished co-authored material.
# List of Acronyms

The list is arranged alphabetically.

<table>
<thead>
<tr>
<th>Acronyms/abbreviations</th>
<th>Full names</th>
</tr>
</thead>
<tbody>
<tr>
<td>aDM</td>
<td>anterior dorsomedial</td>
</tr>
<tr>
<td>ALs</td>
<td>antennal lobes</td>
</tr>
<tr>
<td>APC/C</td>
<td>Anaphase Promoting Complex/Cyclosome</td>
</tr>
<tr>
<td>BDSC</td>
<td>Bloomingston Drosophila Stock Center</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>bIPs</td>
<td>basal intermediate progenitors</td>
</tr>
<tr>
<td>Br-C</td>
<td>Broad Complex</td>
</tr>
<tr>
<td>bRGs</td>
<td>basal radial glia cells</td>
</tr>
<tr>
<td>BUs</td>
<td>bulbs</td>
</tr>
<tr>
<td>CA</td>
<td>calyx</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>CB</td>
<td>central brain</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Co-IP</td>
<td>co-immunoprecipitation</td>
</tr>
<tr>
<td>CX</td>
<td>central complex</td>
</tr>
<tr>
<td>DL</td>
<td>Dorsolateral</td>
</tr>
<tr>
<td>DM</td>
<td>dorsomedial</td>
</tr>
<tr>
<td>DSCP</td>
<td>Drosophila synthetic core promoter</td>
</tr>
<tr>
<td>E(spl)</td>
<td>Enhancer of Split</td>
</tr>
<tr>
<td>EB</td>
<td>ellipsoid body</td>
</tr>
<tr>
<td>EcR</td>
<td>ecdysone receptor</td>
</tr>
<tr>
<td>EGFRe</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGR</td>
<td>early growth response</td>
</tr>
<tr>
<td>EMSAs</td>
<td>performed electrophoretic mobility shift assays</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>EnR</td>
<td>repressor domain of Engrailed</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal Sorting Complex Required for Transport</td>
</tr>
<tr>
<td>ETS or Ets</td>
<td>E26 transformation-specific</td>
</tr>
<tr>
<td>FB</td>
<td>fan-shaped body</td>
</tr>
<tr>
<td>FIMO</td>
<td>Find Individual Motif Occurrences</td>
</tr>
<tr>
<td>GMCs</td>
<td>ganglion mother cells</td>
</tr>
<tr>
<td>HD</td>
<td>homeodomain</td>
</tr>
<tr>
<td>ILPs</td>
<td>insulin/IGF-like peptides</td>
</tr>
<tr>
<td>imINPs</td>
<td>immature intermediate neural progenitors</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>INPs</td>
<td>intermediate neural progenitors</td>
</tr>
<tr>
<td>IPCs</td>
<td>intermediate progenitor cells</td>
</tr>
<tr>
<td>IPC NBs</td>
<td>NBs that generate insulin-producing cells</td>
</tr>
<tr>
<td>IPC NBs</td>
<td>inner proliferation center neuroblasts</td>
</tr>
<tr>
<td>iSVZ</td>
<td>inner SVZ</td>
</tr>
<tr>
<td>LAL</td>
<td>lateral complex</td>
</tr>
<tr>
<td>LNBs, or pNBs</td>
<td>olfactory lateral (projection) NBs</td>
</tr>
<tr>
<td>LOF</td>
<td>loss-of-function</td>
</tr>
<tr>
<td>MARCM</td>
<td>mosaic analysis with a repressible cell marker</td>
</tr>
<tr>
<td>MB</td>
<td>mushroom body</td>
</tr>
<tr>
<td>mINPs</td>
<td>mature intermediate neural progenitors</td>
</tr>
<tr>
<td>modERN</td>
<td>model organism Encyclopedia of Regulatory Networks</td>
</tr>
<tr>
<td>MRGs</td>
<td>master regulator genes</td>
</tr>
<tr>
<td>NBs</td>
<td>neuroblasts</td>
</tr>
<tr>
<td>NE</td>
<td>neuroepithelium</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NO</td>
<td>noduli</td>
</tr>
<tr>
<td>NSCs</td>
<td>neural stem cells</td>
</tr>
<tr>
<td>OL</td>
<td>optical lobe</td>
</tr>
<tr>
<td>OPC</td>
<td>outer proliferation center</td>
</tr>
<tr>
<td>oRGS</td>
<td>outer radial glia cells</td>
</tr>
<tr>
<td>oSVZ</td>
<td>outer SVZ</td>
</tr>
<tr>
<td>PAN</td>
<td>Posterior Asense-Negative</td>
</tr>
<tr>
<td>PB</td>
<td>protocerebral bridge</td>
</tr>
<tr>
<td>pDM</td>
<td>posterior dorsomedial</td>
</tr>
<tr>
<td>Pdm</td>
<td>protocerebral domain</td>
</tr>
<tr>
<td>PED</td>
<td>pedunculus</td>
</tr>
<tr>
<td>PI3K</td>
<td>Class IA phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PN</td>
<td>projection neuron</td>
</tr>
<tr>
<td>pPlm</td>
<td>PI primordium</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>RGCs, or RGs</td>
<td>radial glia cells</td>
</tr>
<tr>
<td>SD</td>
<td>SIX domain</td>
</tr>
<tr>
<td>sfGFP</td>
<td>superfolder GFP</td>
</tr>
<tr>
<td>SIX</td>
<td>sine oculis homebox</td>
</tr>
<tr>
<td>STAP</td>
<td>self-transcribing active regulatory region sequencing</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>TF</td>
<td>transcriptional factor</td>
</tr>
<tr>
<td>tOPC</td>
<td>tips of the OPC</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>TSS</td>
<td>transcription start site</td>
</tr>
<tr>
<td>TTFs</td>
<td>temporal transcription factors</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activating sequence</td>
</tr>
<tr>
<td>VDRC</td>
<td>Vienna Drosophila Resource Center</td>
</tr>
<tr>
<td>VNCs</td>
<td>ventral nerve cords</td>
</tr>
<tr>
<td>VP16AD</td>
<td>VP16 activation domains</td>
</tr>
<tr>
<td>VZ</td>
<td>ventricular zone</td>
</tr>
</tbody>
</table>
### List of Genes

The list is arranged alphabetically.

<table>
<thead>
<tr>
<th>Gene Symbols</th>
<th>Full Names</th>
<th>Type of Protein (or RNA, when noncoding), summarized from Flybase</th>
</tr>
</thead>
<tbody>
<tr>
<td>abd-A</td>
<td>abdominal A</td>
<td>a homeobox-containing transcription factor component of the bithorax complex</td>
</tr>
<tr>
<td>ac</td>
<td>achaete</td>
<td>a basic helix loop helix transcription factor; proneural factor; a component of the achaete-scute complex</td>
</tr>
<tr>
<td>Akt</td>
<td>Akt kinase</td>
<td>the core kinase component of the insulin/insulin-like growth factor pathway</td>
</tr>
<tr>
<td>ana2</td>
<td>anastral spindle 2</td>
<td>a centriole protein that is essential for centriole assembly, promoting the assembly of the central &quot;cartwheel&quot; structure</td>
</tr>
<tr>
<td>Antp</td>
<td>Antennapedia</td>
<td>the distal-most member of the Antennapedia complex; a sequence-specific homeodomain transcription factor</td>
</tr>
<tr>
<td>AP-2α</td>
<td>Adaptor Protein complex 2, α subunit</td>
<td>also known as α-adaptin; a subunit of the AP-2 adaptor complex; required for proper intracellular trafficking and secretion</td>
</tr>
<tr>
<td>Apc2</td>
<td>Adenomatous polyposis coli 2</td>
<td>one of Drosophila adenomatous polyposis coli (APC) family proteins; a negative regulator of Arm; a critical component of the destruction complex that phosphorylates Arm and thus targets it for ubiquitination and proteasomal destruction</td>
</tr>
<tr>
<td>aPKC</td>
<td>atypical protein kinase C</td>
<td>a member of the conserved Par complex and is asymmetrically localized to the apical cortex</td>
</tr>
<tr>
<td>arm</td>
<td>armadillo</td>
<td>the Drosophila homolog of β-catenin; a Wnt-TCF signaling pathway core component</td>
</tr>
<tr>
<td>ase</td>
<td>asense</td>
<td>a basic helix loop helix transcription factor, proneural factor; a component of the achaete-scute complex</td>
</tr>
<tr>
<td>ash2</td>
<td>absent, small, or homeotic discs 2</td>
<td>a component of the histone methyltransferase complex (SET1/MLL) that specifically methylates lysine 4 of histone H3 and a member of the trithorax family</td>
</tr>
<tr>
<td>asl</td>
<td>asterless</td>
<td>a component of the centriole that is required for centrosome function</td>
</tr>
<tr>
<td>aurB</td>
<td>aurora B</td>
<td>a serine-threonine kinase and member of the chromosomal passenger complex</td>
</tr>
<tr>
<td>baz</td>
<td>bazooka</td>
<td>a scaffold protein that forms a complex with the products of par-6 and aPKC and with other cortical, cytoskeletal and regulatory proteins</td>
</tr>
<tr>
<td>brat</td>
<td>brain tumor</td>
<td>a tumor suppressor that regulates proliferation in the brain; Enables mRNA 3'-UTR binding activity and translation repressor activity;</td>
</tr>
<tr>
<td>br</td>
<td>broad</td>
<td>also known as Broad Complex (Br-C); a Zinc finger C2H2 transcription factor</td>
</tr>
<tr>
<td>brm</td>
<td>brahma</td>
<td>an ATPase subunit of the Brahma chromatin-remodeling complex (SWI/SNF complex), which is involved in transcription regulation</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Notes</td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
<td>-------</td>
</tr>
<tr>
<td>btd</td>
<td>buttonhead</td>
<td>a triple C2H2 zinc finger transcription factor; the homolog of mammalian Sp8</td>
</tr>
<tr>
<td>cas</td>
<td>castor</td>
<td>a zinc finger C2H2 transcription factor, repressing the prior redundant genes, pdm2 nub</td>
</tr>
<tr>
<td>Cdk8</td>
<td>Cyclin-dependent kinase 8</td>
<td>a Cyclin-dependent kinase; a component of the Mediator complex; a coactivator involved in regulated gene transcription of nearly all RNA polymerase II-dependent genes</td>
</tr>
<tr>
<td>chinmo</td>
<td>Chronologically inappropriate morphogenesis</td>
<td>putative BTB-zinc finger transcription factor; essential for neuronal temporal patterning</td>
</tr>
<tr>
<td>Chro</td>
<td>Chromator</td>
<td>a chromodomain protein in spindle matrix complex, required for proper microtubule spindle formation</td>
</tr>
<tr>
<td>crb</td>
<td>crumbs</td>
<td>a transmembrane protein that binds to multiple proteins, a negative regulator of Notch activity</td>
</tr>
<tr>
<td>Cull</td>
<td>Cullin 1</td>
<td>a core component of multiple SCF (SKP1-CUL1-F-box protein) E3 ubiquitin-protein ligase complexes</td>
</tr>
<tr>
<td>cycC</td>
<td>Cyclin C</td>
<td>a component of the Mediator complex, a coactivator involved in regulated gene transcription of nearly all RNA polymerase II-dependent genes</td>
</tr>
<tr>
<td>cycE</td>
<td>Cyclin E</td>
<td>Enables cyclin-dependent protein serine/threonine kinase regulator activity</td>
</tr>
<tr>
<td>D</td>
<td>Dichaete</td>
<td>an HMG-domain protein and member of the Sox family of transcription factors</td>
</tr>
<tr>
<td>dap</td>
<td>dacapo</td>
<td>a Cyclin-dependent kinase inhibitor in the CIP/KIP family. It binds to CycE-Cdk2 complexes and thereby inhibits their protein kinase activity</td>
</tr>
<tr>
<td>Det</td>
<td>Deterin</td>
<td>a component of the chromosomal passenger complex involved in regulation of apoptosis and cytokinesis</td>
</tr>
<tr>
<td>Dfd</td>
<td>Deformed</td>
<td>a homeobox-containing transcription factor; regulating apoptosis activator reaper</td>
</tr>
<tr>
<td>dl</td>
<td>dorsal</td>
<td>The Toll-NF-kB Signaling Pathway Core Component, functioning with the product of Dif</td>
</tr>
<tr>
<td>Dl</td>
<td>Delta</td>
<td>a single pass transmembrane EGF family protein; one of two ligands of the Notch signaling pathway</td>
</tr>
<tr>
<td>dlg</td>
<td>discs large 1</td>
<td>a Guanylate kinase, a component of the Scribble complex</td>
</tr>
<tr>
<td>Dll</td>
<td>Distal-less</td>
<td>a homeodomain transcription factor</td>
</tr>
<tr>
<td>dpn</td>
<td>deadpan</td>
<td>a basic helix loop helix transcription factor; maintaining the self-renewal of neuroblasts</td>
</tr>
<tr>
<td>dpp</td>
<td>decapentaplegic</td>
<td>a ligand of the transforming growth factor-β signaling pathway that signals through Smad transcription factors; a BMP Signaling Pathway Core Component</td>
</tr>
<tr>
<td>E(spl)m3</td>
<td>Enhancer of split m3, helix-loop-helix</td>
<td>a basic helix-loop-helix transcriptional repressor as a member the enhancer of split gene (E(spl)) complex; downstream of Notch signaling pathway</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>E(spl)m7</td>
<td>Enhancer of split m7, helix-loop-helix, a basic helix-loop-helix transcrip</td>
<td></td>
</tr>
<tr>
<td>E(spl)m8</td>
<td>Enhancer of split m8, helix-loop-helix, a basic helix-loop-helix transcription factor; repressor; downstream of Notch signaling pathway</td>
<td></td>
</tr>
<tr>
<td>E(spl)mβ</td>
<td>Enhancer of split mβ, helix-loop-helix, a transcriptional repressor that requires a bHLH protein for their transcription (By similarity); downstream of Notch signaling pathway</td>
<td></td>
</tr>
<tr>
<td>E(spl)mγ</td>
<td>Enhancer of split mγ, helix-loop-helix, a basic helix-loop-helix (bHLH) transcription factors; downstream protein of Notch signaling pathway</td>
<td></td>
</tr>
<tr>
<td>E(spl)mδ</td>
<td>Enhancer of split mδ, helix-loop-helix, a basic helix-loop-helix transcriptional repressor as a member the enhancer of split gene (E(spl)) complex; downstream of Notch signaling pathway</td>
<td></td>
</tr>
<tr>
<td>E2f1</td>
<td>E2F transcription factor 1, a Transcriptional activator that binds to E2f site</td>
<td></td>
</tr>
<tr>
<td>EcR</td>
<td>Ecdysone receptor, a C4 zinc finger ligand-dependent DNA-binding transcription factor;</td>
<td></td>
</tr>
<tr>
<td>Eip93</td>
<td>Ecdysone-induced protein 93F, a DNA binding HTH domain, Psq-type transcription factor; programs a cell death response during larval development; transcription is steroid-regulated</td>
<td></td>
</tr>
<tr>
<td>en</td>
<td>engrailed, a homeodomain-containing transcription factor (NK-like homeobox transcription factor, repressor)</td>
<td></td>
</tr>
<tr>
<td>erm</td>
<td>earmuff, Zinc finger (C2H2 subfamily) transcriptional repressor</td>
<td></td>
</tr>
<tr>
<td>ey</td>
<td>eyeless, a paired homeobox transcription factor that is involved in eye development as part of the retinal determination gene network, contributes to brain formation</td>
<td></td>
</tr>
<tr>
<td>foxO</td>
<td>forkhead box, sub-group O, a forkhead box family of transcription factor involved in the regulation of the insulin signaling pathway</td>
<td></td>
</tr>
<tr>
<td>fzr</td>
<td>fizzy-related, a protein that binds to the Anaphase-Promoting Complex/Cyclosome (APC/C) ubiquitin ligase to stimulate its activity during G1 phase</td>
<td></td>
</tr>
<tr>
<td>gcm</td>
<td>glial cells missing, an essential zinc finger transcription factor that determines the fate of the lateral glial cells</td>
<td></td>
</tr>
<tr>
<td>grh</td>
<td>grainy head, a FGFR signaling pathway core component; a polycomb group factor recruiter</td>
<td></td>
</tr>
<tr>
<td>gro</td>
<td>groucho, a global developmental co-repressor in conjunction with manifold DNA-binding repressor partner proteins; downstream of key signaling pathways such as Wg/Wnt and Dpp/TGF-beta</td>
<td></td>
</tr>
<tr>
<td>Gai</td>
<td>G protein α i subunit, a G protein α subunit displaying sequence homology to mammalian Gia that inhibits adenylate cyclase activity, the component of pins/Gai complex</td>
<td></td>
</tr>
<tr>
<td>ham</td>
<td>hamlet, a PRDM family transcription factor;</td>
<td></td>
</tr>
<tr>
<td>hb</td>
<td>hunchback, a zinc finger C2H2 transcription factor; a gap class segmentation protein</td>
<td></td>
</tr>
<tr>
<td>hdac1</td>
<td>Histone deacetylase 1, also known as Rpd3; a histone deacetylase, catalyzes the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4)</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td></td>
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<tr>
<td>-------</td>
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<td></td>
</tr>
<tr>
<td>hdac3</td>
<td>Histone deacetylase 3, a histone deacetylase involved in chromatin silencing</td>
<td></td>
</tr>
<tr>
<td>hh</td>
<td>Hedgehog, the Hh signaling pathway ligand</td>
<td></td>
</tr>
<tr>
<td>hth</td>
<td>Homothorax, a homeodomain transcription factor that allows the nuclear import of the protein encoded by exd by direct binding</td>
<td></td>
</tr>
<tr>
<td>HUWE1</td>
<td>HECT, UBA and WWE domain containing E3 ubiquitin protein ligase 1, enables ubiquitin protein ligase activity</td>
<td></td>
</tr>
<tr>
<td>Imp</td>
<td>IGF-II mRNA-binding protein, a protein that regulates the stability, translation and/or transport of its associated mRNAs, a large number of them encoding F-actin regulators</td>
<td></td>
</tr>
<tr>
<td>InR</td>
<td>Insulin-like receptor, a ligand-stimulated tyrosine-protein kinase</td>
<td></td>
</tr>
<tr>
<td>insc</td>
<td>Inscuteable, an adaptor protein in apical cortex, binding to the apical complex proteins</td>
<td></td>
</tr>
<tr>
<td>IntS1</td>
<td>Integrator complex subunit 1, a component of the Integrator complex which is involved in the transcription of small nuclear RNAs (snRNA) and their 3'-box-dependent processing</td>
<td></td>
</tr>
<tr>
<td>IntS5</td>
<td>Integrator complex subunit 5, also known as omd, a component of the Integrator complex which is involved in the transcription of small nuclear RNAs (snRNA) and their 3'-box-dependent processing</td>
<td></td>
</tr>
<tr>
<td>IntS8</td>
<td>Integrator complex subunit 8, a component of the Integrator complex which is involved in the transcription of small nuclear RNAs (snRNA) and their 3'-box-dependent processing</td>
<td></td>
</tr>
<tr>
<td>ix</td>
<td>Intersex, Mediator complex subunit 29</td>
<td></td>
</tr>
<tr>
<td>klu</td>
<td>Klumpfuss, a zinc finger - early growth response family transcription factor</td>
<td></td>
</tr>
<tr>
<td>Kr</td>
<td>Krüppel, a zinc finger C2H2 transcription factor; a gap class segmentation protein</td>
<td></td>
</tr>
<tr>
<td>kto</td>
<td>Kohtalo, Mediator complex subunit 12</td>
<td></td>
</tr>
<tr>
<td>l(1)sc</td>
<td>Lethal of scute, a basic helix loop helix transcription factor; proneural factor; a component of the achaete-scute complex</td>
<td></td>
</tr>
<tr>
<td>Lgl</td>
<td>Lethal (2) giant larvae, a tumor suppressor protein that regulates cell polarity and asymmetric cell division</td>
<td></td>
</tr>
<tr>
<td>lin-28</td>
<td>Lin-28, a cold shock and RNA-binding protein; a positive regulator of insulin-like receptor or JAK-STAT signaling pathway</td>
<td></td>
</tr>
<tr>
<td>MED1</td>
<td>Mediator complex subunit 1, Mediator complex subunit 1</td>
<td></td>
</tr>
<tr>
<td>MED10</td>
<td>Mediator complex subunit 10, Mediator complex subunit 10</td>
<td></td>
</tr>
<tr>
<td>MED11</td>
<td>Mediator complex subunit 11, Mediator complex subunit 11</td>
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</tr>
<tr>
<td>MED14</td>
<td>Mediator complex subunit 14, Mediator complex subunit 14</td>
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<td>Mediator complex subunit 15, Mediator complex subunit 15</td>
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<td>MED17</td>
<td>Mediator complex subunit 17</td>
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<td>MED8</td>
<td>Mediator complex subunit 8</td>
<td>Mediator complex subunit 8</td>
</tr>
<tr>
<td>MED9</td>
<td>Mediator complex subunit 9</td>
<td>Mediator complex subunit 9</td>
</tr>
<tr>
<td>Mi-2</td>
<td>Mi-2</td>
<td>a nuclear ATP-dependent nucleosome remodeler of the CHD family</td>
</tr>
<tr>
<td>mira</td>
<td>miranda</td>
<td>a cytoplasmic and cortical scaffolding protein transporting pros, brat, stau</td>
</tr>
<tr>
<td>mir-ban</td>
<td>bantam</td>
<td>a small noncoding RNA; enables mRNA 3'-UTR binding activity and mRNA binding activity involved in posttranscriptional gene silencing</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Gene Name</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td>-------------</td>
</tr>
<tr>
<td>mor</td>
<td>moira</td>
<td>a member of the trithorax group of homeotic gene regulators; a chromatin remodeling protein and functions as the Swi3 component of the Brahma complex in SWI/SNF complex</td>
</tr>
<tr>
<td>mud</td>
<td>mushroom body defect</td>
<td>a component of Pins/ Gαi complex, regulating spindle orientation via interactions with the dynein complex</td>
</tr>
<tr>
<td>Myc</td>
<td>Myc</td>
<td>a transcription factor that is homologous to vertebrate Myc proto-oncogenes</td>
</tr>
<tr>
<td>N</td>
<td>Notch</td>
<td>a receptor for membrane-bound ligands Delta and Serrate</td>
</tr>
<tr>
<td>nab</td>
<td>nab</td>
<td>a transcriptional co-factor that acts in combination with the transcriptional factors encoded by rin in proximal-distal wing patterning and the product of sqz in CNS development</td>
</tr>
<tr>
<td>neur</td>
<td>neuralized</td>
<td>an E3 ubiquitin ligase of the RING family, part of the Notch signaling pathway, contributing to the endocytosis-dependent activation of the ligand</td>
</tr>
<tr>
<td>nip</td>
<td>numb binding protein</td>
<td>also called moladietz, located in basal part of cell and plasma membrane and interacting with numb</td>
</tr>
<tr>
<td>ns3</td>
<td>Nucleostemin 3</td>
<td>a GTPase required for the nuclear export of the 60S ribosomal subunit</td>
</tr>
<tr>
<td>numb</td>
<td>numb</td>
<td>a membrane-associated inhibitor of Notch signaling</td>
</tr>
<tr>
<td>omd</td>
<td>oocyte maintenance defects</td>
<td>also known as IntS5; a component of the Integrator complex which is involved in the transcription of small nuclear RNAs (snRNA) and their 3'-box-dependent processing</td>
</tr>
<tr>
<td>osa</td>
<td>osa</td>
<td>a subunit of the BAP chromatin remodeling complex (one of the two SWI/SNF complexes)</td>
</tr>
<tr>
<td>par6</td>
<td>par-6</td>
<td>a scaffold protein that forms a complex with the products of baz and aPKC and with other cortical, cytoskeletal and regulatory proteins; enabling protein kinase C binding activity</td>
</tr>
<tr>
<td>pav</td>
<td>pavarotti</td>
<td>a microtubule motor protein from the Kinesin superfamily; functions in spindle formation, cortical cytoskeleton reorganization and cytokinesis</td>
</tr>
<tr>
<td>pdm</td>
<td>POU domain protein 2</td>
<td>a POU homeobox transcription factor</td>
</tr>
<tr>
<td>pins</td>
<td>partner of inscuteable</td>
<td>a GDP-dissociation inhibitor involved in asymmetric cell division and mitotic spindle orientation,</td>
</tr>
<tr>
<td>pntP1</td>
<td>pointed P1</td>
<td>Ets transcription factor Pointed isoform P1; downstream of Ras signaling</td>
</tr>
<tr>
<td>pon</td>
<td>partner of numb</td>
<td>a protein contributing to asymmetric localization of the numb product and the subsequent suppression of Notch signaling</td>
</tr>
<tr>
<td>pros</td>
<td>Prospero</td>
<td>a homeodomain-containing transcription factor; cell-cycle exit determinant; the cargo protein of Miranda</td>
</tr>
<tr>
<td><strong>Rbbp5</strong></td>
<td>Retinoblastoma binding protein 5</td>
<td>a component of the SET1 complex that specifically di- and trimethylates lysine 4 of histone H3 and of the MLL3/4 complex which also methylates histone H3 lysine 4</td>
</tr>
<tr>
<td>---</td>
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<td>---</td>
</tr>
<tr>
<td><strong>rho</strong></td>
<td>rhomboid</td>
<td>an intra-membrane serine protease that processes the membrane precursors of Egrf ligands.</td>
</tr>
<tr>
<td><strong>Roc1a</strong></td>
<td>Regulator of cullins 1a</td>
<td>a C2H2 type zinc finger protein and component of several Cullin-Ring E3 ubiquitin ligase complex; binds directly to the Cullin subunit</td>
</tr>
<tr>
<td><strong>SAK</strong></td>
<td>Sak kinase</td>
<td>a serine/threonine protein kinase member of the Polo-like kinase family; phosphorylates centrosomal proteins and self-regulates by autophosphorylation</td>
</tr>
<tr>
<td><strong>Sas-4</strong></td>
<td>Spindle assembly abnormal 4</td>
<td>a centriole protein that is essential for centriole assembly; recruited to centrioles through an interaction with the centriole protein encoded by ana2, and it helps recruit microtubules to the centriole</td>
</tr>
<tr>
<td><strong>sc</strong></td>
<td>scute</td>
<td>a basic helix loop helix transcription factor; proneural factor; a component of the achaete-scute complex</td>
</tr>
<tr>
<td><strong>scrib</strong></td>
<td>scribble</td>
<td>a scaffolding protein that is part of the scribble complex in apical cortex</td>
</tr>
<tr>
<td><strong>sdt</strong></td>
<td>stardust</td>
<td>a membrane-associated guanylate kinase (MAGUK) protein, involved in positive regulation of ubiquitin-dependent endocytosis</td>
</tr>
<tr>
<td><strong>shg</strong></td>
<td>shotgun</td>
<td>also known as E-cadherin; a calcium-dependent cell adhesion protein</td>
</tr>
<tr>
<td><strong>six4</strong></td>
<td>six4</td>
<td>a homeodomain-containing transcription factor</td>
</tr>
<tr>
<td><strong>skd</strong></td>
<td>skuld</td>
<td>Mediator complex subunit 13</td>
</tr>
<tr>
<td><strong>SkpA</strong></td>
<td>SKP1-related A</td>
<td>a subunit of Skp, Cullin, F-box (SCF)-containing ubiquitin ligase complexes</td>
</tr>
<tr>
<td><strong>slif</strong></td>
<td>slimfast</td>
<td>a cationic amino-acid transporter by facilitated diffusion across membranes</td>
</tr>
<tr>
<td><strong>slimb</strong></td>
<td>supernumerary limbs</td>
<td>an essential, conserved F-box protein and a component of the SCF (Skp/Cullin/F-box) E3 ubiquitin-ligase, providing substrate specificity to the SCF</td>
</tr>
<tr>
<td><strong>slp1</strong></td>
<td>sloppy paired 1</td>
<td>a transcription factor of the fork-head family that functions by interacting with the corepressor encoded by gro</td>
</tr>
<tr>
<td><strong>slp2</strong></td>
<td>sloppy paired 2</td>
<td>a transcription factor of the fork-head family. Together with the product of slp1</td>
</tr>
<tr>
<td><strong>Snrl</strong></td>
<td>Snf5-related 1</td>
<td>a core component of the ATP-dependent SWI/SNF chromatin-remodeling complex (Brahma complex)</td>
</tr>
<tr>
<td><strong>sog</strong></td>
<td>short gastrulation</td>
<td>a secreted BMP antagonist</td>
</tr>
<tr>
<td><strong>SoxN</strong></td>
<td>SoxNeuro</td>
<td>an HMG-domain transcription factor, a negative regulator of Wnt-TCF signaling pathway, a proneural factor</td>
</tr>
<tr>
<td><strong>sp1</strong></td>
<td>Sp1</td>
<td>a member of the Sp-family of Cys2His2-type zinc finger transcription factor</td>
</tr>
<tr>
<td><em>spdo</em></td>
<td>sanpodo</td>
<td>a four-pass transmembrane domain containing protein that interacts with both the product of numb and the Notch signaling pathway</td>
</tr>
<tr>
<td><em>spi</em></td>
<td>spitz</td>
<td>the cardinal Egfr ligand that is produced as a transmembrane precursor and processed by the products of S and rho</td>
</tr>
<tr>
<td><em>sqz</em></td>
<td>squeeze</td>
<td>a C2H2-type zinc-finger transcription factor; regulating neuropeptidergic cellular identity and axon/dendrite pathfinding</td>
</tr>
<tr>
<td><em>stg</em></td>
<td>string</td>
<td>a CDC25 family protein phosphatase; a tyrosine protein phosphatase required for progression of the cell cycle</td>
</tr>
<tr>
<td><em>Su(H)</em></td>
<td>Suppressor of Hairless</td>
<td>the coactivator (the DNA-binding partner) of Notch; acts as a transcriptional repressor when it is not associated with Notch proteins</td>
</tr>
<tr>
<td><em>svp</em></td>
<td>seven up</td>
<td>a nuclear receptor (NR) which is C4 zinc finger ligand-dependent DNA-binding transcription factor</td>
</tr>
<tr>
<td><em>Syp</em></td>
<td>Syncrip</td>
<td>an RNA-binding protein that regulates the localization and translation of mRNAs involved in multiple processes</td>
</tr>
<tr>
<td><em>tll</em></td>
<td>tailless</td>
<td>Orphan receptor, nuclear hormone receptor family, NRE2 subfamily, repressor</td>
</tr>
<tr>
<td><em>Tor</em></td>
<td>Target of rapamycin</td>
<td>the components of TORC1 complex and TORC2 complex; enables chromatin DNA binding, protein binding or protein kinase activity</td>
</tr>
<tr>
<td><em>trx</em></td>
<td>trithorax</td>
<td>a chromatin-modifying enzyme methylating the histone encoded by His3 on Lys-4, promoting its further acetylation and antagonizing the epigenetic silencing by Polycomb group proteins</td>
</tr>
<tr>
<td><em>tum</em></td>
<td>tumbleweed</td>
<td>a GTPase activating protein for Rho family GTPases involved in Wnt signaling regulation; part of centralspindlin complex.</td>
</tr>
<tr>
<td><em>Ubx</em></td>
<td>Ultrabithorax</td>
<td>a homeodomain (HOX-like (HOXL) homeobox) transcription factor;</td>
</tr>
<tr>
<td><em>wds</em></td>
<td>will die slowly</td>
<td>a component of the histone methyltransferase complex (SET1/MLL) that specifically methylates lysine 4 of histone H3</td>
</tr>
<tr>
<td><em>wg</em></td>
<td>wingless</td>
<td>a ligand of the Wnt/Wg signaling pathway</td>
</tr>
<tr>
<td><em>wor</em></td>
<td>worniu</td>
<td>a zinc finger C2H2 transcription factor, involved in nervous system development including neuroblast asymmetric cell division</td>
</tr>
<tr>
<td><em>yki</em></td>
<td>yorkie</td>
<td>a transcriptional co-activator protein that is negatively regulated by Hippo signaling</td>
</tr>
<tr>
<td><em>zld</em></td>
<td>zelda</td>
<td>a C2H2 zinc finger transcription factor;</td>
</tr>
</tbody>
</table>
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CHAPTER I. General Introduction

This chapter aims to provide fundamentals of neural stem cell (NSC) biology focusing on those in the *Drosophila* central nervous system (CNS), by introducing the anatomy, developmental regulation and functional dissection of *Drosophila* NSCs (or called neuroblasts, NBs), particularly the type II NB system, summarizing some of the significant discoveries in genetics of *Drosophila* NSC field, and limitedly promising directions which would further elucidate the underlying mechanisms that regulate NSC development. Based on prior research progress, this chapter also articulates critical questions remaining in the field of *Drosophila* NSC biology, in particular outstanding questions in the development of type II NB lineages studied in this dissertation.
1. Brief Caption

*Drosophila* neuroblasts (NBs) offer significant advantages as a model system for studying stem cell development in the central nervous system (CNS). One type of NSCs, called types II NBs, plays an important role in promoting brain complexity. One outstanding feature of type II NBs is their capability to generate intermediate neural progenitors (INPs), which undergo self-renewal divisions to produce terminally dividing ganglion mother cells (GMCs). Studies on type II NBs, INPs and their derived lineages have provided key insights into mechanisms underlying type II NB specification and lineage progression, which guarantee increased neuronal or glia output and diversity.

INPs, also known as intermediate progenitor cells (IPCs) in mammalian nervous system, drastically boost brain complexity by increasing both output and diversity of neurons/glia. *Drosophila* INPs are transit-amplifying progenitor cells that can further produce a large number of neurons and glia with diverse subtypes. These INP-derived neurons or glia contribute to specific neural circuits, which perform diverse functions in *Drosophila* brains (Alvarez & Diaz-Benjumea, 2018; Bayraktar & Doe, 2013; Ren et al, 2018; Ren et al, 2017; Viktorin et al, 2011; Walsh & Doe, 2017; Wang et al, 2014). In developing gyrencephalic brains, INPs, which are also called outer/basal radial glia cells (oRGs or bRGs) localized in the ventricular zone (VZ), are produce by radial glia cells (RGCs, NSCs in mammals) and possess a long basal process extending to the pial surface like RGCs (Penisson et al, 2020). oRGs are highly proliferative and can dramatically amplify neurogenesis and gliogenesis, which are crucial for the rapid expansion and folding of the cortical surface (Fietz et al, 2010; Hansen et al, 2010; Pinson et al, 2019; Reillo et al, 2011; Shitamukai et al, 2011; Wang et al, 2011b).
On the other hand, dysregulation of INP development accounts for various neurogenetic disorders. Disruption of basal radial glia production results in defects in progenitor cell proliferation and neuronal migration, which potentially contributes to the pathogenesis of microcephaly (reduction of cerebral volume), lissencephaly (absence of or abnormal folds) or heterotopias (presence of grey matter within the white matter) (Heng et al., 2017; Penisson et al., 2020). Over-proliferation or dedifferentiation of INPs, on the other hand, could lead to development of brain tumors, such as glioblastoma in mammals (Wang et al., 2020) or tumor-like growth in Drosophila (Bowman et al., 2008; Komori et al., 2014b; Mukherjee et al., 2016). Therefore, understanding the underlying mechanisms regulating INP development and proliferation is crucial for understanding the generation of the brain complexity and brain tumor formation. In this chapter I will review some key findings of the molecular mechanisms involved in type II NB specification/maintenance and INP generation/maintenance, and how their progeny are produced and integrated into the functional circuits for higher-level brain activity and, when appropriate, compare to the mechanisms regulating the development of type I NBs, which have been studied extensively and most of our understanding of fly neural stem cell biology has been learned from.

2. A snapshot of Type II NB lineages

Let’s first take a snapshot of the anatomy of type II NB lineages. In each lobe of larval central brains, there are totally eight type II NB lineages. Six type II NB lineages are located in the dorsomedial (DM) region of the brain and they are called DM1-6 lineages. The other two are in the dorsolateral (DL) region and are called DL1-2 lineages. During embryonic stages when they are first specified (beginning at stage 11) and in early larval stages, eight type II NBs are clustered in three groups, an anteromedial cluster (or called anterior dorsomedial (aDM) cluster) containing three NBs (corresponding to DM1-3 above), a medial cluster (or called posterior
dorsomedial (pDM) cluster) containing 3 NBs (corresponding to DM4-6 above), and a posterior lateral cluster (or called dorsolateral (DL) clusters) containing two NBs (corresponding to DL1-2 above) (Alvarez & Diaz-Benjumea, 2018; Walsh & Doe, 2017). After type II NBs undergo several rounds of self-renewing divisions and establish their lineages, all type II NB lineages are well separated from each other and can be individually identified (Bayraktar et al., 2010; Bayraktar & Doe, 2013) (Fig. I-1).

For any given typical type II NB lineage in the third instar larval brain, the cell type composition may be characterized by a combination of transcriptional markers, the proneural protein Asense (Ase), the self-renewing factor Deadpan (Dpn, a basic helix-loop-helix (bHLH) protein), the Notch (N) downstream target, Enhancer of Split-mγ (E(spl)mγ), and cell cycle exit factor Prospero (Pros). A typical DM type II NB lineage in third instar larvae is composed of a single, large Ase- Dpn+ E(spl)mγ+ Pros- NB, 1-2 Ase- Dpn- E(spl)mγ- Pros- immature INPs (imINPs) next to the NB, 2-3 Ase+ Dpn- E(spl)mγ- Pros- imINPs, 20-30 Ase+ Dpn+ E(spl)mγ+ cytoplasmic Pros+ mature INPs (mINPs), multiple Ase+ Dpn+ E(spl)mγ- nuclear Pros+ GMCs and Ase- Dpn- E(spl)mγ- nuclear Pros+ neurons, and/or Ase- Dpn- Pros- glial cells missing (gcm)+ glia cells (Bayraktar et al., 2010; Berger et al., 2012; Izergina et al., 2009; Viktorin et al., 2011; Zhu et al., 2011). The DL type II NB lineages have the same cell types but with a smaller number of mINPs (only around 1/5 of the INP number in a DM lineage), GMCs and maybe neurons or glia. Due to the lack of Ase expression and their posterior location in brain lobes, they are also referred to as Posterior Asense-Negative (PAN) NBs, with the six medial NBs having their progeny along the lobe surface and the two lateral ones having progeny located in interior brain regions (Bowman et al., 2008). Because of the presence of mINPs which undergo multiple but limited (about 4-6, up to 10) rounds (Xie et al., 2014) of asymmetric divisions to produce self-renewing INPs and terminally dividing GMCs, the number of neurons or glia is dramatically boosted. Based on
results from mosaic analysis with a repressible cell marker (MARCM)-based clonal analyses, each type II NB at 96hrs after clone induction generates an average of more than 450 cells in each lineage, which is more than twice the average number of progeny in a type I NB lineage or a mushroom body NB lineage (Bello et al., 2008).

Since INPs are mitotically active and self-renewing, the overall number of mINPs are gradually increased during the development of type II NBs until they reach a constant level. During the embryonic stages 13-17, the total number of INPs is relatively constant, possibly due to the low mitotic rate of INPs and eventually going to quiescence at the end of embryogenesis (Walsh & Doe, 2017). Therefore, during embryogenesis, an average of total 4-6 INPs, 8-10 INPs, or 2-4 INPs are produce by the aDM, pDM, or DL cluster, respectively (Walsh & Doe, 2017). INPs also resume proliferation in early second instar larvae (Alvarez & Diaz-Benjumea, 2018) as NBs do. While in larval stages, the average number of INPs per lineage is gradually increased and reaches to a plateau of around 20 to 30 (Komori et al., 2014a; Zhu et al., 2011). In regards to cell size, type II NBs usually are 10-15µm in diameter and INPs are around 5-7µm in diameter (Bello et al., 2008; Boone & Doe, 2008a; Bowman et al., 2008) while GMCs and neurons have a diameter of about 4-5µm which is similar to those in type I NB lineages (Boone & Doe, 2008b) (Fig. I-1 and I-2). In order to establish individual type II NB lineages, asymmetric NB divisions, self-renewal/proliferation, imINP differentiation/maturation must be delicately regulated. A type II NB takes 80-90mins to perform one round of asymmetric division to produce one self-renewing type II NB and one Ase⁻ imINP which takes 3-5hrs to become an Ase⁻ committed imINP. Then within 4hrs the Ase⁺ imINP becomes a mitotic mINP which undergoes up to 10 rounds of asymmetric division in a time window of 7-9hrs to produce a new self-renewing mINP and a GMC and the GMC stays for 8-10hrs before terminally dividing once to produce a pair of
neurons and/or glia, with each INP producing around 8-12 neurons (Bowman et al., 2008; Walsh & Doe, 2017).

In contrast, a type I NB lineage is also well characterized based on cell morphology and markers, including Ase, Dpn, E(spl)mγ, or cytoplasmic or nuclear Pros protein (Fig. I-1 and I-2). A type I NB lineage contains a largest Ase⁺ Dpn⁺ E(spl)mγ⁺ type I NBs with a diameter of 10-15µm and low levels of cytoplasmic Pros (Bayraktar et al., 2010; Weng & Cohen, 2015), 4-6 Ase⁺ Dpn⁻ E(spl)mγ⁻ terminally dividing GMCs with a diameter of 5-7µm and nuclear Pros expression, and smaller postmitotic neurons with nuclear Pros expression. Schematic diagrams of both type II and type I NB lineages with simplified lineage progression are illustrated in Fig. I-1 and Fig. I-2.

Figure I-1. Diagrams of Drosophila larval brains with type I NB lineages and type II NB lineages illustrated.
(Upper) The illustration of *Drosophila* CBs and VNCs, showing most type I NB lineages are localized on ventral surface of VNCs and both ventral and dorsal surface of CBs, a small subset of type I NBs on dorsal surface of VNCs, and all type II NB lineages on dorsal surface of CBs. (Left Lower) Ventral CBs and VNCs contain only type I NB lineages, which are visualized by labelling with mCD8-GFP or mCD8-RFP (in green) driven by pan-NB driver *insc-GAL4* or type I NB lineage-specific driver *ase-GAL4*, and contain a Dpn*+* Ase*+* (in magenta) type I NB, several Ase*+* (in red) GMCs, and multiple postmitotic neurons in each lineage. (Right Lower) Dorsal brains contain eight type II NB lineages per lobe which are visualized and distinguished from type I NBs by labelling with mCD8-GFP or mCD8-RFP (in green) driven by type II NB lineage-specific *pntP1-GAL4*, and contain a Dpn*+* Ase*−* (in blue) type II NB, Dpn*+* Ase*+* (in magenta) mINPs, Ase*+* (in red) GMCs, and multiple postmitotic neurons in each lineage.

CB, central brain; VNC, ventral nerve cord; OL, optical lobe; aDM, anterior dorsomedial; pDM, posterior dorsomedial; DL, dorsolateral.

**Figure I-2. Lineage progression of type I NB lineages and type II NB lineages.**

The expression patterns and regulations of some key molecules involved in each step of type I or II NB lineage development are indicated. (Left) A schematic diagram depicts the developmental progression of a type I NB lineage. A type I NB produces a self-renewed type I NB and a daughter GMC, which divides once to produce a pair of neurons. (Right) A schematic diagram depicts the developmental progression of a type II NB lineage. PntP1 (in yellow) is a master regulator and expressed in type II NBs to suppress Ase (in red), in newly born imINPs to suppress Pros (in gray), and in late imINPs to activate Erm (in magenta).
expression. Self-renewal factors, including Dpn or E(spl)mγ (in blue), maintain type II NB proliferation and self-renewal partially through inhibiting Erm in NBs. Silence of self-renewal factors (through pathways un-indicated here) and termination of PntP1 expression through negative feedback inhibition by Erm ensure imINPs differentiation/maturation to become fate committed mlINPs, which resume proliferation.

3. Specification and origin of type II NB lineages

As a prerequisite to produce neurons/glia, both type II NBs and type I NBs need to be properly specified from the ventral neuroectoderm by utilizing specific mechanisms during embryonic stages.

Although how the selection and delamination of type II NBs from neuroepithelium (NE), such as how the lateral inhibition mediated by Notch signaling is involved (see below) or how the their proneural cluster pattern differs from type I NBs, remain unclear, some of the mechanisms of type II NB specification have been elucidated. In the first study on type II NB specification during embryogenesis, it shows that one type II NB originates in a subregion of the Pdm placode corresponding to the medial PI primordium (pPIm). The specification and survival of this NB is dependent on an active Notch pathway or Epidermal growth factor receptor (EGFR) activity, respectively, during around embryonic stages 12-15. This type II NB is converted to a type I NB that generates insulin-producing cells (referred to IPC_NBs) in the absence of Notch, whereas in the absence of EGFR activity it undergoes apoptosis (Hwang & Rulifson, 2011). Later studies revealed that all eight type II NBs are among the last wave of NB formation to be specified from stages 11-12 to 14-15 and begin to produce INPs, GMCs and neurons during embryogenesis (Alvarez & Diaz-Benjumea, 2018; Walsh & Doe, 2017). Spitz (Spi) ligand-activated EGFR signaling pathway along with its downstream effector Pointed P1 (PntP1) are required to specify the initial formation of type II NBs during embryogenesis. However, additional factors, including Buttonhead (Btd) and Sp1 which are expressed in the brain neuroectoderm but not dependent on
EGFR pathway activation, are also required for the specification of type II NBs (Alvarez & Diaz-Benjumea, 2018). Both embryonic type II NBs and INPs undergo quiescence and re-entry into the cell cycle (Alvarez & Diaz-Benjumea, 2018; Walsh & Doe, 2017), possibly utilizing mechanisms similar to those in type I NBs (see Section 4 below).

As a comparison, a canonical model of “lateral inhibition” illustrates how type I NBs are specified from the ventral neuroectoderm during early embryonic development (around stage 8). The delamination of potential type I NBs from the neuroectoderm, a process being coordinated with the specification of NBs, spans a time window of around 4hrs from stage 8 to stage 11 and occurs in up to five waves, generating lineages with distinct progeny pools, including a mixture of interneurons, and/or motoneurons, and/or glia cells (Bossing et al, 1996; Doe, 1992; Schmidt et al, 1997). First, multiple signaling pathways, including EGFR (Hwang & Rulifson, 2011; Skeath, 1998), Dorsal (Dl), Decapentaplegic (Dpp) (Von Ohlen & Doe, 2000), and Short gastrulation (Sog) (François et al, 1994; Zusman et al, 1988), act cooperatively in a dose/concentration-dependent manner to trigger the formation of clusters (called proneural clusters) of 6-8 neuroectodermal cells competent to generate NBs (Cubas et al, 1991; Hartenstein & Wodarz, 2013). Cells of the proneural clusters express a combination of proneural factors (Skeath & Carroll, 1992), including the Achaete (Ac)-Scute (Sc) complex (which generally includes Lethal of scute (L(1)sc) and Ase, however Ase is not expressed in proneural clusters in the embryo) (Brand et al, 1993; Cabrera et al, 1987; Campuzano & Modolell, 1992; Cubas et al., 1991; Ghysen & Dambly-Chaudiere, 1989; Skeath & Carroll, 1994; Skeath & Doe, 1996). Next, lateral inhibition plays roles through Notch signaling via cell-cell interaction that is regulated by a delicate feedback loop. Delta (Dl), the ligand of Notch, is activated by Ac-Sc (Haenlin et al, 1994; Kunisch et al, 1994) in cells of proneural clusters. Then Delta interacts with Notch receptors on the neighboring cells, and then E(spl) genes are activated and function together with
their cofactor Groucho (Gro) to directly suppress Ac-Sc in the neighboring cells (Gigliani et al., 1996; Heitzler et al., 1996; Oellers et al., 1994). Thus, through lateral inhibition, only the cell with the highest Dl levels or the earliest Dl expression would be selected to have stable proneural gene expressions and be specified as a NB, while its neighboring cells losing Ac-Sc expression would commit the epidermal fate and become epidermoblasts. Consistent with this mechanism, loss of Notch function leads to defects in embryonic neurogenesis. However, mutations in the proneural genes or Notch pathway do not fully block NB formation, suggesting that additional genes are involved. Notch signaling interplays with Epithelial-mesenchymal transition (EMT) genes, including crumbs (crb), which modulates Notch signaling (Das & Knust, 2018), and stardust (sdt), which is a target of E3 ubiquitin ligase Neuralized (Neur) and mediates Crb down-regulation (Arefin et al., 2019; Perez-Mockus et al., 2017). Two additional proneural genes, soxNeuro (soxN) and worniu (wor), are identified to bridge the interplay between Notch and EMT genes to promote NB selection (Arefin et al., 2019). Furthermore, these pathways may be involved in not only specifying type I NBs but also regulating their survival or maintenance in a cell type-dependent manner. For example, the type I NBs that generate small cholinergic neurons rely on the Notch pathway for their specification and the EGFR pathway for avoiding apoptosis, whereas the type I NBs that generate insulin-producing cells rely on neither Notch signaling nor EGFR activity for their specification or survival, even though both groups of NBs are derived from placodes in the head midline dorsomedical procephalic (Pdm) NE (Hwang & Rulifson, 2011).

4. From embryogenesis to larval neurogenesis: the hibernation and the reactivation

At the end of embryogenesis, although most of type I NBs in abdominal neuromeres and a large number of NBs in gnathal segments are eliminated from the developing CNS through apoptosis, a programmed cell death mediated by caspases, or through an apoptosis independent loss (Harding
with type II NBs in central brains and the majority of the cephalic and thoracic type I NBs go into quiescence by arresting in the G0 or G2 phase of cell cycle (Bray et al., 1989; Harding & White, 2018; Hartenstein & Wodarz, 2013; Otsuki & Brand, 2018; Peterson et al., 2002). For type I NBs, the quiescence and apoptosis seem independent from each other (Harding & White, 2019), although there are models suggesting that both of them follow cell cycle exit, which is regulated by four key cell-cycle genes: *cyclin E* (*cycE*), *string* (*stg, cdc25*), *e2f1*, and *dacapo* (*dap*) (Bahrampour et al., 2017; Baumgardt et al., 2014). Apoptosis of those eliminated type I NBs at the end of embryogenesis is triggered and mediated by the Reaper activator Deformed (Dfd) in gnathal NBs (Urbach et al., 2016) and by glia resourced DI-Notch signaling and promoted by the Hox gene *abdominal-a* (*abd-a*) in abdominal segments (Arya et al., 2015; Pinto-Teixeira et al., 2016).

Mechanisms regulating the entrance or exit of quiescence of NBs involves multiple different factors. Studies have demonstrated that the switches to trigger NB quiescence include Hox proteins, temporal transcription factors (TTFs, see Section 5 and 6.1 below for more information) and the cell-cycle exit factor Pros. Hox proteins Antennapedia (*Antp*) and Abd-a expressed spatially in thoracic segments (for type I NBs only) promote NB entry into quiescence and TTF Castor (*Cas*) induces quiescence, while TTF Pou domain protein (*Pdm*) acts downstream of *Cas* and inhibits NB quiescence through inhibiting Squeeze (*Sqz*) and Nab (Tsuji et al., 2008). Despite the spatial or temporal regulation on NB quiescence, a more generic factor Pros is shown to be required and sufficient to induce NBs to go dormant at the end of embryonic stages only at low levels in nucleus (Lai & Doe, 2014). Also, as a generic control, the Hippo pathway not only promotes the timely entry of NBs into quiescence but also maintains hibernation by preventing precocious exit from quiescence through its downstream co-activator Yorkie (*Yki*) (Poon et al., 2016). In addition, TTF Grainyhead (*Grh*) mutant NBs prematurely exit quiescence suggesting
that Grh in late embryonic stage, as well as during quiescence, promotes quiescence (Cenci & Gould, 2005) through an unclear mechanism given that Grh also shows an opposite effect by repressing Pros to reactivate the hibernate NBs (see below).

The quiescent NBs re-enter the cell cycle and begin proliferation in response to extrinsic or intrinsic cues at late first instar or early second instar stage (Hartenstein & Wodarz, 2013). Extrinsic cues may include those from diet, such as amino acids (Britton & Edgar, 1998). Amino acids are sensed by the cationic amino-acid transporter Slimfast (SLIF) in the fat body and signals are relayed through Target of rapamycin (TOR), causing the fat body to produce systemic growth factors/mitogens (Colombani et al, 2003; Okamoto et al, 2009), including those that can be sensed by stellate surface glia which further activates the expression and secretion of insulin-like/IGF-like peptides (ILPs). And then the ILPs are finally detected by insulin-like receptors and the Phosphatidylinositol 3-kinase (PI3K)/Akt/TOR pathway in NBs to trigger the exit of quiescence (Chell & Brand, 2010; Sousa-Nunes et al, 2011). Meanwhile, the intrinsic machinery within NBs is required to accomplish the reactivation. One critical intrinsic factor identified is the spindle matrix complex that contains Chromator (Chro), which functions downstream of insulin/PI3K/Akt/TOR pathway to suppress Pros directly or indirectly through activating TTF Grh (Li et al, 2017a).

5. Contribution to glio/neurogenesis by type II NBs and INPs

Lineage tracing of postembryonic type II NB or INP lineages has shown that they generate both neurons and glia, which contribute to neuropil substructures of the adult brain to support its functions. For gliogenesis, one model indicates that INPs in DM1-DM3 lineages produce a mix of neuronal/glial cells, INPs in DM4 and DM6 lineage prefer generating neurons only and INPs in DM5 clones seem to generate only glia cells, which is dependent on gcm expression (Viktorin et
However, a later study supports a disparate model that DM2-3, DM5-6 and DL1 lineages are capable of generating glia with multiple subtypes including outer chiasm glia, inner chiasm glia, lateral cell body rind glia, astrocyte-like glia or ensheathing glia, while lineages DM1, DM4 and DL2 are not capable of producing any glia cells, in which the absence of glia in DM1 or DM4 (not DL2) is likely in part due to premature loss of glia precursor cells mediated by apoptosis (Ren et al., 2018). The latter study also showed 1) that distinct extent of apoptosis present in each differential type II NB lineage contributes to distinct gliogenic potentials in individual lineages, since antagonizing apoptosis generally rescues or increases the glia cell number in a distinct extent in DM1-6 and DL1 (except DL2) lineages, and 2) that INPs are multipotent precursors that can generate both neurons as well as glia cells with diverse subtypes, including astrocyte-like and ensheathing glia, and require active Delta-Notching signaling to promote gliogenesis and expansion via switching off neurogenesis (Ren et al., 2018). The glia cells produced by INPs proliferate and differentiate locally in the developing central complex (CX) of pupae to ensheath/wrap/infiltrate various neuropils in adult brains, including (but not limited to) four central complex neuropils (the protocerebral bridge (PB), the fan-shaped body (FB), and the noduli (NO) and the ellipsoid body (EB)) of the central complex, the calyx (CA) and pedunculus (PED) of the mushroom body, the lateral complex (LAL), the superior neuropils, inferior neuropils, and ventromedial neuropils, the lateral horn, the ipsilateral optic lobe, the lobula complex (lobula and lobula plate) or the medulla, in a lineage-dependent manner (Ren et al., 2018; Viktorin et al., 2011).

Each DM type II NB lineage contributes a subset of postembryonic neurons (also known as secondary neurons) whose axons form commissural and longitudinal axon tracts to target larval brain neuropils. During the pupal brain development the secondary neurons form widespread arborizations and innervate the PB, FB, and NO, but not EB of the developing central complex,
(Izergina et al., 2009), a major neuropil devoted to several behaviors and functions, including multimodal sensory processing, flight, visual pattern memory and locomotion (Bayraktar et al., 2010; Walsh & Doe, 2017). In adult brains, the majority of neuronal cell bodies produced by INPs (traced by earmuff (erm) enhancer fragment R9D11) localize in the dorsal posterior cortex (i.e., dorsal posterior complex lateral or medial to the mushroom body calyces) and cell bodies of the rest of the neurons localize to the anterior cortex lateral to the anterior lateral accessory lobes (LAL) and other areas such as bulbs (BUs, also known as lateral triangles), while their axons project to all four central complex neuropils, the two central complex accessory areas, LAL and BUs and several other regions including those dorsal to the LALs, posterior to the mushroom body medial lobes, and lateral to the anterior EB in central brains, those central and anterior parts of medial protocerebrum and those in the mushroom body vertical and medial lobes as well as specific glomeruli in the antennal lobes (ALs) (Bayraktar et al., 2010). Using two pointed-GAL4 (pntP1-GAL4, i.e., GAL414-94, and R45F08-GAL4) drivers in combination with flippase/FRT based lineage-tracing to label early-born neurons (labeled when heat shock-Flp was induced in the early embryo (between 2 hrs and 4 hrs after egg laying), and identified based on their midline associated and distal position to NBs or INPs compared to those later/recently-born neurons) in type II NB lineage reveals that the central complex primordia (i.e., the immature fan-shaped body or called immature protocerebral bridge in the developing larval brains, a large commissural neuropil domain consisting of the DPC1 and trCM and probably part of the DPC2 commissure) is established by early-born neurons produced by at least four type II NBs (DM1, DM2, DM3, DM6) through innervation by projections of these early-born neurons and that these neurons continue projection/process growth and differentiation and are integrated into the mature modular circuitry of the adult brain central complex (mainly FB) (Izergina et al., 2009; Riebli et al, 2013). These early-born neurons are at least produced by temporally late Ey+ INPs (see below) (Bayraktar & Doe, 2013). A more comprehensive study shows that each lineage of DM1 to DM4
produces morphologically similar neurons populating a unique quadrant of the EB and jointly constitute a specific repertoire of small-field CX neurons and DM5, DM6, and DL1 lineages generate lineage-specific large-filed CX neurons (Yang et al., 2013). The embryonic-born INPs produce neurons which persist into adult brains and their projections innervate neuropils of PB, FB, EB (but not the NO) of the central complex (Walsh & Doe, 2017).

The diversity of the neurons and glia generated from type II NBs and INPs at both embryonic and postembryonic stages is boosted by the expression of a series of TTFs. Type II NBs start the expression of TTFs from Pdm (not like type I NBs starting the expression from Hunchback (Hb), krüppel (Kr)), and then Cas, Nab, and Grh subsequentially in early specified type II NBs or from Cas to (maybe Nab and) Grh subsequentially in late specified type II NBs during embryogenesis (Alvarez & Diaz-Benjumea, 2018; Walsh & Doe, 2017), suggesting that they may coordinate with type I NBs in response to common intrinsic or extrinsic cues to trigger the expression of TTFs. Although the exact TTF order in larval type II NBs during or after quiescence has not been fully determined, a similar model as in larval type I NBs i.e., Dichaete (D)/Cas/Grh→ Seven-up (Svp)/Grh has been demonstrated (Bayraktar & Doe, 2013; Maurange et al., 2008). The unique feature for boosting neuronal output/diversity in type II NB lineages is the generation of diverse INPs which are temporally regulated to enhance the output. In a given type II NB lineage, about 40 INPs derived from the first larval-born INP could be individually distinguishable with each INP generating sibling INPs to produce a morphologically similar but temporally regulated series of distinct neuron types (Wang et al., 2014). Thus, this feature of INPs makes them behave like oRGs which promote brain complexity in mammals. Embryonic and larval INPs adopt a similar TTF expression flow of D→Grh→ Eyeless (Ey) from early/young INPs to late/old INPs to generate diverse types of neurons and glia over time, which target to distinct adult brain regions in a INP age-dependent manner (Alvarez & Diaz-Benjumea, 2018; Bayraktar & Doe, 2013;
Walsh & Doe, 2017; Wang et al., 2014). The fate of early or late born INPs is also governed by type II NBs intrinsically through the Imp to Syncrip (Syp) switch, which follows and is controlled by a Cas→Svp transition, thus coarsely patterning the INPs fate temporally during postembryonic neurogenesis (Ren et al., 2017). Therefore, through coordination of temporal patterning in both type II NBs and INPs, the diversity and output of neurons and/or glia are further increased (Bayraktar & Doe, 2013; Ren et al., 2017).

Similarly, type I NBs also express TTFs to increase the heterogeneity of NBs and diversity of their progeny. After delamination, Hb, Kr, Pdm, Cas and Grh are sequentially activated in central brain type I NBs and sequentially produce unique associated lineages with distinct progeny pools (Brody & Odenwald, 2000; Kohwi & Doe, 2013; Verma et al, 2019). The expression of temporal factors is coordinated in all NBs from the time of the first birth of type I NBs, i.e., late delaminated type I NBs start expression of the corresponding late factors, such as from Kr (Tsuji et al., 2008). One intriguing aspect is that the earlier factor activates the next factor which in turn represses the earlier factor in a negative feedback loop (Baumgardt et al, 2009; Grosskortenhaus et al, 2006; Tran & Doe, 2008). The TTF cascade utilized by the central brain type I NBs to control the generation of specific neuron types is also broadly adopted by other NB systems. For example, although how TTF cascades in the visual system regulate proliferation of optic NBs is unknown, Homothorax (Hth), Klumpfuss (Klu), Ey, Sloppy paired (Slp), D, and Tailless (Tll) are sequentially expressed from young to old NBs in the main outer proliferation center (OPC) in the optic lobe (Apitz & Salecker, 2014; Li et al, 2013b; Suzuki et al, 2013), and Distalless (Dll) to Ey to Slp to D in NBs in the tips of the OPC (tOPC), or D to Tll in inner proliferation center NBs (referred to IPC NBs). The similar temporal strategy is also unitized by vertebrates or mammals to generate diverse progeny in the CNS (Cleary & Doe, 2006; Isshiki et al, 2001; Kohwi & Doe, 2013; Maurange, 2020; Rossi et al, 2017).
6. Regulations on the stability of type II NBs and INPs at Drosophila larval stages

6.1 Regulations by temporal transcription factors

Drosophila larval stages are the window in which ~90% of neurons of the adult CNS are produced. NBs divide ~50 times over 4-5 days until early/mid-pupal stages when they are eliminated (Maurange et al., 2008). After reactivation from quiescence, the NBs re-enter into G1 from arrest at G0 or G2 phase and become mitotically active to generate diverse types of neurons/glia, through recalling the expression of TTFs which are paused during quiescence, as well as subsequent activation of Cas/Grh, then Svp/Grh or additional unknown TTFs (Li et al., 2013a; Maurange et al., 2008; Tsuji et al., 2008). These TTFs enhances the diversity of neurons generated postembryonically not only by maintaining the mitotic activity of NBs but also by preventing the abnormal overproliferation of NBs in response to intrinsic or extrinsic cues. The regulation through TTFs in both type II NBs and other NBs shares a lot of commonalities but may also differ in details. First, to produce a sufficient number of neurons/glia, mis-regulation of temporal identity or termination of self-renewal of NBs needs to be avoided at multiple levels. TTFs need to be maintained precisely in line with the corresponding developmental stages or with their correct locations. For example, Imp, which is expressed in the NBs at early larval stages (corresponding to the mid-point of the entire neurogenesis process including embryonic stages) and knockdown of which gives rise to precocious termination of neurogenesis (Liu et al., 2015), functions to maintain proliferation/self-renewal of early NBs by stabilizing myc mRNA through direct binding (Maurange, 2020; Samuels et al., 2020). Additionally, Imp post-transcriptionally promotes Chronologically inappropriate morphogenesis (Chinmo) expression, also through directly binding to chinmo mRNA, to not only determine the progeny fate of NBs in mushroom body (Zhu et al., 2006) and central brain (Dillard et al., 2018), but also promote NB growth and proliferation (Genovese et al., 2019; Narbonne-Reveau et al., 2016; Samuels et al., 2020). Grh promotes proliferation of type I NBs in thorax likely through directly regulating E-Cadherin
(Dumstrei et al., 2003) but antagonizes proliferation of type I NBs in the abdomen by providing the competence for NBs to undergo Abd-a-dependent apoptosis (Almeida & Bray, 2005; Cenci & Gould, 2005). Grh may also function similarly to regulate the proliferation of type II NBs.

Second, in order to prevent overproliferation or over-growth, the termination of NBs needs to be tightly controlled. The termination of NB self-renewal is also mainly mediated by the TTFs (for more information please see Section 7 pertaining to the end of the neurogenesis). However, there are clues suggesting that many other postembryonic TTFs remain to be discovered. For example, the pNBs give rise to at least 40 types of antennal lobe projection neurons (PNs) (Yu et al., 2010), which is hard to be explained with known postembryonic TTFs. In addition, NBs still divide a few rounds before exiting the cell cycle or before the transition from Chinmo to Broad Complex (Br-C) (see below) (Maurange et al., 2008), suggesting that there may be unidentified TTFs involved.

6.2 Regulations of the formation or maintenance of type II NBs

For the specification of type II NBs, in addition to EGFR/Sp1/Btd (the homolog of mammalian Sp8), a major finding has been the identification of PntP1, one member of the E26 transformation-specific (ETS) transcription factor family which is required to specify and maintain type II NB identity, although it is still unclear if or how PntP1 initially triggers the specification of type II NBs during embryogenesis. PntP1 has a conserved ~85 amino acid Ets domain in the C-terminus which binds the purine-rich consensus DNA sequence 5'-GGAA/T-3' (Hollenhorst et al., 2011; Karim et al., 1990). Specific antibodies against PntP1 demonstrate specific expression of PntP1 in type II NBs and all the derived imINPs (newly born Ase^- imINPs, early Ase^- imINPs and late Ase^+ imINPs). Functionally, PntP1 specifies and maintains type II NBs by suppressing Ase expression. Ectopic expression of PntP1 is sufficient to transform type I NB lineages to type II NB-like lineages, which behave similarly to non-transformed type II NB
lineages (Zhu et al., 2011). Btd is expressed in type II NB lineages and functions cooperatively with PntP1 to specify type II NB lineages by repressing Ase and also to promote INP generation (Xie et al., 2014). The transcriptional repressor, Tll is specifically expressed in type II NBs and imINPs and binds to ase enhancer to suppress Ase expression in type II NBs (Hakes & Brand, 2020; Rives-Quinto et al., 2020), however a role of Tll downstream of PntP1 has not yet been identified.

Maintenance of the identity/self-renewal/proliferation of type II NBs has been well characterized. Suppressing of Ase is a prerequisite to maintain type II NBs identity, as ectopic expression of Ase in type II NBs transforms type II to type I NBs and eliminates INPs (Bowman et al., 2008). Self-renewal factor Dpn regulates the proliferation of type II NBs. Loss of Dpn in type II NBs results in a gradual loss to a complete depletion of type II NBs by 3\textsuperscript{rd} instar larval brains, which is 1) partially mediated by the ectopic expression of Ase and nuclear Pros, which acts to inhibit cell self-renewal and to promote cell cycle exit and differentiation, and 2) partially by the derepression of Erm expression in type II NBs (also see below), which leads to a gradual loss of type II NBs by antagonizing PntP1 (Janssens et al., 2017; Jiang & Reichert, 2014; Li et al., 2017b; Li et al., 2016; Zhu et al., 2012). The bHLH protein Dpn is also required to maintain self-renewal and proliferation of type I NBs, partially by repressing the cell cycle terminator Pros, although the vulnerability to loss of Dpn is higher for optic NBs or type II NBs but lower for central brain type I NBs (San-Juán & Baonza, 2011; Wallace et al., 2000; Zhu et al., 2012). The exact mechanism of how Dpn maintains the proliferation/self-renewal of type I NBs remains unclear, though it is likely through repressing the expression of Cyclin dependent kinase (Cdk) inhibitor Dap to control mitotic activity as demonstrated in outer proliferation center NBs in optic lobes (Wallace et al., 2000). The unequal sensitivity of type I NBs compared to type II NBs in response to Dpn down-regulation is not fully understood and may be partially due to a disparity in the inherent
self-renewal potential induced by the presence of type II NB identity factors, such as Pointed P1 (PntP1) (Li et al., 2017b; Li et al., 2016; Zhu et al., 2012), Tll (Hakes & Brand, 2020; Rives-Quinto et al., 2020) or Zelda (Zld) (Larson et al., 2021; Reichardt et al., 2018) and/or other unknown mechanisms, such as autophagy or pro-apoptosis pathways. Notch signaling has been demonstrated to be necessary and sufficient to promote type II NB self-renewal as well as cell growth by activating its canonical downstream targets E(spl)mγ, E(spl)mβ and E(spl)m8 (Janssens & Lee, 2014; Li et al., 2017b; Li et al., 2016; Zhu et al., 2012). Inhibition of Notch signaling in type II NBs shows gradually reduced cell growth and cell size leading to eventual elimination of type II NBs (Li et al., 2016; Song & Lu, 2011). The effect of Notch is mediated by directly activating transcription of the downstream growth regulator myc through Notch coactivator, Suppressor of Hairless (Su(H)), and Myc further interacts with eukaryotic translation initiation factor 4E (eIF4E) to promote the transcription of downstream genes and NB growth or self-renewal (Song & Lu, 2011). Although Notch signaling is activated in type I NBs and is essential for maintaining NSC identity in various species (Imayoshi et al., 2010; Lasky & Wu, 2005; Wang et al., 2011a), Notch inhibition does not affect either the cell-size or cell fate maintenance of type I NBs, suggesting that either Notch signaling is not involved in maintaining larval type I NB identity or other redundant pathways may exist (Song & Lu, 2011; Zacharioudaki et al., 2012). In addition, studies have shown that maintaining self-renewal by Notch is independent of Dpn, as loss of Notch does not change Dpn expression and loss of Dpn does not inhibit the overproliferation of type II NBs caused by aberrantly activated Notch (Zacharioudaki et al., 2012; Zhu et al., 2012). Further studies on maintaining type II NBs identity demonstrated that inhibition of the precocious activation of Erm by Notch or Dpn in type II NBs is the underlying mechanism for them to prevent the gradual NB loss (Janssens et al., 2017; Li et al., 2017b; Li et al., 2016). The zinc finger protein of the early growth response (EGR) family protein Klu is required for NB growth, self-renewal and maintenance of type I NBs and type II
NBs, as loss of Klu leads to gradual loss of type I NBs and type II NBs due to premature differentiation (Berger et al., 2012; Xiao et al., 2012). The underlying mechanism of how Klu maintains the identity of NBs is still unclear, though in *Drosophila* enterocyte progenitors Klu has been shown to repress the activation of the proneural gene sc to restrict the fate of enteroblasts (EBs) (Korzelius et al., 2019). Dpn and some E(spl) proteins (including E(spl)m3, E(spl)m7, E(spl)mβ, E(spl)mδ, and E(spl)mγ), and Klu all directly bind to the erm enhancer to suppress its activation in type II NBs (Janssens et al., 2017; Li et al., 2017b). Along with the transcriptional inactivation mediated by Dpn/Klu/E(spl), histone modification in the form of deacetylation modification of multiple lysine residues of the erm enhancer chromatin is involved in suppressing Erm expression in type II NBs. Recruitment of histone deacetylase 1 (Hdac1)/Rpd3 confers the erm enhancer with a poised state for its inactivation in type II NBs but rapid activation in imINPs (Janssens et al., 2017). In addition, Klu maintains the identity and self-renewal of type II NBs possibly by functioning downstream of Notch (Xiao et al., 2012), or likely through interacting with Notch components (Korzelius et al., 2019), or mediated by β-catenin/Armadillo (Arm) activity (Komori et al., 2014b). The SET1/MLL complex, maintains type II NB identity by maintaining an active chromatin state at the btd locus (and maybe also the pnt locus) by methylating histone H3 Lysine (H3K4) at btd and/or pnt gene loci. This requires Trithorax (Trx), and the core components of the SET1/MLL complex, Absent, small, or homeotic discs 2 (Ash2), Retinoblastoma binding protein 5 (Rbbp5), and Will die slowly (Wds) (Komori et al., 2014a; Xie et al., 2014). The zinc-finger protein Zld, a key activator of early zygotic transcription (Liang et al., 2008) is expressed in type II NBs and absent in all the progeny, and maintains the type II NB undifferentiated state and promotes proliferation by working synergistically with Notch and likely directly activating Dpn expression (Larson et al., 2021; Reichardt et al., 2018). The microRNA *bantam* is required to maintain cell growth and proliferation of type II NBs (as well as mINPs), by directly targeting mRNAs of the cell polarity
proteins Brain tumor (Brat), Pros and Numb to suppress their expression levels to promote cell
growth and proliferation (Weng & Cohen, 2015; Wu et al, 2017). Reduction of the Lsg1 family
member Nucleostemin 3 (Ns3), a putative GTPase, which was previously shown to regulate
insulin signaling in serotonergic neurons and organismal body growth (Kaplan et al, 2008), leads
to proliferation arrest in type II NBs in a Pros-independent mechanism, and likely functions by
establishing correct NB cortical polarity to promote NB proliferation (Johnson et al, 2018).

6.3 Regulations of the asymmetric division of type II NBs.

Type II NBs undergo self-renewing asymmetric divisions to produce a new type II NB and a
daughter imINP. The mechanisms regulating asymmetric divisions are well conserved in type I
and type II NBs, but there are some features that are unique to the asymmetric division of type II
NBs.

Once delaminated from the neuroectoderm, NBs start proliferations to establish their own
lineages through asymmetric divisions. Asymmetric cell division involves two main steps: 1)
rotating the division machinery including centrosome and spindle fibers so that they are
perpendicular to neuroepithelium (Kaltschmidt et al, 2000; Rebollo et al, 2009); 2) setting up cell
polarity with distinct complexes at the apical and basal poles, which specify the future NB and
the differentiating daughter cell, respectively. The two processes are mutually dependent and
interlinked. For example, Par complex and Pins/Gai complex, the two cell polarity complexes on
apical side, regulate the mitotic spindle orientation as indicated by their capability to direct
mitotic spindle asymmetry and displacement (Cai et al, 2003; Mauser & Prehoda, 2012; Siegrist
& Doe, 2005), with Pins complex having a predominant role (Izumi et al, 2004). Apical complex
scaffold protein Insuteable (Insc) functions actively to regulate the binding of Pins preferentially
to Discs large (Dlg). Dlg then causes the astral microtubules to attach to cortex in interphase or
early prophase, or further to coiled-coil Mushroom body defect protein (Mud), which generates the astral spindle shortening forces to pull the centrosome (Mauser & Prehoda, 2012).

To establish the polarity for initiating asymmetric divisions, both the apical and basal complex are well segregated, recruited and assembled, respectively. The apical complex on the apical side of NBs specifies the NB fate of the apical daughter cell. This complex is composed of the Par complex, the Pins/Gαi complex and the Scribble complex. And the functions of these complexes are concerted. **The typical Par complex** comprises atypical protein kinase C (aPKC), PDZ domain proteins Bazooka (also called Par3) and Par6 (Betschinger et al., 2003). Par proteins were the first complex shown to be localized to the apical cortex and its most important role is to restrict the basal fate-determinant to their destination, such as Miranda (Mira) and Prospero (Pros) (Betschinger et al., 2003; Izumi et al., 2004). **The Pins/Gαi complex** is composed of Partner of Inscuteable (Pins), a subunit (Gi) of the heterotrimeric G protein (Gαi) and Mud (Mauser & Prehoda, 2012; Siller et al., 2006). Neuroblast-specific protein Insc functions as a partner of Pins and is recruited by Par complex, and co-localizes the whole complex at the apical side of NBs in mitosis (Saini & Reichert, 2012). **The Scribble complex** is composed of Scribble (Scrib), Lethal giant larvae (Lgl) and Dlg. This complex has been shown to co-localize to the apical cortex and regulates multiple aspects of the asymmetry of type I NBs, including targeting components of the basal complex or keeping mitotic spindle asymmetry (Albertson & Doe, 2003; Ohshiro et al., 2000; Peng et al., 2000). On the basal side of NBs, the complex comprising Numb, Partner of Numb (Pon), Pon adaptor protein Miranda, and its cargo Brat or Prospero (Pros, which is only in type I NBs but not in type II NBs) is localized (Betschinger et al., 2003; Saini & Reichert, 2012). The opposite localization of the basal complex is directed by apical complexes in mitosis (Atwood & Prehoda, 2009; Barros et al., 2003; Betschinger et al., 2003; Qin et al., 2004; Wirtz-Peitz et al., 2008; Zhang et al., 2016), including through a serial cascade of phosphorylation to
translocate the protein or mRNA of specific basal components (Barros et al., 2003; Bello et al., 2006; Betschinger et al., 2003; Betschinger et al., 2006; Broadus et al., 1998; Ikeshima-Kataoka et al., 1997; Lee et al., 2006; Matsuzaki et al., 1998; Petritsch et al., 2003; Qin et al., 2004; Shen et al., 1997; Smith et al., 2007; Sousa-Nunes et al., 2009; Wirtz-Peitz et al., 2008).

Following the establishment of the polarity, NBs will then undergo cytokinesis. Cytokinesis of NBs depends on both the polarity-dependent pathway and spindle-dependent pathway (Connell et al., 2011; Roth et al., 2015). The conserved cytokinesis machinery and its assembly which has been well summarized by some reviews (Glotzer, 2009; Green et al., 2012) and will not be discussed here in detail. Instead, I will briefly review some key regulations on asymmetric cytokinesis pertaining to the Drosophila NBs. First, although both the cell-polarity and spindle orientation are largely established before cytokinesis, cytokinesis itself in asymmetric division of NBs is a relatively independent process related to astral spindle, which is dispensable for the correct cleavage furrowing or the accomplishment of asymmetric cytokinesis (Giansanti et al., 2001). Second, during cytokinesis of NBs, the polarity-dependent pathway and the (central) spindle-dependent pathway are actually independent of each other. The Gαi/Pins activity facilitates positioning the contractile ring/cleavage furrow unequally to the basal side independent of the central spindle structure and independently induce their own contractile ring/cleavage furrow (Cabernard et al., 2010; Connell et al., 2011). Correspondingly, the cleavage furrow constriction that relies on the central spindle, including chromosomal passenger complex component Survivin/Determin (Det), Aurora B (AurB) and/or Pavarotti (Pav) and tumbleweed (Tum), is also decoupled from the polarity-dependent cytokinesis (Roth et al., 2015).

One of the most prominent features in type II NB asymmetric divisions, compared to those in type I NBs, is the lack of Pros in type II NBs (and also in imINPs). Pros does not function to
specify type II NB identity because ectopic expression of Pro in type II NBs does not transform them to type I NBs (Bayraktar et al., 2010) and removal of Pros in type I NBs does not induce ectopic INPs in the lineages either (Bowman et al., 2008). Therefore, the asymmetric division of type II NBs does not involve asymmetric segregation of Pros like in type I NBs. Disrupting the normal mitotic machinery of type II NBs leads to defects in asymmetric division of NBs and the failure of imINP specification or production of two type II NBs instead. SCF{Supernumerary limbs} E3 ubiquitin ligase complex, which is composed of Cullin 1 (Cul1), SKP1-related A (SkpA), Regulator of cullins 1a (Roc1a) and the F-box protein Supernumerary limbs (Slimb), inhibits ectopic NB formation by promoting asymmetric division of NBs. It does so by targeting SAK kinase to prevent centrosome amplification as well as by in part preventing hyperactivation of Akt, which is a critical regulator of cell proliferation, metabolism or NB quiescence (Li et al, 2014a).

Improper mitotic spindle orientation due to Pins/Gαi complex related deficits, such as mutations in mud/anastral spindle 2 (ana2)/asterless (asl)/spindle assembly abnormal 4 (sas-4)/pins, results in two daughter cells that inherit equal amount of cortical proteins and are both capable of self-renewal (Li et al, 2014b).

In addition to segregating fate determinants to imINPs, a few mechanisms specific to the generation of imINPs have also been identified. For example, Trx likely keeps the pntP1 locus active also through SET1/MLL complex-mediated H3K4 methylation of chromatin to specify INP identity (Komori et al., 2014a). PntP1 as well as Btd function synergistically to promote specification of imINPs and the generation of mINPs (Xie et al., 2014), whereas Tll is sufficient to promote generation of imINPs but not mINPs, as demonstrated by its capability to induce imINP-like cells when misexpressed in type I NBs, and to trigger dedifferentiation of imINPs to supernumerary type II NBs when overexpressed in imINPs (Rives-Quinto et al., 2020).
6.4 Mechanisms promoting INP maturation and cell fate commitment

Newly born imINPs need to become genetically mature through 1) depletion of both proliferation/self-renewal genes and NSC identity factors, and 2) turning on the expression of differentiation genes. Dysregulation of either of the two aspects can lead to reversion of INPs to type II NBs.

Once imINPs are produced, self-renewal factors including Dpn, the Notch downstream targets, Zld, and Klu need to be removed immediately, by mRNA decay or protein sequestration/degradation. To achieve this, Brat (inherited by the imINPs from NBs) directly binds to the 3’UTR of zld mRNA or to the 3’UTR of dpn mRNA in a complex with Tis11 to mediate mRNA degradation (Komori et al., 2018; Reichardt et al., 2018). The Cul1 based ubiquitin E3 ligase complex promotes proteasome-dependent degradation of Dpn in imINPs or hairy and enhancer of split 1 (Hes1), the Dpn homolog in vertebrate (Chen et al., 2017; Imayoshi & Kageyama, 2014; Komori et al., 2018). In a complementary mechanism, Insensible (Insb), a Hes family protein, may function to sequester Dpn protein in an inactive complex (Komori et al., 2018). The elimination of Notch or its downstream effector activity in imINPs is also multilayered. First, the Numb-Notch axis, which also functions in the transition from type I NB to GMCs to inhibit Notch signaling, may play important roles. Inhibition of Notch is mainly achieved by the Numb inherited during the asymmetric divisions of NBs. Numb promotes endocytosis of the Notch receptor and/or Sanpodo (Spdo, the trafficking partner of Notch) through α-Adaptin (Berdnik et al., 2002; Hutterer & Knoblich, 2005; O’Connor-Giles & Skeath, 2003), thus lowering Notch signaling in basal daughter cells. In addition, Numb independently inhibits protein levels of Myc, a known transcriptional target of Notch by reducing protein stability mediated by HECT, UBA and WWE domain containing E3 ubiquitin protein ligase 1 (Huwe1) based protein degradation (Wu et al., 2017). Second, given that either the endocytosis of
Notch receptors mediated by Numb or the lysosomal degradation of Notch receptors which are polyubiquitinated by E3 ubiquitin ligases and sorted through the Endosomal Sorting Complex Required for Transport (ESCRT) pathway may be insufficient to dampen Notch in imINPs, retromer-mediated trafficking of Notch receptor acts as a safeguard mechanism to retrieve the hypoubiquitinated Notch which escapes the ESCRT-lysosomal degradation (Li et al., 2018). Third, Brat and Numb may attenuate Notch nuclear import to dampen Notch intracellular domain (NICD) initiated downstream gene transcription (Mukherjee et al., 2016). In addition, Cullin3 (Cul3) promotes differentiation and prevents the reversion of progeny, including imINPs (as well as GMCs), back to NBs, by promoting aPKC-directed asymmetric segregation and localization of Numb to downregulate Notch signaling (Komori et al., 2020). The inhibition of Klu expression and activity in imINPs is not fully understood yet, while there is evidence demonstrating that Brat functions in imINPs to antagonize Klu either directly or through attenuating Arm by activating Adenomatous polyposis coli 2 (Apc2, a negative regulator of Arm), a key destruction complex component (Komori et al., 2014b). Due to the interactions between Brat and above factors, loss of Brat causes a dramatic overproliferation of type II NBs at the expense of neurons by compromising the differentiation of INPs (Bello et al., 2006; Bowman et al., 2008). The Numb mutant phenocopies the Brat mutant in larval brains (Cai et al., 2003; Wang et al., 2006).

To dismiss type II NB identity genes in imINPs, pntP1 or tll are two master regulator genes which must be targeted. PntP1 protein has its peak expression in newly born imINPs to activate fez zinc finger family repressor dFez/Erm which in turn suppresses activity and expression of PntP1 in imINPs (Janssens et al., 2017; Li et al., 2016; Xie et al., 2016; Zhu et al., 2011). Tll, a putative target activated by Su(H) (the DNA-binding partner of Notch) (Zacharioudaki et al., 2016), is attenuated or silenced sequentially in imINPs by Erm and Hamlet (Ham), or in mINPs by Ham, through transcriptional repression by recruiting histone deacetylase 3 (Hdac3) (Hakes &
Brand, 2020; Rives-Quinto et al., 2020). In addition to suppressing PntP1 and Tll, Erm also maintains the developmental potential of Ase+ imINPs by activating Pros-dependent cell cycle exit and prevents INPs from dedifferentiating to NBs by antagonizing the self-renewal factors Dpn, Klu or Notch, leading to the attenuation of the competence of INPs to respond to these factors (Janssens et al., 2014; Weng et al., 2010). However, the Erm-dependent attenuation or antagonization of self-renewal factors is more likely a safeguard mechanism, since in physiological condition these self-renewal factors have been decommissioned in imINPs. Erm also functions through regulating chromatin remodeling by forming a suppressor complex with Hdac3 and Brahma (Brm), which is shown to promote differentiation and cell cycle arrest (Koe et al., 2014). In addition to Brm, other components of SWI/SNF complex including Osa, Mor and Snr1 are required to prevent dedifferentiation of INPs and Osa directly activates Ham transcription to limit the proliferation of imINPs and prevent their fate reversion (Eroglu et al., 2014). Activation of Erm and Brm is promoted by IntS5, IntS8, and IntS1, which are components of the integrator complex, an evolutionarily conserved complex that contains 14 subunits and regulates RNA processing and gene transcription (Zhang et al., 2019). Thus, it demonstrates the role of the integrator complex in preventing INP dedifferentiation. Brm and Erm also genetically interact with Frizzy-related (Fzr), a known co-activator of Anaphase Promoting Complex/Cyclosome (APC/C) ubiquitin ligase, to promote NB to INP transition (Ly & Wang, 2020).

INPs also need to avoid premature differentiation and cell cycle exit in order to maintain their progenitor state and become fate committed mitotic mINPs. This requires not only the reactivation of self-renewing programs such as Dpn expression and Notch signaling like in any type II NBs, but also more importantly the inhibition of expression of homeodomain protein Pros in newly born
imINPs (Bayraktar et al., 2010; Zacharioudaki et al., 2012) by Btd and PntP1 (Bayraktar et al., 2010; Xie et al., 2016; Xie et al., 2014).

7. Mechanisms involved in terminating NBs proliferation or self-renewal

The self-renewal and proliferation of NBs needs to be terminated in a timely manner through cell cycle exit (Chai et al., 2013; Mao et al., 2014), apoptosis (Bello et al., 2003; Siegrist et al., 2010), or autophagy (specifically by mushroom body NBs) (Pahl et al., 2019; Pinto-Teixeira et al., 2016) to ensure only a precise number of neurons are generated during development. In this section I will review mechanisms involved in terminating the self-renewal/proliferation of type II NBs and compare to those of type I NBs.

For timely termination, postembryonic NBs including type II NBs and most type I NBs sequentially undergo a series of events, including reduced energy metabolism, reduction in cell growth, terminally symmetrical divisions and then cell cycle exit or programmed cell-death at late larval or early pupal stages. The cell-autonomous growth reduction, reflected by reduced cell size and prolonged NB cell cycle, is mediated not through the common Insulin-like receptor (InR)/TOR pro-growth pathway or autophagy/apoptosis pro-death pathway, but through mediator complex subunits (Med4, Med6, Med9, Med10, Med11, Med22, Med27, Med31). The mediator complex regulates ecdysone-induced transcription by directly binding to ecdysone receptor (EcR) (Homem et al., 2014; Pahl et al., 2019). By regulating ecdysone signaling and the expression of ecdysone targets and metabolic enzymes related to glucose metabolism and energy production, the mediator complex increases oxidative phosphorylation levels, which result in the subsequent cell-growth arrest and termination of symmetrical divisions of NBs (Homem et al., 2014).
The majority, if not all, of the NBs in late larval or pupal central brains do not utilize programmed cell death but a cell cycle exit, which depends on the nuclear Pros expression, for terminating their self-renewal and proliferation (Maurange et al., 2008). Before the termination of the self-renewal, the TTF cascade of (Hb-Kr-)Pdm-Cas-Svp from early embryonic stages to larval stages is scheduled to timely promote cell cycle exit or apoptosis by following these steps: 1) embryonic Cas activates and maintains Grh expression, inhibiting premature nuclear Pros and permitting continued mitotic activity; 2) late expressed Svp antagonizes Grh activity to trigger a pupal burst of nuclear Pros; 3) Cas/Svp and other unknown later factors facilitate the transcriptional switch from activating switch from Chinmo (an early NB identity marker expressed during embryonic stages and larval stages prior to 60hrs after hatching) to Br-C and Ecdysone-induced protein 93F (Eip93, or E93) (relative late NB identity markers expressed during larval stages at 60-120, or 72-120hrs after hatching, respectively), which promotes shrinkage of the cell size of neurons and adoption of later neuronal identity (Genovese et al., 2019; Maurange et al., 2008; Syed et al., 2017). In addition to the Chinmo to Br-C transition, another accompanied temporal transition occurs between two mRNA-binding proteins, Imp (an early NB identity marker expressed in the similar stage as Chinmo) and Syp (a relative late NB identity marker expressed in the similar stage as Br-C/E93), which was demonstrated first in mushroom body NBs (Liu et al., 2015). The transition of Imp to Syp functions upstream to activate Chinmo in early NBs and repress Chinmo in late NBs (Syed et al., 2017; Yang et al., 2017). Syp promotes NB cell cycle exit by permitting nuclear Pros accumulation likely through direct binding to pros mRNAs to stabilize the mRNAs and enhancing transcription, while Imp acts in an opposite way (McDermott et al., 2014; Yang et al., 2017). Further studies have shown that the TTF cascade, particularly Cas-Svp, triggers the termination of proliferation when NBs get old. The expression of EcR is activated by Svp and the active ecdysone-signaling is required to complete the transition of Chinmo/Imp/lin-28 to Br-c/Syp/E93 (Syed et al., 2017), which partially accounts for cell cycle exit mediated by ecdysone
signaling. Additionally, Hedgehog (Hh) signaling functions downstream of Cas to control the NB termination by regulating Grh expression and is likely the bridge between the TTF cascade and the asymmetric division machinery (Chai et al., 2013). There are several tumorigenicity models related to mis-regulation of the TTF cascade. For example, the temporal patterning by Chinmo delineates the early window of malignant susceptibility of brain tumors. Temporally delayed Chinmo expression leads to hyperplastic NB tumor generation by establishing a positive feedback loop, in which Chinmo boosts protein biosynthesis and expression of the mRNA binding proteins Imp and Lin-28. Imp and Lin-28 in turn post-transcriptionally sustain Chinmo expression directly or indirectly to prevent differentiation (Narbonne-Reveau et al., 2016). Furthermore, temporal patterning genes are redeployed in Pros LOF NB tumors to trigger and govern the cellular hierarchy, heterogeneity and metabolism within NB tumors (Genovese et al., 2019). Most postembryonic type I NBs in abdominal segments of the ventral nerve cord and some in the thoracic segments undergo canonical Reaper, Hid, and/or Grim (RHG)-dependent apoptosis during late third instar stage by maintaining Hox gene abd-a expression through Grh in the abdomen or by activating the expression of Antp or Ultrabithorax (Ubx) in the thorax (Bello et al., 2003; Cenci & Gould, 2005; Maurange et al., 2008). The apoptosis is also governed by the temporal cascade of Cas to Svp, and Chinmo to Br-C (orchestrating with Imp/Lin-28 to Syp/E93) to cease the proliferation of NBs in response to ecdysone (Liu et al., 2015; Maurange et al., 2008; Syed et al., 2017; Yang et al., 2017).

In addition to cell cycle exit and apoptosis, termination of NB self-renewal can also be mediated by autophagy. This mostly occurs in the mushroom body NBs. Autophagy promotes the death of mushroom NBs by restricting cell growth and proliferation of NBs and downregulating PI3K signaling pathway including its downstream effector Forkhead box sub-group O (Foxo) via E93. In addition, the temporal identity gene Syp activates pro-autophagy E93 to terminate neurogenesis.
by downregulating PI3K levels. This provides a mechanism that terminates mushroom body NBs step by step following early neurogenesis (Pahl et al., 2019).

8. The rationales of the research in this dissertation

Although prior studies have deciphered comprehensive mechanisms pertaining to the regulation of development of larval NSCs in *Drosophila*, those have mainly focused on type I NBs. Many aspects of how type II NBs are regulated still need to be studied and are expected to generate a new understanding of how to generate the brain complexity, as well as new insights into the mechanisms underlying neurogenetic disorders.

**First**, how the identity of type II NBs is specified and maintained, which is the premise for generating INPs and the neurons/glia, is still not fully understood. As stated above, one of the type I NB identity markers, Ase, needs to be silenced in type II NBs. Although it has been shown that PntP1 is necessary and sufficient to suppress Ase in type II NBs, the underlying mechanisms are still unclear. Thus, in order to address these questions, work in CHAPTER II dissected the transcriptional properties of PntP1 in specifying and maintaining the identity of type II NBs, and identification of its novel downstream target, TII which directly suppresses Ase.

**Second**, how the two unique cell types, imINPs and mINPs are stabilized and balanced in type II NB lineages remains poorly understood, although a main regulator Erm and its partial function have been identified. To this end, work in both CHAPTER III and CHAPTER IV adopted an RNA interference (RNAi)-based screen approach to identify three additional factors, Six4, Skuld (Skd) and Kohtalo (Kto) which are involved in governing the correct fate commitment of imINPs, and deciphered their underlying mechanisms. In order to properly reduce function of the type II NB identity gene *pntP1* in imINPs, homeodomain protein Six4 (CHAPTER III) is shown to
function redundantly and interacts with Erm, which is shown to be activated by PntP1 and its cofactors of Skd (mediator complex subunit 13) and Kto (mediator complex subunit 12) (CHAPTER IV).
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CHAPTER II. The Ets protein Pointed P1 represses Asense expression in type II neuroblasts by activating Tailless

Running Title: PntP1 represses Asense by activating Tailless

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Contribution Statement

R.C. and S.Z. conceived the idea, designed the project and approaches. R.C. carried out the experiments, collected and analyzed the data, generated figures, and wrote and revised the manuscript. X. D. performed partial experiments of generating plasmids used in Figure II-3B-G. S.Z revised the manuscript.
Abstract

Intermediate neural progenitors (INPs) boost the neuronal output and diversity from neural stem cells (NSCs) by undergoing transient proliferation. In the developing *Drosophila* brains, INPs are generated from type II neuroblasts (NBs). In order to maintain type II NB identity and their capability to produce INPs, the proneural protein Asense (Ase) needs to be silenced by the Ets transcription factor pointed P1 (PntP1), a master regulator of type II NB development. However, the molecular mechanisms underlying the PntP1-mediated suppression of Ase are still unclear since the repression has long been demonstrated. In this study, we utilized genetic and molecular approaches to determine the transcriptional property of PntP1 and identify the *cis*-DNA elements and the direct downstream effector of PntP1 that mediate the suppression of *ase*. Our results demonstrate that PntP1 directly activates the expression of the transcriptional repressor, Tailless (Tll), by binding to seven Ets-binding sites, whereas Tll in turn suppresses the expression of Ase in type II NBs by binding to two hexameric core half-site motifs. We further show that Tll provides positive feedback to maintain the expression of PntP1 and the identity of type II NBs. Thus, our study identifies a novel direct target of PntP1 and reveals mechanistic details of the specification and maintenance of the type II NB identity by PntP1.
Introduction

One of the common strategies to contribute to the construction of the complex central nervous system (CNS) is generating a large number of diverse types of neurons or glia that can integrate into the neural circuits. Neural progenitors play pivotal roles during the process of amplifying neuronal or glia output and diversity from neural stem cells (NSCs). In *Drosophila* larval CNS, one type of neural progenitors, called intermediate neural progenitor cells (INPs) is exclusively produced by a specific type of NSCs, called type II neuroblasts (NBs), which were discovered over a decade ago (Bello *et al.*, 2008; Boone & Doe, 2008; Bowman *et al.*, 2008). Compared to other types of NBs in *Drosophila* CNS, including type 0 NBs that directly produce neurons at embryonic stages (Baumgardt *et al.*, 2014; Bertet *et al.*, 2014; Karcavich & Doe, 2005) or type I NBs that produce neurons by generating terminally dividing ganglion mother cells (GMCs) (Datta, 1995; Truman & Bate, 1988), type II NBs produce 4 times more neurons with more diverse subtypes by generating INPs, which undergo several rounds of self-renewal divisions before/while producing GMCs (Bayraktar & Doe, 2013; Bello *et al.*, 2008; Izergina *et al.*, 2009; Wang *et al.*, 2014; Xie *et al.*, 2014; Zhu *et al.*, 2011).

Type II NBs differ from type I and type 0 NBs by the lack of the expression of the proneural protein Asense (Ase). The identification of type II NBs corrects the long-standing view that all NBs express Ase (Brand *et al.*, 1993; Jarman *et al.*, 1993). The exact roles of Ase in type 0 and type I NBs is still unclear because loss of Ase does not generate obvious phenotypes (Bayraktar *et al.*, 2010; Bowman *et al.*, 2008). Nevertheless, the absence of Ase is a prerequisite for specification/maintenance of the type II NB identity and the generation of INPs (Zhu *et al.*, 2011). Ectopic Ase expression in type II NBs leads to transformation of type II NBs to type I NBs, depletion of INPs, and direct production of GMCs from the NBs (Bowman *et al.*, 2008; Zhu...
et al., 2011). Our previous studies have shown that Pointed P1 (PntP1), which is a member of the E26 transformation-specific (Ets) family, is the master regulator of type II NBs specification and is responsible for the suppression of Ase (Xie et al., 2014; Zhu et al., 2011). Mis-expression of PntP1 is sufficient to transform type I NBs into type II NBs by suppressing Ase expression and promote the production of INPs. The transformed type II NB lineages are equally susceptible to tumorigenic overproliferation resulting from loss of tumor suppressors such as Brain Tumor (Brat) and Earmuff (Erm), the latter of which is a direct target of PntP1 in immature INPs (imINPs) (Janssens et al., 2017; Li et al., 2017; Li et al., 2016; Zhu et al., 2011). However, it is not clear if PntP1 acts as a transcriptional repressor to directly suppress Ase expression or acts as a transcriptional activator to suppress Ase indirectly by activating other transcription factor(s).

In this study, we showed that PntP1 functions as a transcriptional activator to suppress Ase expression indirectly in type II NBs by activating the expression of the transcriptional repressor Tailless (Tll). We provide evidence to demonstrate that Tll is a direct target of PntP1 in type II NBs, whereas Tll suppresses Ase expression directly. We further identified PntP1 and Tll binding sites in the regulatory regions of tll and ase genes, respectively. Therefore, our work reveals a novel PntP1→Tll → Ase pathway and elucidates mechanistic details of PntP1-mediated specification and maintenance of the type II NB identity.

**Results**

**PntP1 functions as a transcriptional activator to indirectly suppress Ase expression in type II NBs**

Our previous studies have demonstrated that PntP1 is both necessary and sufficient for the suppression of Ase expression in type II NBs (Li et al., 2016; Xie et al., 2014; Zhu et al., 2011).
In order to elucidate how PntP1 suppresses Ase expression, we first wanted to determine if PntP1 functions as a transcriptional repressor to directly suppresses Ase expression or as a transcriptional activator to indirectly suppress Ase by activating the expression of other transcriptional repressors. To this end, we first generated an artificial repressor construct \textit{UAS-NLS-EnR-Ets}, which utilizes the upstream activating sequence (UAS) to drive the expression of a chimeric protein that contains a PKKKRKV nuclear localization signal (NLS) of simian virus 40 (SV40) large T antigen (short for NLS) (Kalderon \textit{et al}, 1984), the repressor domain of Engrailed (aa. 1-298) (EnR) (Jaynes & O'Farrell, 1991; Vickers & Sharrocks, 2002), and the PntP1 Ets DNA binding domain (aa. 512-597) (Klambt, 1993; O'Neill \textit{et al}, 1994) and tested if this artificial chimeric repressor protein could functionally mimic endogenous PntP1 or antagonize PntP1’s function. Three other constructs, \textit{UAS-pntP1} (Zhu \textit{et al}, 2011), \textit{UAS-NLS-Ets} and \textit{UAS-NLS-EnR} were generated as controls (Fig. II-1A). To test if the artificial EnR-Ets repressor protein could functionally mimic PntP1, we expressed these constructs in type I NBs using the pan-NB driver \textit{insc-GAL4} (Luo \textit{et al}, 1994). As we showed previously (Li \textit{et al}, 2016; Xie \textit{et al}, 2014; Zhu \textit{et al}, 2011), expressing \textit{UAS-pntP1} suppressed Ase expression in more than 80% of type I NBs and induced generation of Ase+ Dpn+ mINP-like cells in over 15% of type I NB lineages in the VNC (Fig. II-1C-C’, M). However, expression of \textit{UAS-NLS-EnR-Ets} or other control vectors \textit{UAS-NLS-Ets} or \textit{UAS-NLS-EnR} neither suppressed Ase expression in type I NBs nor induced any INP-like cells in the VNC (Fig. II-1D-F’, M). These results suggest that the artificial EnR-Ets chimeric repressor protein could not functionally mimic PntP1.

Next, we examined if the EnR-Ets repressor could antagonize PntP1’s activity. We expressed \textit{UAS-NLS-EnR-Ets} in type II NBs using type II NB lineage-specific \textit{pntP1-GAL4} (Zhu \textit{et al}, 2011). In a wild type brain lobe, there are always eight type II NB lineages and each lineage contains an Ase+ Dpn+ type II NB, 3-4 Dpn- immature INPs (imINPs), and 20-30 Ase+ Dpn+
mINPs (Fig. II-1G-G’, N). Type II NB lineages developed normally when PntP1 was overexpressed (Fig. II-1H-H’, N). However, expression of EnR-Ets but not EnR or Ets domain alone in type II NBs resulted in ectopic Ase expression in over 80% of type II NBs and a reduction in the number of mINPs in all type II NB lineages. On average 20% of type II NB lineages lost all INPs and were transformed to type I NB-like lineages. Moreover, there were more than total 20 type II NB lineages per brain lobe (Fig. II-1K-K’, N). These phenotypes were similar to those caused by PntP1 knockdown in type II NBs (Fig. II-1L-L’ and N), indicating that EnR-Ets antagonizes PntP1 activity. Therefore, PntP1 likely does not function as a transcriptional repressor in type II NB lineages.

To determine if PntP1 acts as a transcriptional activator, we then generated a construct UAS-NLS-VP16AD-Ets, which expresses a chimeric activator by fusing SV40 NLS coupled with VP16 activation domains (VP16AD) (Cousens et al., 1989; Triezenberg et al., 1988) to the Ets domain of PntP1 (Fig. II-2A 2). To test if VP16AD-Ets can functionally mimic PntP1 (Fig. II-2A 1) UAS-pntP1, we expressed it in type I NBs using insc-GAL4 as a driver. To our surprising, expression of UAS-NLS-VP16AD-Ets did not suppress Ase or promote generation of INP-like cells in any type I NB lineages as expression of UAS-pntP1 did (Fig. II-1C-C’, II-2C-D’, I). We reasoned that the lack of the phenotypes could be due to the missing of protein-protein interaction domains, which are common for Ets family proteins and essential for recruiting co-activators to activate target gene expression (Hollenhorst et al., 2011; Li et al., 2000). Therefore, we generated other three constructs, UAS-NLS-pntP1(1/2N)-VP16AD-Ets, UAS-NLS-VP16AD-pntP1(1/2N)-Ets, UAS-NLS-VP16AD-pntP1(1/2C)-Ets, which express chimeric activators harboring either the N-terminal half (pntp1(1/2N) (aa. 1-255)) or the C-terminal half of PntP1 (pntp1(1/2C) (aa. 256-511)) at either the N-terminus or C-terminus of VP16AD (Fig. II-2A 3) -5)). Our results showed that expression of UAS-NLS-VP16AD-pntP1(1/2C)-Ets led to the suppression of Ase in an
average of 45% of type I NBs and induction of Ase+ Dpn+ mINP-like cells in over 6% of type I NB lineages in VNCs (Fig. II-2G-G’, I), whereas expression of either UAS-NLS-pntP1(1/2N)-VP16AD-Ets or UAS-NLS-VP16AD-pntP1(1/2N)-Ets did not (Fig. II-2E-F’, I). To ensure that the effects of NLS-VP16AD-pntP1(1/2C)-Ets were not due to inclusion of endogenous activation domain of PntP1 in the pntP1(1/2C) fragment, we also generated a control construct UAS-NLS-pntP1(1/2C)-Ets, which does not express the VP16AD. However, expressing UAS-NLS-pntP1(1/2C)-Ets neither suppressed Ase expression in type I NBs nor induced any INP-like cells (Fig. II-2H-I), indicating that the pntP1(1/2C) fragment does not contain the endogenous activation domain.

Taken together, results from the artificial chimeric repressor and activator proteins demonstrate that PntP1 functions as a transcriptional activator (Fig. II-2J-K) in type II NB lineages to indirectly suppress Ase expression.
Figure II-1. Expressing the EnR-Ets chimeric repressor transforms type II NB lineages to type I NB lineages.

NB lineages are labeled with mCD8-GFP (in green) driven by insc-GAL4 for type I NB lineages (B-F) or by pntP1-GAL4 for type II NB lineages (G-L), and counterstained with anti-Dpn (in red) or anti-Ase (in blue) antibodies. White arrows point to some Ase⁺ Dpn⁺ type I NBs or Ase⁻ Dpn⁺ type II NBs as examples. White arrowheads point to Ase⁺ Dpn⁺ mINPs or mINP-like cells. Yellow arrowheads point to Ase⁺ Dpn⁻ GMCs. Dashed lines highlight an example NB lineage which is enlarged in insets. The brain lobes are oriented so that the midline is to the right and the anterior side of the brain up. Scale bars equal 50µm.

(A) Schematic diagrams show indicated constructs used to express PntP1, NLS-EnR-Ets, NLS-Ets fragment only, or NLS-EnR fragment only, respectively.
(B-B’) VNCs of wild type brains contain only Ase⁺ Dpn⁺ type I NBs which directly produce Ase⁺ Dpn⁻ GMCs.

(C-C’) Misexpressing UAS-pntP1 in type I NBs represses Ase expression and induces Ase⁺ Dpn⁺ mINP-like cells.

(D-F’) Misexpressing UAS-NLS-EnR-Ets (D-D’), UAS-NLS-Ets (E-E’) or UAS-NLS-EnR (F-F’) in type I NBs does not repress Ase expression or induce any mINP-like cells.

(G-H’) A wild type (G-G’) or UAS-pntP1 overexpressing lobe (H-H’) has eight type II NB lineages, each of which contains a largest Ase⁻ Dpn⁺ type II NB and smaller Ase⁺ Dpn⁻ mINPs.

(I-I’) Type II NBs with UAS-NLS-EnR-Ets misexpression ectopically expresses Ase and directly produces Ase⁺ Dpn⁻ GMCs other than INPs. Note that UAS-NLS-EnR-Ets misexpression also leads to the generation of supernumerary GFP⁺ type II NB lineages (see all dashed lines).

(J-K’) Misexpressing UAS-NLS-Ets or UAS-NLS-EnR does not affect the normal development of type II NB lineages, including the repression of Ase, the number of type II NBs, or the composition of cell types.

(L-L’) Knocking down PntP1 by expressing UAS-pnt RNAi results in ectopic Ase expression in type II NBs, loss of INPs and generation of supernumerary GFP⁺ type II NBs.

(M-N) Quantifications of percentage of Ase⁻ type I NBs or percentage of type I NB lineage with mINPs in VNCs (M), and percentage of Ase⁺ type II NBs, percentage of type II NB lineage with mINPs or number of total GFP⁺ type II NBs per brain lobe (N) with indicated genotypes. ***, P < 0.001. The number on top of, or within each bar in graphs of this figure and all following figures indicates the number of samples examined.
Figure II-2. PntP1 functions as a transcriptional activator to repress Ase expression and promote INP generation.

In all images, type I NB lineages in VNCs are labeled with mCD8-GFP (in green) driven by insc-GAL4, and counterstained with anti-Dpn (in red) or anti-Ase (in blue) antibodies. White arrows point to those example type I NBs in which Ase is repressed. White arrowheads point to induced Ase+ Dpn+ mINP-like cells and yellow arrowheads point to Ase+ Dpn- GMCs. Scale bars equal 50µm.

(A) Schematic diagrams show indicated constructs used to express PntP1, or sub-fragments of PntP1 with or without being fused with VP16AD domain, respectively.

(B-B') VNCs of wild type brains contain only Ase+ Dpn+ type I NBs (appearing magenta) which directly produce Ase+ Dpn- GMCs (appearing blue).

(C-C') Misexpressing UAS-pntP1 in type I NBs represses Ase expression and induces Ase+ Dpn+ mINP-like cells.
(D-F') Misexpressing chimeric activators of NLS-VP16AD-Ets (D-D'), NLS-pntP1(1/2N)-VP16AD-Ets (E-E') or NLS-VP16AD-pntP1(1/2N)-Ets (F-F') does not suppress Ase expression in type I NBs.

(G-H') Misexpressing the chimeric activator of NLS-VP16AD-pntP1(1/2C)-Ets represses Ase expression and induces Ase’ Dpn’ mINP-like cells (G-G’), while misexpressing NLS-pntP1(1/2C)-Ets does not (H-H’).

(I) Percentage of type I NBs with Ase being suppressed and with mINPs in VNCs with or without indicated artificial fusion proteins being expressed. ***, P < 0.001.

(J) Summary of phenotypes resulting from the expression of PntP1, or indicated chimeric proteins in type I NB or type II NB lineages.

(K) A diagram of functional domains of the PntP1 protein based on phenotypic analyses of the chimeric activator proteins. The potential activation domain is likely localized in the aa.1-255 region of PntP1, while the region of aa.256-511 is probably necessary for PntP1’s activity, potentially involved in recruiting co-factors, and the Ets domain (aa.512-597) is required for binding to target DNAs.

**Identification of cis-elements mediating the suppression of ase in type II NBs**

Next, we hypothesized that a transcriptional repressor acts downstream of PntP1 to suppress Ase through directly binding to ase enhancer in type II NBs. To identify such a repressor, we planned to take a bottom-up approach by mapping cis-elements in the ase regulatory region that mediate ase suppression in type II NBs so that the cis-elements could be used to identify the transcriptional repressor using biochemical approaches or other genomic approaches. To identify the cis-element, we adopted a well-established enhancer-activity-assay (Pfeiffer et al., 2008) by inserting the ase enhancer fragment upstream of the Drosophila synthetic core promoter (DSCP), which drives GAL4 transcription in pBPGUW vector. The expression pattern of the GAL4 reporter lines, which are named pDes-(ase)enhancer-GAL4, will then be examined by driving the expression of UAS-mCD8-GFP (Fig. II-3A).
Previous studies have shown that two overlapping *ase* enhancer segments, the *F:2.0* fragment (Jarman *et al.*, 1993) and the *GMR20B05* fragment (Pfeiffer *et al.*, 2008), activate reporter expression only in type I NBs but not type II NBs (data not shown), suggesting that the overlapping region contains the *cis*-repressive element. The overlapping region is a 675-base pair (bp) fragment located at -1,734bp to -1,060bp from the transcription start site (TSS) of *ase* (Fig. II-3A). Indeed, a GAL4 transgene under the control of this 675bp enhancer segment is expressed in all type I NBs but not any type II NBs (Fig. II-3B and W). To map the *cis*-repressive element, we then made a series of deletions of the 675bp fragment and fused these smaller fragments with the GAL4 transgene. By examining expression of these GAL4 reporter lines, we found that deleting the region from -1,295bp to -1,280bp led to activation of the GAL4 in all type II NBs (Fig. II-3B-H and W), indicating that this 16bp region contains the *cis*-repressive element. Further, our GAL4 reporter lines also showed that deleting the region from -1,138bp to -1,086bp resulted in a complete loss of GAL4 expression in all NBs in the larval brains (Fig. II-3I-W), indicating that this region contains a *cis*-active element that is essential for activating *ase* expression in all NBs.
Figure II-3. Identification of cis-elements mediating the repression and activation of Ase expression.

(A) A diagram showing the strategy of the enhancer-activity-assay used to determine the activity of test ase enhancer fragments in type I NBs or in type II NBs. Distinct test ase enhancer fragments of interests are amplified and cloned to upstream of Drosophila synthetic core promoter (DSCP)-GAL4 cassette in pBPGUW vector and the resulted pDES-(ase)enhancer-
GAL4 is integrated into *Drosophila* genome for the combination with UAS-mCD8-GFP reporter to determine the transcriptional activity of corresponding test *ase* enhancer fragments. Fragments used for identifying potential cis-elements are indicated as blue strips and named according to their positions relative to *ase* transcription start site (TSS).

(A1-A3) Schematic diagrams of possible mechanisms of how Ase is activated in type I NBs (A1) and two possible mechanisms of how Ase is suppressed in type II NBs (A2 or A3) which can be distinguished by the enhancer-activity-assay as shown in (A).

(B-G) Determining the activity of indicated test *ase* enhancer cloned to upstream of *DSCP-GAL4* by driving mCD8-GFP expression in type I NBs (Ase+ Dpn+, appearing magenta) or type II NBs (Ase- Dpn+, appearing red). Test *ase* enhancer of TSS (-1734 ~ -1060) (B) or TSS (-1295 ~ -1060) (C) activates mCD8-GFP expression in all type I NBs but not in any type II NBs, whose lineages are highlighted with white dashed lines. Test *ase* enhancer of TSS (-1287 ~ -1060) (D) or TSS (-1282 ~ -1060) (E) activates mCD8-GFP expression in all type I NBs and in a subset of type II NBs, whose derived lineages are highlighted with yellow dashed lines, while the rest of type II NB lineages have no mCD8-GFP expression (highlighted with white dashed lines). Test *ase* enhancer of TSS (-1279 ~ -1060) (F) or TSS (-1276 ~ -1060) (G) activates mCD8-GFP expression in all type I NBs and in all type II NBs, whose derived lineages are highlighted with yellow dashed lines.

(H) Quantifications of percentage of type II NBs without mCD8-GFP expression when indicated test *ase* enhancer was used.

(I-V) Determining the activity of indicated test *ase* enhancer which is cloned to upstream of *DSCP-GAL4* by driving mCD8-GFP in type I NBs (Ase+ Dpn+, appearing magenta) or type II NBs (Ase- Dpn+, appearing red). Upper panels show dorsal brains and bottom panels show VNCs. Test *ase* enhancer of TSS (-1212 ~ -1060) (I, J), TSS (-1212 ~ -1086) (O, P), TSS (-1180 ~ -1060) (Q, R) or TSS (-1138 ~ -1060) (S, T) activates mCD8-GFP expression in all type I NBs and type II NBs, while test *ase* enhancer of TSS (-1212 ~ -1166) (K, L), TSS (-1212 ~ -1138) (M, N) or TSS (-1087 ~ -1060) (U, V) does not activate mCD8-GFP reporter expression in any type I NBs in VNCs, or in the majority of type I NBs in dorsal brains.

Scale bars equal 50µm.

(W) Summary of the expression of mCD8-GFP driven by the *pDes-(ase)enhancer-GAL4* constructs with indicated *ase* enhancer fragments in type I NBs or type II NBs.

**Tll suppresses Ase expression directly by binding to *ase* enhancer**
Based on the sequence of the mapped 16bp region that contains the repressive element, then we predicted potential binding transcription factors using a combination of the programs MEME (Mutiple Em for Motif Elicitation, https://meme-suite.org/meme/tools/meme) (Timothy & Elkan, 1994) and Tomtom (https://meme-suite.org/meme/tools/tomtom) (Gupta et al, 2007). We identified a total of 83 candidates that could potentially bind to the 16bp sequence, including the transcriptional repressor Tailless (Tll), which has been shown recently to repress Ase expression in type II NBs (Hakes & Brand, 2020). Therefore, we next asked whether Tll binds to the 16bp repressive element we mapped from the ase regulatory region. To this end, we first compared the suppression of Ase in type I NBs by the expression of UAS-Tll driven by pDes-(ase)enhancer-GAL4 reporter lines with or without the 16pb cis-repressive element. The underlying rational is that if Tll suppresses Ase expression through interacting with the repressive element, misexpressing Tll driven by pDes-(ase)enhancer-GAL4 containing the repressive element would lead to attenuated GAL4 expression and subsequent weaken suppression of Ase (Fig. II-4H) due to negative-feedback inhibition of GAL4 expression by Tll, while using pDes-(ase)enhancer-GAL4 lines that do not contain the repressive element would result in stronger GAL4 expression and subsequently stronger suppression of Ase expression (Fig. II-4I) for the lack of suppression of GAL4 by Tll. Indeed, our results showed that expression of UAS-Tll driven by two pDes-(ase)enhancer-GAL4 lines containing the repressive element resulted in Ase suppression only in less than 3% of type I NBs (Fig. II-4A-B’, E and Fig. II-51A-D) and very subtle overproliferation of type I NBs (Fig. II-4A-B’, F and Fig. II-51A-D). However, misexpression of UAS-Tll using two pDes-(ase)enhancer-GAL4 lines that do not contain the repressive element led to loss of Ase in more than 40% of type I NBs (Fig. II-4C-D’ and E) and a drastic increase in the number of NBs, which often clustered together, in the VNC (Fig. II-4C-D’ and F). Furthermore, the expression of UAS-mCD8-GFP driven by pDes-(ase)enhancer-GAL4 lines containing the repressive element was much weaker than that driven by pDes-(ase)enhancer-GAL4 lines that do
not contain the repressive element (Fig. II-4A1-D1 and G). Since all the pDes-(ase)enhancer-GAL4 transgenes were inserted in the same genome locus, the difference of the expression of mCD8-GFP and suppression of Ase are unlikely due to positional effects of these GAL4 transgenes (Fig. II-4G). Therefore, these data support that Tll could potentially bind to the 16bp repressive element we mapped. Indeed, when we searched potential Tll bind sites in the 675bp region from -1,734bp to -1,060bp using the software FIMO (Find Individual Motif Occurrences) (Grant et al., 2011), we identified two potential Tll binding motifs that are adjacent to each other with a space of 11nt in between and have sequences similar to the consensus Tll binding sequence 5’-AAGTCA half-site (Noyes et al., 2008). One motif (5’-CGTCGTCAAA, named as “Tll_site_L” here) is located from -1,292bp to -1,283bp, which is within the 16bp repressive element we mapped. The other (5’-CCGAGTCAAA, named as “Tll_site_R”) is located further downstream, from -1,271bp to -1,262bp (Fig. II-5A). We further examined if Tll_site_R was required to suppress reporter expression in type II NBs. To this end, we generated a pDes(ase)enhancer-GAL4 driver which contains ase enhancer region from -1,295 to -1,060 containing wild type Tll_site_L but mutations in Tll_site_R and used it to drive the expression of UAS-mCD8-GFP. We found that mCD8-GFP was indeed activated by this driver not only in type I NBs but also in type II NBs (Fig. II-5A’), indicating that Tll_site_R in ase enhancer is essential for the suppression activity in type II NBs.

To determine if Tll binds to these two motifs, we then performed electrophoretic mobility shift assays (EMSAs). We first examined if DNA fragments containing either one or both of these motifs could compete with a 25bp DNA fragment containing the canonical AAGTCA Tll-binding core site from krüppel (kr) enhancer, which has been demonstrated to bind strongly to Tll (Ruth et al., 1994), for the binding with Tll. We expressed Xpress-tagged Tll in vitro for EMSAs and the DNA fragment with the canonical Tll binding core site was labeled with Cy5 at the 5’-end and
used as a probe (probe 1) (Fig. II-5A). We verified the specific binding between Xpress-Tll protein and Cy5-probe1 (Fig. II-5B, lane #3), by the specific competition by the cold competitor (competitor 1) containing the same sequence as Cy5-probe1 (Fig. II-5B, lane #4) and the presence of a supershifted band when anti-Xpress antibody was present (Fig. II-5B, lane #9). Then we tested competition with DNA fragments that contain either one (competitors 4, 5, 6, and 8) or both (competitors 2 and 10) of the predicted binding motifs. As negative controls, we also used DNA fragments that didn’t contain a complete predicted motif (competitor 3) or contained mutations in one (competitors 7, 9, 11, and 12) or both (competitor 13) of the motifs, for the competition. Our results showed that all DNA fragments (competitors 2, 4, 5, 6, 8, and 10) that contain either one or both of the motif could successfully compete with probe 1 in a dose-dependent manner (Fig. II-5B, lanes #6-8, #10-18, #19, #21, and #23), whereas all the DNA fragments (competitors 3, 7, 9, and 13) that didn’t contain either of the wild type motifs showed no competition at all (Fig. II-5B, lanes #5, #20, #22, #26). However, the DNA fragments that contain the wild type Tll_site_L motif alone (competitors 4, 6, and 12) (Fig. II-5B, lanes #6, #10-12, and #25) could not compete as strongly as DNA fragments that contains the wild type Tll_site_R motif alone (competitors 5, 8, and 11) (Fig. II-5B, lanes #7, #13-15, and #24) or both motifs (competitors 2 and 10) (Fig. II-5B, lanes #8, #16-18, and #23), strongly suggesting that the Tll_site_L motif has lower affinity with Tll than the Tll_site_R motif.

In order to directly visualize the binding of the predicted motifs with Xpress-Tll, we also synthesized Cy5-labeled probes containing either one (probe 2 and 3) or both of the predicted motifs (probe 4) for EMSAs. Consistent with the above competition results, we were able to detect binding of probe 3 and 4 with Xpress-Tll (Fig. II-5D-E, lanes #29 and #41), which could be completely competed by cold competitors (competitor 8 and 10) with the same sequences (Fig. II-5D-E, lanes #30 and #42) or the competitor 1 that carried the canonical Tll binding motif from
kr (Fig. II-5E, lane #46), but not by the competitors that carried mutations in the corresponding motifs (competitors 9 and 13) (Fig. II-5D-E, lanes #31 and #45). Furthermore, we observed that the binding of Xpress-Tll with either probe3 or probe4 generated two separate bands when we run the gels for a long time (Fig. II-5F, lanes #47 and 48), suggesting the Tll can bind to the motifs either as a monomer or homodimer as reported previously (Benod et al, 2014; Qu et al, 2010; Ruth et al., 1994; Ruth & Umesono, 1996). However, we could not detect direct binding of probe 2 that carried the Tll_site_L motif alone ((Fig. II-5C, lane #27) likely due to its relatively low affinity with Tll. In any event, the binding of probe 3 with Xpress-Tll could be successfully competed by DNA fragments that contains the wild type (competitor 6) but not mutated (competitor 7) sequence of the Tll_site_L motif in dose-dependent manner (Fig. II-5D, lanes #32-37). The binding of probe 4 with Xpress-Tll could also be partially competed by competitor 12 that carried a wild type Tll_site_L motif and a mutated Tll_site_R motif (Fig. II-5E, lane #44), although the competition was not as strong as the competitor 11 that carried a mutated Tll_site_L motif and a wild type Tll_site_R motif (Fig. II-5E, lane #43).

Taken together, all the EMSA results demonstrate that the both predicted Tll binding motifs can bind to Tll, but the Tll_site_R motif has higher affinity than the Tll_site_L motif.
Figure II-4. *UAS-Tll* expression driven by *pDes-(ase)enhancer-GAL4* is compromised when the repressive element is included in the GAL4 promoter.
In images (A-D) type I NBs are labeled with mCD8-GFP (in green) driven by pDes-(ase)enhancer-GAL4 drivers containing indicated test ase enhancer fragments, and counterstained with anti-Ase (in red) or anti-Dpn (in blue) antibodies. In images (A1-D1), type I NBs are visualized by live imaging for mCD8-GFP. Scale bars equal 10µm.

(A-B') Expressing UAS-Tll driven by pDes-(ase)enhancer-GAL4 containing the test ase enhancer of TSS (-1734 ~ -1060) (A-A') or TSS (-1295 ~ -1060) (B-B'), which contains both repressive and active elements, leads to the suppression of Ase in a very few type I NBs (open arrows). The majority of type I NBs are still Ase+ (white arrows).

(C-D') Expressing UAS-Tll driven by pDes-(ase)enhancer-GAL4 containing the test ase enhancer of TSS (-1279 ~ -1060) (C-C') or TSS (-1212 ~ -1060) (D-D'), which contains active elements only, leads to the suppression of Ase in an average of 42% (C-C') or 57% (D-D') of type I NBs (open arrows) and the formation of supernumerary type I NB clusters (dashed lines).

(A1-D1) Live mCD8-GFP is expressed at low levels in type I NBs which are still Ase+ when UAS-Tll is expressed driven by pDes-(ase)enhancer-GAL4 containing the test ase enhancer of TSS (−1734 ~ -1060) (A1) or TSS (−1295 ~ -1060) (B1), while mCD8-GFP is drastically boosted in Ase− type I NBs when UAS-Tll is expressed driven by pDes-(ase)enhancer-GAL4 containing the test ase enhancer of TSS (−1279 ~ -1060) (C1) or TSS (−1212 ~ -1060) (D1).

(E-G) Quantifications of percentage of Ase+ type I NBs in VNCs (E), number of total type I NBs per VNC (F), or mCD8-GFP intensity of type I NBs (G) with indicated genotypes. The average mCD8-GFP expression driven by the pDes-(ase)enhancer-GAL4 with the fragment -1,734 ~ -1,060 bps in both the WT and UAS-Tll groups is normalized to 1. *** , P < 0.001. NS, no significance.

(H-I) Schematic diagrams show how the suppression of Ase by Tll is compromised (H) in type I NBs corresponding to (A-B1), or maintained (I) in type I NBs corresponding to (C-D1). pDes-(ase)enhancer-GAL4 first drives the expression of Tll. The resulted exogenous Tll or its possible downstream effectors (if any) bind the repressive ase enhancer region in the driver of pDes-(ase)enhancer-GAL4 and attenuates the subsequent GAL4 expression, leading to attenuated mCD8-GFP expression and Tll levels which are not adequate to suppress Ase efficiently (H). However, pDes-(ase)enhancer-GAL4 drivers containing only active ase enhancer regions drive stable GAL4 expression and subsequently constitutive mCD8-GFP or Tll expression, which does not inhibit the GAL4 expression and in turn efficiently suppresses Ase (I).
Figure II-5. Till protein directly binds to ase regulatory regions.
Open arrowheads point to free Cy5 labeled probes at the bottom of gels, while solid arrowheads point to the lagged bands of complex of Tll-probes or supershifted bands of complex of Xpress Ab-Tll-probes. Bottom numbers under corresponding lanes indicate the ratio of competitors to probes.

(A) The sequence of Cy5 labeled probes and distinct competitors used in EMSAs are shown as indicated. Cy5-probe1 contains sequence of a kr enhancer sub-fragment, which contains the underlined hexameric 5’-AAGTCA half-site core sequence which has been previously confirmed to be directly bound by Tll protein. Green square brackets indicate the positions related to ase TSS. Boxed sequences indicate the TSS (-1295 to -1280) region which we have mapped and shows the activity to suppress reporter expression in type II NBs. Sequences in green indicate two potential Tll binding sites, Tll_site_L and Tll_site_R, respectively. Underlined sequences in green show the core half-site of DNA binding motif of Tll in ase enhancer. Cy5-probe2 or Cy5-probe3 contains Tll_site_L or Tll_site_R, respectively. Cy5-probe4 contains both Tll_site_L or Tll_site_R. The mutated nucleotides within or adjacent to Tll_site_L and/or Tll-site_R in competitors 7, 9, and 11-13 appear red. (A’) When Tll_site_R is mutated in the ase enhancer fragment TSS (-1295 ~ -1060), the derived fragment was used to generate corresponding p(Des)enhancer-GAL4 driver which can activate mCD8-GFP expression in both type I NB lineages and type II NB (white arrows) lineages.

(B) The bindings between Xpress tagged Tll protein and Cy5-probe1, and the competitive bindings between Tll and indicated competitors. Specific binding between Tll and Cy5-probe1 is detected in lane #3 and verified in lane #9 indicated by a supershifted band when the antibody against Xpress is present. Specific competition is detected between Tll protein and Tll_site_L (lanes #6, #10-12, and #19) but not between Tll protein and mutated Tll_site_L (lane #20). Specific competition is also detected between Tll protein and Tll_site_R (lanes #7, #13-15, #21), but not between Tll proteins and mutated Tll_site_R (lane #22). Although the competition is not obviously enhanced when both Tll_site_L and Tll_site_R are present (lanes #8 and #16-18), or the competition is not obviously decreased when Tll_site_L is mutated alone (lane #24) compared to that when only Tll_site_R is present (lanes #7 and #13-15), mutations in Tll_site_R leads to a decreased competition (by comparing lanes #25 Vs #24) and mutations in both Tll_site_L and Tll_site_R further decrease the competition (by comparing lanes #26 Vs #25).

(C) No obvious binding is detected between Tll and Cy5-Probe2 (lane #27), which contains Tll_site_L, possibly due to the binding below the detection threshold.

(D) The bindings between Tll and Cy5-Probe3, and competitive bindings between Tll and indicated competitors. Lane #29 shows the band of Tll-Cy5-probe3, which is specifically competed by competitor 8 containing wild type Tll_site_R (lane #30) but not by competitor 9 containing mutated Tll_site_R (lane #31). Lane #38 shows the supershifted band of Xpress-Ab-Tll-Cy5-probe3. Competition is detected between Tll and competitor 6 containing Tll_site_L while
the competition is largely lost between Tll and competitor 7 containing mutated Tll_site_L (by comparing lanes #32 Vs 33, #34 Vs #35, #36 Vs #37).

(E) Cy5-probe4 which contains both sites of Tll_site_L and Tll_site_R is specifically bound by Tll protein (lane #41). Competitor 10 with the same sequence as Cy5-probe4 (lane #42), specific competitor 1 (lane #46), or competitor 11 containing mutated Tll_site_L and wild type Tll_site_R (lane #43) completely competes with Cy5-probe4 for binding to Tll protein. Competitor 12 containing wild type Tll_site_L and mutated Tll_site_R (lane #44) weakly competes with the probe, and the competition is further reduced when both Tll_site_L and Tll_site_R are mutated in competitor 13 (lane #45).

(F) Electrophoretic separation for a longer time reveals two bands of indicated probes which are bound by Tll.

**PntP1 suppresses Ase expression in type II NBs by activating Tll**

We then asked whether Tll is a downstream target of PntP1 in type II NB lineages. We first examined if PntP1 was necessary for Tll expression in type II NBs. We knocked down PntP1 in type II NBs and examined Tll expression using a Tll-EGFP reporter line, which carries an EGFP coding sequence in the Tll locus and expresses Tll-EGFP fusion proteins (Hakes & Brand, 2020; Venken et al., 2009). Tll-EGFP is robustly expressed in wild type type II NBs (Fig. II-6A-A” and I). However, when PntP1 was knocked down in type II NBs, Tll-EGFP expression was drastically reduced by 80% on average and Ase was ectopically turned on in an average of 90% of type II NBs (Fig. II-6B-B”, I and J). Restoring Tll levels by expressing UAS-Tll in PntP1 knockdown type II NBs led to the suppression of Ase (Fig. II-6C-D”, J and Fig. II-S2A-E). Furthermore, co-expression of UAS-pnt RNAi and UAS-Tll induced more supernumerary type II NBs than the expression of either one of them (Fig. II-6B-D” and K) probably because both knockdown of PntP1 and overexpression of Tll would promote dedifferentiation of imINPs into type II NBs (Fig. II-S2A-E) as previously reported (Hakes & Brand, 2020; Rives-Quinto et al., 2020; Xie et al, 2016). These results demonstrate that PntP1 is necessary for Tll expression in type II NBs and Tll acts downstream of PntP1 to repress Ase expression in type II NBs.
Next, we examined if PntP1 is sufficient to activate Tll expression. We misexpressed *UAS-PntP1* in type I NBs using *insc-GAL4* as a driver and examined Tll expression using the Tll-EGFP reporter line. In wild type animals, Tll-EGFP is weakly expressed in a small subset of type I NBs in the VNC (Fig. II-6E-E” and L). When PntP1 was misexpressed in type I NBs, it dramatically increased Tll-EGFP expression by about 14-fold on average in all type I NBs in the VNC, and the expression of Tll-EGFP protein also persisted their progeny. Consistently, Ase expression was abolished in about 90% of type I NBs in the VNC (Fig. II-6F-F”, and L-M). In order to determine if the loss of Ase expression was due to activation/increase in Tll expression, we simultaneously knocked down Tll while misexpressing PntP1 in type I NBs. We found that Ase expression was restored in an average of 93% of type I NBs (Fig. II-6G-H” and M). These results suggest that misexpressing PntP1 is sufficient to activate the expression of Tll, which in turn suppresses Ase expression, in type I NBs.
Figure II-6. TII is the downstream effector of PntP1 that represses Ase expression in type II NBs.

In all images, white arrows and open arrows point to some type I NBs or type II NBs as examples. Dashed lines highlight some type I NB or type II NB lineages as examples. White arrowheads
point to some Ase+ Dpn+ mINP-like cells and yellow arrowheads point to some Ase+ Dpn+ GMCs as examples. Scale bars equal 10µm.

(A-B") Type II NB lineages are labeled with mCD8-RFP (in green) driven by \textit{pntP1-GAL4} and counterstained with anti-Ase (in red), anti-Dpn (in blue) and anti-GFP (in white) antibodies. Tll is specifically expressed in wild type type II NBs which are Ase- (A-A"), while knockdown of PntP1 by expressing \textit{UAS-pnt RNAi} leads to the abolishment of Tll expression and ectopic Ase expression in type II NBs (B-B").

(C-D") Type II NB lineages are labeled with mCD8-GFP (in green) driven by \textit{pntP1-GAL4} and counterstained with anti-Ase (in red) and anti-Dpn (in blue) antibodies. Expressing \textit{UAS-Tll} in type II NBs results in dramatic generation of supernumerary type II NBs which are Ase- (C-C"), and knockdown of PntP1 by expressing \textit{UAS-pnt RNAi} does not turn on Ase expression in the presence of restored Tll (D-D").

(E-F") \textit{UAS-transgenes} are expressed driven by \textit{insc-GAL4}. Type I NB lineages are labeled with Phalloidin (in green) and counterstained with anti-Ase (in red), anti-Dpn (in blue) or anti-GFP (in white) antibodies. Type I NBs are Ase positive and have no Tll-GFP expression (open arrows) or low expression levels (white arrows) in VNCs of wild type brains at late 3\textsuperscript{rd} instar larval stage (E-E"), while misexpressing \textit{UAS-PntP1} drastically activates ectopic Tll-GFP expression in all type I NBs and represses Ase (F-F").

(G-H") Type I NB lineages are labeled with mCD8-GFP (in green) driven by \textit{insc-GAL4} and counterstained with anti-Ase (in red) or anti-Dpn (in blue) antibodies. Suppression of Ase in type I NBs and generation of Ase+ Dpn+ mINP-like cells resulting from misexpressing \textit{UAS-PntP1} (G-G") are inhibited by reducing Tll levels through expressing \textit{UAS-Tll RNAi} (H-H").

(I-K) Quantifications of Tll-GFP intensity in type II NBs with or without expressing \textit{UAS-pnt RNAi} (I), percentage of Ase+ type II NBs in brain lobes (J), or number of GFP+ type II NBs per lobe (K) with indicated genotypes. ***, $P < 0.001$. NS, no significance.

(L-M) Quantifications of Tll-GFP intensity in type I NBs with or without \textit{UAS-PntP1} misexpression (L) or quantifications of percentage of Ase+ type I NBs in brains with indicated genotypes (M). ***, $P < 0.001$.

\textbf{PntP1 activates Tll expression directly by binding to its enhancer region}

Next, we wanted to determine if PntP1 transactivates Tll through directly binding to \textit{tll} enhancer. A previous study (Jenett \textit{et al}, 2012) has shown that a 3.1kb enhancer fragment, \textit{R31F04}, located at -4,912 bp to -1,855 bp upstream of the \textit{tll} transcription start site, drives the expression of the
GAL4 transgene specifically in type II NBs, whereas another overlapping fragment R31D09, located at -2,893 bp to -157 bp, does not (Fig. II-7A-B), suggesting that the enhancer region from -4,912 bp to -2,893 bp contains cis-elements required for Tll expression in type II NBs. We used the software FIMO to search PntP1 binding site in this 2 kb enhancer region and found seven putative PntP1 binding sites (sites #1-7) containing the consensus 5’-GGAA/T core sequence (Fig. II-7A).

To determine if PntP1 binds to these putative binding sites, we performed EMSAs and Chromatin Immunoprecipitation (ChIP) in combination with quantitative PCR (qPCR). For EMSAs, we used a previously confirmed DNA fragment containing the sequence bound by PntP1 from the *erm* enhancer R9D11 (Janssens et al., 2017) as a probe (Cy5-probe5) and the DNA fragments containing the individual predicted PntP1 binding sites and their franking sequences as competitors (competitors T1-T7) (Fig. II-7C). We confirmed the binding of the probe with PntP1 by the presence of a band of the Xpress-PntP1-probe complex, which was supershifted by the presence of the anit-Xpress antibody and was fully competed by a competitor containing the same sequence as the probe (competitor P1) (Fig. II-7C-D, lanes #1-3 and 24). Our results showed that all the competitors T1-T7 competed with the probe for binding to PntP1 to various degrees (Fig. II-7C-D, lanes #4-5, 8-10, and 13-14), but the competitors containing mutations in the predicted binding sites (competitors T1Mu-T7Mu) or containing no predicted PntP1 binding sites (competitors neg1 and neg2 and the competitor 1 from the kr regulatory region) did not (lanes #6-7, 11, and 15-23). Based on the extent of competition, the affinity of the predicted binding sites for PntP1 is in the following order from high to low: site #1, site #4, site #6, site #7 > site # 5> site #2 > site #3. Similar EMSA results were also obtained when a DNA fragment with the exact same sequence as competitor T4, which contains the predicted PntP1 binding site 4, was used as a probe (Cy5-probe 6) (Fig. II-7C and E).
In order to examine if PntP1 directly binds the *tll* enhancer region *in vivo*, we performed ChIP-qPCR assays using larval brains enriched with type II NBs due to Brat knockdown and 5 distinct sets of primers flanking the predicted PntP1 binding sites (Fig. II-7A). Consistent with EMSA results, our ChIP-qPCR results showed that the anti-PntP1 antibody could pull down DNAs from the *tll* enhancer region containing the predicted binding sites, but not DNAs from the *tll* enhancer regions (neg #3 and neg #5) that do not contain the predicted PntP1 binding sites or *ase* enhancer regions, the latter of which provides additional evidence that PntP1 doesn’t suppress *ase* expression directly (Fig. II-7F). These ChIP-qPCR results demonstrate that endogenous PntP1 proteins bind to the *tll* enhancer region directly *in vivo.*
Figure II-7. PntP1 directly binds to the \textit{tll} enhancer in type II NBs.

(A) A diagram of putative PntP1 binding sites or negative control sites in \textit{tll} enhancer region of \textit{R31FD04} or \textit{R31D09} are shown. Green square brackets indicate PntP1 binding sites (green strips, sites #1-7) and those sequence lacking of PntP1 binding sites (blue strips, neg#1-2) to be tested by EMSAs. Orange square brackets indicate those loci bound by PntP1 (orange strips, Pnt\textsubscript{13}, Pnt\textsubscript{1}, Pnt\textsubscript{3}, Pnt\textsubscript{4}, Pnt\textsubscript{12}) and negative control loci (magenta strips, neg#3, neg#5) not bound by PntP1 to be tested by ChIP-qPCR.
(B) GAL4 under the control of \( tll \) enhancer subfragment \( R31FD04 \), other than \( R31D09 \), specifically activates mCD8-GFP expression in type II NBs. White arrows point to type II NBs. Scale bars equal 50µm.

(C) Sequences of competitors and probes are illustrated. Green sequences under green arrows indicate putative PntP1 binding motifs and sequences under red arrows indicate corresponding mutated PntP1 binding motifs (mutations in nucleotides appearing red). Underlined sequence in probes indicates the GGAA core sequence of PntP1 binding motifs. The direction of arrows is consistent with the consensus 5'-'GGAA/T-'3' core sequence.

(D) PntP1 (tagged with Xpress) binds Cy5-probe5 which contains PntP1 binding motif sequence in \( erm \) enhancer fragment \( R9D11 \) (lanes #2 and 12). The binding is competitively inhibited to an invisible level by competitor P1 containing the same sequence as Cy5-probe5 (lane #3), competitor T1 containing site #1 (lane #9), competitor T4 containing site #4 (lane #5), competitor T6 containing site #6 (lane #13) and competitor T7 containing site #7 (lane #14), or is partially competed by competitor T5 containing site #5 (lane #4), competitor T3 containing site #3 (lane #8) or competitor T2 containing site #2 (lane #10). When PntP1 binding sites #1-7 are mutated, corresponding competitors T1Mu-T7Mu (lanes #15-16 or #19-23) barely compete with Cy5-probe5 for the bindings to Tll. Negative competitors neg#2 (lanes #6 and #17), neg#1 (lanes #7 and #18) or competitor 1 with the sequence for \( kr \) enhancer (lane #11) does not competitively bind Cy5-probe5. The supershifted band in lane #24 indicates a further retardation on the mobility of the complex of Xpress-Ab-PntP1-Cy5-probe5, when anti-Xpress Ab is present. Open arrowheads point to free probes while solid arrowheads point to bands of PntP1-probe complex or supershifted bands of Xpress-Ab-PntP1-probe complex. Bottom numbers under corresponding lanes indicate the ratio of competitors to probes. The affinity of tested PntP1 binding sites is summarized in the table.

(E) PntP1 protein (tagged with Xpress) binds Cy5-probe6 whose sequence contains PntP1 binding site #4 (lane #28) in \( tll \) enhancer. The supershifted band in lane #25 indicates a further retardation on the mobility of the complex of Xpress-Ab-PntP1-Cy5-probe6, when anti-Xpress Ab is present. Specific competitor T4 (lane #29) which contains the same sequence as Cy5-probe6 or specific competitor P1 (lane #45) completely competes with Cy5-probe6 for the binding to PntP1, while non-specific competitor T4Mu which contains mutated site #4 (lane #30) does not. Competitor T1, T2, T3, T5, T6 or T7 which respectively contains PntP1 binding site #1, 2, 3, 5, 6 or 7 (corresponding to lanes #31, #33, #35, #37, #39, and #41) competes with Cy5-probe6 for the binding to PntP1, while competitor T1Mu, T2Mu, T3Mu, T5Mu, T6Mu or T7Mu which contains mutated binding site #1, 2, 3, 5, 6 or 7 (corresponding to lanes #32, #34, #36, #38, #40, and #42) or non-specific competitor neg#1 (lane #43) or neg#2 (lane #44) does not. Open arrowheads point to free probes while solid arrowheads point to bands of PntP1-probe complex or
supershifted bands of Xpress-Ab-PntP1-probe complex. Bottom numbers under corresponding lanes indicate the ratio of competitors to probes.

(F). ChIP-qPCR results indicate PntP1 was enriched in loci Pnt_13, Pnt_1, Pnt_3, Pnt_4, Pnt_12 which contain corresponding predicted PntP1 binding sites in \textit{tll} enhancer fragment \textit{R31FD04}, but not in negative loci neg#3 or neg#4 in \textit{R31D09} fragment, or the negative locus in \textit{ase} enhancer. The quantification represents the average fold enrichment normalized to IgG controls of three biological replicates. ***, \( P < 0.001 \).

\textbf{Tll is required to maintain PntP1 expression in type II NBs}

Though we have shown that Tll is activated by PntP1, there is evidence showing that ectopic expression of Tll in type I NBs also induces PntP1 expression (Hakes & Brand, 2020), leading us to hypothesize that Tll may provide a positive feedback loop to maintain PntP1 expression in type II NBs. To test this hypothesis, we examined PntP1 expression in Tll knockdown type II NBs by examining the expression of \textit{UAS-mCD8-GFP} driven by \textit{pntP1-GAL4} or by immunostaining of endogenous PntP1 proteins. Since the \textit{pntP1-GAL4} is an enhancer trap line, in which the \textit{GAL4} transgene is inserted at 347 bps upstream of the TSS of \textit{pntP1} (Zhu et al., 2011), the levels of mCD8-GFP driven by \textit{pntP1-GAL4} should reflect the activity of the \textit{pntP1} enhancer/promoter and endogenous PntP1 levels in type II NB lineages. We found that when Tll was knocked down, and the expression of mCD8-GFP in type II NBs were significantly reduced by around 75\% (Fig. II-8A-B’’’). Our immunostaining results also showed that endogenous PntP1 expression was almost abolished in Tll knockdown type II NB lineages (Fig. II-8C-D” and P). Consistent with the reduction of PntP1 expression, type II NB lineages were transformed into type I-like NB lineages as indicated by ectopic Ase expression in the NBs (Fig. II-8B-B” and O) and the loss of Ase\textsuperscript{+} mINPs and imINPs and the expression of \textit{R9D11-mCD8-GFP}, which reflects endogenous Erm expression in the lineages (Fig. II-8H-J”). Therefore, Tll is required for maintaining the expression of PntP1 and there is a positive feedback loop between PntP1 and Tll in type II NBs.
Although we have demonstrated that Tll is the downstream of PntP1 to suppress Ase, it is still possible that PntP1 may repress Ase in parallel with to or even downstream of Tll, considering that PntP1 is abolished when Tll is knocked down. To exclude this possibility, we overexpressed UAS-PntP1 to restore PntP1 levels in Tll knockdown type II NBs and checked if ectopic Ase expression would be inhibited. When UAS-PntP1 was artificially overexpressed in wild type type II NBs driven by pntP1-GAL4, PntP1 levels were elevated by 2.2 folds without affecting normal development of type II NB lineages (Fig. II-8E-E” and P). However, to our surprising, overexpressing UAS-PntP1 construct failed to restore PntP1 protein levels in 76.8% of Tll knockdown type II NBs and Ase is still ectopically turned on (Fig. II-8F-F” and P). We speculated it is partially because that GAL4 levels controlled by pntP1 promoter/enhancer was decreased due to reduced pntP1 enhancer/promoter activity, which is reflected by reduced level of mCD8-GFP (Fig. II-8B”). Nevertheless, PntP1 levels were restored to comparable wild type levels in the rest 23.2% of Tll knockdown type II NBs but failed to suppress Ase (Fig. II-8G-G” and P). These results were in agreement with the conclusion that PntP1 is not parallel to Tll or downstream of Tll when suppressing Ase expression. In addition, the conclusion is also supported by the results of misexpression of Tll with or without knockdown of PntP1 in type I NBs. First, we misexpressed UAS-Tll in type I NBs driven by insc-GAL4 and found that more than 93% of NBs, including those overproliferative ones, are Ase negative and only a small subset of these Ase+ type I NBs have ectopic PntP1 expression (Fig. II-8K-L” and Q). Sconed, we reduced ectopic PntP1 by expressing UAS-pnt RNAi in Tll misexpressing type I NBs and found it did not restore Ase expression caused by Tll misexpression (Fig. II-8M-N” and Q).

All these results together demonstrate that in type II NBs Tll is required to maintain PntP1 levels. However, PntP1 is not the redundant or downstream effector of Tll in the suppression of Ase.
Figure II-8. PntP1 is maintained by Tll in type II NBs.

(A-B'”) Type II NB lineages are labeled with mCD8-GFP (in green) driven by pntP1-GAL4 and counterstained with anti-Ase (in red) or anti-Dpn (in blue) antibodies. White arrows point to type II NBs. In wild type type II NB lineages, Ase in absent in type II NBs which are labeled with mCD8-GFP, whose intensity is normalized as 1 (A-A’”). Knockdown of Tll in type II NBs leads to ectopic Ase expression in type II NBs and a decrease in mCD8-GFP intensity of type II NBs by 75% (B-B’”).

(C-G”) Type II NB lineages are labeled with mCD8-GFP (in green) driven by pntP1-GAL4 and counterstained with anti-PntP1 (in red) or anti-Ase (in blue) antibodies. White arrows point to type II NBs. Wild type type II NBs have PntP1 expression and lack of Ase expression (C-C’”), while knockdown of Tll leads to loss of PntP1 expression and ectopic Ase expression in type II NBs (D-
Artificial expression of PntP1 in type II NBs shows enhanced PntP1 levels (E-E’), whereas PntP1 levels are not restored in 76.8% of Tll knockdown type II NBs when UAS-PntP1 is expressed (F-F’). PntP1 levels which are restored to comparable levels as those in wild type type II NBs in 23.2% of Tll knockdown type II NBs fail to suppress Ase (G-G’).

(H-J’) Type II NB lineages are labeled with mCD8-RFP (in red) driven by pntP1-GAL4 and R9D11(II)-mCD8GFP reflecting the activation of Erm and indicating INPs, and counterstained with anti-RFP (in red) or anti-Ase (in blue) antibodies. R9D11(II)-mCD8-GFP is expressed from Ase⁺ imINPs in wild type type II NB lineages (H-H’). Knocking down Tll in type II NBs by expressing UAS-Tll RNAi (I-I”) or UAS-tll-miRNA[S] (J-J”) results in loss of R9D11(II)-mCD8-GFP expression.

(K-N”) Type I NBs are labeled with mCD8-GFP (in green) driven by insc-GAL4 and counterstained with anti-Dpn and anti-PntP1 or anti-Ase antibodies. PntP1 is never expressed in wild type type I NBs (open arrows) (K-K”) and expressing UAS-Tll is sufficient to induce PntP1 expression in a subset of type I NBs (white arrows) but not in the rest of type I NBs (open arrows) (L-L”). Expressing UAS-Tll suppresses Ase expression and promotes the generation of supernumerary type I NBs (M-M”), and reducing PntP1 by expressing UAS-pnt RNAi does not restore Ase expression or inhibit supernumerary type I NB generation (N-N”).

(O-Q) Quantifications of percentage of Ase⁺ type II NBs with indicated genotypes (O), PntP1 intensity in type II NBs with indicated genotypes (P) and percentage of Ase⁺ type I NBs with indicated genotypes in VNCs (Q). ***, P < 0.001. NS, no significance.

Scale bars equal 10µm in A-J, or 50 µm in K-N.

Discussion

In this study, we dissected the molecular mechanism of how PntP1 suppresses Ase expression to specify the type II NB identity. We demonstrate that PntP1 acts as a transcriptional activator to indirectly suppress Ase expression by activating the expression of Tll, whereas Tll provides a positive feedback to maintain the expression of PntP1 and the type II NB identity (Fig. II-9). We further mapped the cis-elements that mediate the suppression of Ase by Tll and the activation of Tll by PntP1. Thus, our work reveals mechanistic details of PntP1-mediated suppression of Ase expression and specification of type II NBs and identifies a novel direct target of PntP1 in type II NBs.
Figure II-9. A proposed working model of the suppression of Ase expression by PntP1 in type II NBs.

A schematic diagram shows that PntP1 is specifically expressed in type II NBs and directly activates Tll expression. Tll binds two suppression sites in ase enhancer to suppress its transcription but maintains the expression of PntP1 through an unknown pathway.

PntP1 functions as a transcriptional activator in type II NB lineages

In this study, we have demonstrated that PntP1 functions as a transcriptional activator by showing that the artificial chimeric repressor protein EnR-Ets antagonizes the function of endogenous PntP1 proteins when expressed in type II NB lineages and that the artificial chimeric activator proteins VP16AD-pntP1(1/2C)-Ets could functionally mimic endogenous PntP1 protein when expressed in type I NBs. Our results are in line with a previous in vitro study showing that PntP1 substantially activates bacterial chloramphenicol acetyltransferase (CAT) reporter expression under the control of Ets binding sites (O’Neill et al., 1994). Interestingly, our results show that the chimeric protein VP16AD-Ets is not sufficient to functionally mimic endogenous PntP1 proteins even though the Ets domain can bind to PntP1 target DNAs as demonstrated by the antagonization of PntP1’s function by the EnR-Ets protein. Only when the C-terminal sub-fragment (aa. 256 - 511) of PntP1 is included can the chimeric activator protein functionally mimic endogenous PntP1 proteins. The Ets family proteins usually recruit additional cofactors to
activate/repress target gene expression. We think that this C-terminal fragment likely contains protein-proteins interaction domains that are essential to recruit its cofactors. Since neither the pntP1(1/2C)-Ets truncated protein nor the chimeric proteins VP16AD-pntP1(1/2N)-Ets is able to mimic wild type PntP1 protein’s function, it is unlikely that this C-terminal sub-fragment contains the activation domain of PntP1 and the other N-terminal sub-fragment [pntP1(1/2N)] contains the protein-proteins interaction domains instead. Otherwise, with the VP16AD to function as an activation domain and the N-terminal sub-fragment to recruit cofactors, the chimeric VP16AD-pntP1(1/2N)-Ets protein would be able to functionally mimic wild type PntP1 protein. Given that a large number of Ets family transcription factors share highly Ets domains that bind to DNAs containing a consensus 5’-GGAA/T core but have diverse functions and activities in distinct cell types, the C-terminal sub-fragment of PntP1 may recruit cell-type-specific cofactors to regulate the expression of specific target genes in type II NB lineages, such as Erm (Janssens et al., 2017; Zhu et al., 2011) and Tll, as it has been proposed as a general strategy for Ets family proteins to regulate specific target gene expression (Hollenhorst et al., 2011; Wei et al., 2010). Therefore, one interesting direction to further dissect the molecular mechanisms of PntP1-mediated specification of type II NBs could be to map the protein-protein interaction domains of PntP1 and identify the cofactors that are recruited by PntP1 in type II NB lineages.

**Tll is a novel direct target of PntP1 that mediates the suppression of Ase**

PntP1 performs diverse functions in different cell types in type II NB lineages. For example, in type II NBs, PntP1 is required to suppress Ase expression. In newly generated imINPs, PntP1 prevents premature differentiation of INPs, whereas late during imINP development, it promotes INP cell fate commitment and prevents dedifferentiation of imINPs (Xie et al., 2016; Zhu et al., 2011). Therefore, as a transcriptional activator, PntP1 must activate the expression of many
different target genes in type II NB lineages. However, only one direct target of PntP1 has been identified previously, which is Erm (Janssens et al., 2017). Here we identify Tll as another direct target of PntP1 that function primarily in type II NBs to suppress Ase expression. We demonstrate that PntP1 is both necessary and sufficient for Tll expression. We further identify 7 putative binding sites and demonstrate by EMSAs and ChIP-qPCR assays that all these sites can bind to PntP1 both in vitro and in vivo, although individual sites may have different affinity with PntP1. These binding sites may work cooperatively to mediate efficient activation of Tll by PntP1 in type II NBs.

Our work further demonstrate that Tll is the direct target of PntP1 that mediates the suppression of Ase in type II NBs by showing that simultaneous knockdown of Tll essentially blocks the suppression of Ase by mis-expressed PntP1 in type I NBs. By fine mapping the cis-repressive elements in ase regulatory regions, we identified two Tll binding sites located at -1,292bp to -1,262bp upstream from the ase TSS, which is consistent with a recent study showing that Tll binds to a 5kb region upstream of the ase TSS (Hakes & Brand, 2020). Although the core hexameric sequences of these two binding sites are not exactly the same as the typical Tll binding hexamer AAGTCA, our EMSA results demonstrate that Tll can indeed bind either as a monomer to one of the sites or as a homodimer to both sites, the latter of which is common for orphan nuclear receptors (King-Jones & Thummel, 2005).

In addition to PntP1 binding sites, other studies (Larson et al., 2021; Rives-Quinto et al., 2020; Zacharioudaki et al., 2016) report that Suppressor of Hairless (Su(H)), a binding partner of the intracellular domain of Notch, and Zelda (Zld) also bind to the enhancer region of ill, implicating that Notch and Zld could be upstream activator of Tll. However, Su(H) and Zld are not just expressed in type II NBs but also in type I NBs (Reichardt et al., 2018). Su(H) and Zld are
unlikely to be sufficient to activate Tll, but whether PntP1 acts together with Su(H) and Zld to activate Tll in type II NBs is worth further investigation.

However, considering that PntP1 is not only expressed in type II NBs but also has even stronger expression in imINPs, whereas Tll is strongly expressed in type II NBs but only very weekly in imINPs, and that ectopic expression of Tll in imINPs reverts imINPs to type II NBs (Hakes & Brand, 2020; Rives-Quinto et al., 2020), therefore, there must be a mechanism to inhibit the activation of Tll by PntP1 in imINPs. A recent study proposed that Erm and Hamlet (Ham) function sequentially to suppresses Tll expression in imINPs based on 1) decreasing or increasing the copy number of the \( \text{tll} \) gene suppresses or enhances the supernumerary type II NB phenotype in \( \text{ham} \ text{erm} \) double heterozygous mutants; and 2) overexpressing Erm or Ham in type II NBs inhibits the expression of Tll in type II NBs (Rives-Quinto et al., 2020). However, these data could be also explained by changes in the expression of PntP1 as we demonstrated here that there is a positive feedback loop between PntP1 and Tll. For example, the reduction in the Tll expression resulting from Erm overexpression in type II NBs could be due to inhibition of PntP1 by Erm in type II NB as we reported previously rather than direct inhibition of Tll by Erm. Furthermore, Erm and Ham are not expressed in the newly generated imINPs, whereas Tll expression in the newly generated imINPs is already largely suppressed. Therefore, the suppression of Tll in the newly generated imINPs cannot be explained by the inhibition by Erm and Ham. Other mechanisms are likely also involved in suppressing Tll in imINPs.

A positive feedback loop between PntP1 and Tll

Our results not only demonstrate that PntP1 is a direct upstream activator of Tll but also show that Tll is required to maintain PntP1 expression in type II NBs. We show that PntP1 expression
is lost in Tll knockdown type II NBs. Therefore, there is a positive feedback loop between PntP1 and Tll that is essential for maintaining the type II NB identity. Considering that PntP1 misexpression induces Tll expression in all type I NBs and generation of mINPs in a subset of type I NB lineages, whereas Tll misexpression induces PntP1 expression only in a small subset of type I NB lineages and does not induce the generation of mINPs, we think that PntP1 functions as a master regulator of type II NB lineage development and acts upstream of Tll, which in turn suppresses Ase expression in type II NBs. Since Tll mainly functions as a transcriptional repressor (Hakes & Brand, 2020; Morán & Jiménez, 2006) as also demonstrated in this study, it is unlikely that Tll directly activates PntP1 expression. Our previous studies suggest that there might be an unknown feedback signal from INPs that could be required for maintaining PntP1 expression (also see CHAPTER III) (Xie et al., 2014). Thus, one potential explanation for the loss of PntP1 expression in Tll knockdown type II NBs could be the loss of this unknown feedback signal from INPs due to the loss of INPs resulting from the ectopic Ase expression in type II NBs. However, since Tll misexpression is able to induce PntP1 expression albeit only in a very small subset of type I NBs, it is more likely that Tll suppresses the expression of another unknown transcriptional suppressor that is normally suppressed by Tll in type II NBs. When Tll is knocked down, this unknown transcriptional repressor may be turned on in type II NBs to suppress PntP1 expression. Whereas in type I NBs, this unknown repressor may be normally expressed to suppress PntP1 expression and misexpression of Tll may relieve the suppression of PntP1 expression.

**Activation of Ase in type I NBs**

Our GAL4 reporter assays also identified in ase enhancer a ~50bp fragment that is sufficient for activation of Ase expression in both type I and type II NBs. Earlier studies show that the achaete-scute (AS-C) complex proteins together with Daughterlesss (Da) activate Ase expression in NBs
during the initial specification of the NBs at embryonic stages by directly binding to four E-boxes in the 5’-UTR of ase (Culí & Modolell, 1998; Jarman et al., 1993). But how Ase is maintained in NBs once they are specified is not known. Our study identified a distinct enhancer region for activating/maintaining Ase expression in NBs, suggesting that factor(s) other than AS-C proteins may be involved in activating/maintaining Ase expression in NBs after they are specified. Therefore, the lack of Ase expression in type II NBs is not because of the absence of an activation mechanism, but rather this activation mechanism is actively suppressed by Tll. Identification the transcriptional activator(s) involved in activating/maintaining Ase expression after NBs are specified may shed light on the mechanisms regulating the development of type I and type II NB lineages.

**Material and methods**

**Fly stocks**

UAS-mCD8-GFP or UAS-mCD8-RFP lines were used for visualizing type I NB or type II NB lineages in combination with appropriate GAL4 lines, including pntP1-GAL4 (i.e., GAL414-94) (Zhu et al., 2011), insc-GAL4 (Luo et al., 1994), ase(F:2.0)-GAL4 (Brand et al., 1993; Jarman et al., 1993), GMR20B05-GAL4 (#49843, Bloomington Drosophila Stock Center (BDSC)), GMR31F04-GAL4 (#46187, BDSC) and GMR31D09-GAL4 (#49676, BDSC) (Jenett et al., 2012). R9D11(II)-mCD8-GFP line (Zhu et al., 2011) was used as erm report and to label INPs. Tll-EGFP line (#30874, BDSC) was used to examine the expression of Tll. For loss-of-function analysis of Tll or PntP1 by RNAi knockdown, UAS-pnt RNAi (#35038, BDSC), UAS-Tll RNAi (also called UAS-tll-shRNA, #330031, Vienna Drosophila Resource Center (VDRC), a gift from Dr. A. H. Brand), UAS-tll-miRNA[s] (Lin et al, 2009) (a gift from Dr. A. H. Brand) were used. UAS-Tll (#109680, KYOTO Stock Center, a gift from Dr. A. H. Brand) or UAS-PntP1 (Zhu et al., 2011) were used to mis/over-express or restore Tll or PntP1 respectively. Other lines
including UAS-HA-PntP1 (a gift form Dr. C. Y. Lee) (Komori et al, 2014) and UAS-Brat RNAi (#34646, BDSC) were used to expand the pool of type II NBs for ChIP assay.

**UAS-transgene expression**

To express artificial activators/repressors or corresponding control constructs, embryos were collected for 8~10hrs at 25°C and shifted to 30°C right away to boost the efficiency till late 3rd instar stage for phenotypic analysis. To test or map the activity of ase enhancer fragments, lines of pDes-(ase)enhancer-GAL4 were crossed with UAS-mCD8-GFP lines and embryos were collected for 8~10hrs and raised at 25°C till late 3rd instar stage for phenotypic analysis. To knock down PntP1 and/or restore Tll in type II NBs by using pntP1-GAL4, embryos were collected for 8~10hrs at 25°C and shifted to 30°C right away till late 3rd instar stage for phenotypic analysis. To knock down Tll and/or restore PntP1 in type II NBs by using pntP1-GAL4, embryos were collected for 8~10hrs at 25°C, raised for 2 days and then shifted to 30°C for extra 6hrs for dissection. For misexpressing PntP1 or Tll, and/or knocking down Tll or PntP1 in type I NBs by using insc-GAL4, temperature sensitive tub-Gal80ts (#7017, BDSC) was used to avoid the lethality at embryonic stages: embryos were collected for 8~10hrs and raised to hatch at 18°C, then shifted to 30°C right away for 3~4 days to maximize the efficiency. For phenotypic analysis of misexpressing UAS-Tll in type I NBs by distinct pDes-(ase)enhancer-GAL4 drivers or ase(F:2.0)-GAL4, embryos were collected for 8~10hrs at 25°C and shifted to 30°C right away till 3rd instar stage for dissection.

**Immunostaining and confocal microscopy**

_Drosophila_ larvae at desired developmental stages were sacrificed and dissected. Brain lobes along with VNCs were immunostained as previously described (Lee & Luo, 1999) except that the fixation duration was increased to 35 mins. Primary antibodies used were chicken anti-GFP.
(Catalog #GFP-1020, Aves Labs, Tigard, Oregon; 1:500-1000), rabbit anti-DesRed (Catalog #632392, Takara Bio USA, Inc., Mountain View, CA; 1:250), rabbit anti-Dpn (1:500) (a gift from Dr. Y.N. Jan) (Bier et al., 1992), guinea pig anti-Ase (a gift from Dr. Y.N. Jan, 1:5000) (Brand et al., 1993), rabbit anti-PntP1 (a gift of J. B. Skeath; 1:500) (Alvarez, 2003). Secondary antibodies conjugated to Daylight 405 (1:300-400), Daylight 488 (1:100), Cy3 (1:500), Rhodamine Red-X (1:500), Daylight 647 (1:500) or Cy5 (1:500) used for immunostaining are from Jackson ImmunoResearch (West Grove, PA). The F-actin probe of phalloidin labeled with Alexa Fluor 555 (Catalog #A34055, Thermo Fisher Scientific, Waltham, MA) was used to label type I or type II NB lineages. Images were collected using a Carl Zeiss LSM780 confocal microscopy and processed with Adobe Photoshop. Two-tailed student’s T-test was used for statistical analyses. Graphs were generated with Microsoft Excel or GraphPad Prism 8.

Plasmid construction and transgenic fly generation

To generate chimeric repressors with or without Ets domain, the sequence coding SV40-NLS was synthesized (Integrated DNA Technologies, Inc., Coralville, Iowa) and cassettes of repressor domain of Engrailed (aa. 1-298) (EnR) or Ets domain of PntP1 were amplified by CloneAmp HiFi PCR Premix (Catalog# 639298, Takara Bio., Mountain View, CA) from genomic DNAs of UAS-PntP1 line. To generate chimeric activators containing Ets domain, VP16 activation domain were amplified from genomic DNAs of transgenic line with VP16AD, and sub-fragments of PntP1 as shown in Fig. II-2A were amplified from UAS-PntP1 line. The above cassettes were cloned into pUAST in the order as designed in Fig. II-1A or 2A through corresponding restriction sites. Primers or oligos used are listed as below.

SV40NLS_sense: AATTCATGCCAAAAAAAGAAGAGAAAGGTAA
SV40NLS_Antisense: GATCTTACCTTTCTCTTCTTTTTGGCATG
PntPIEts_F: AAATCTCGAGTTCACGGGATCGGGTCCCATT
PntPIEts_R: ACAATTCTAGACTAATCACAGACAAAGCGGTAGACATATCG
The derived constructs were verified by sequencing (Genewiz, South Plainfield, NJ) and injected into *Drosophila* embryos by the Rainbow Transgenic Flies Inc. (Camarillo, CA) or Bestgene Inc (Chino Hills, CA) and the transformants were generated following standard P-element transposon protocols.

The gateway technology was used to generate distinct pDes-(ase)enhancer-GAL4 constructs as shown in Fig. II-3A. Briefly, each *ase* enhancer fragment was amplified by PCR from genomic DNAs and incorporated into pDONOR221 vector by BP reactions to get entry clones. Then LR reactions were performed to transit the *ase* enhancer fragments from entry clones into destination vector pBPGUW to get desired pDes-(ase)enhancer-GAL4 constructs. Primers used to amplify attB flanking *ase* enhancer fragments are listed as below (the underlined are parts of attB1 in forward (F) oligos and attB2 in reverse (R) oligos).

(-1734~1060)_F: GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATCCAGTATGTTTCCACG
(-1295~1060)_F: GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCGTCGTCAAAGTGGGAC
(-1287~1060)_F: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAAAGTGGGACGCAACCGA
(-1282~1060)_F: GGGGACAAGTTTGTACAAAAAAGCAGGCTGTGGGACGCAACCGAGTCAA
For generating pDes-(ase)enhancer-GAL4 containing mutations in Tll_site_R, the primer set of Tll_site_R_Mutant_F (see below sequence) and (-1734~1060)_R were used to amplify the ase fragment containing mutations in Tll_site_R and then the primer set of (-1295~1060)_F and (-1734~1060)_R were used to add attB sites.

**Tll_site_R_Mutant_F:**

ATGCGTCGTCAAAGTGGGACGCAACCtAtctAcATCCTCTAGGACAACAAAGGAGCCGA

Lower cases indicate mutations in Tll_site_R.

All the pDes-(ase)enhancer-GAL4 constructs were integrated into the same attp2 docking sites at 3rd chromosome (line #8622, BDSC) through φ31 integration system by Rainbow Transgenic Flies, Inc. (Camarillo, CA) or Bestgene Inc. (Chino Hills, CA).

**Electrophoretic mobility shift assays (EMSAs)**

EMSAs experiments were performed as previously described (Li et al., 2017). Briefly, Tll or PntP1 coding region was amplified by using CloneAmp HiFi PCR Premix (Catalog# 639298, Takara Bio., Mountain View, CA) form a cDNA library and cloned into pcDNA™3.1/His
expression vectors (Catalog #V38520, Life Technologies Co., Grand Island, NY). Proteins were expressed from the pcDNA™3.1/His construct by the TNT® T7 Quick Coupled Transcription/Translation kit (Catalog #L1170, Promega Co., Madison, WI) according to manufacturer's instructions. Empty pcDNA™3.1/His vectors were used as negative controls. Previously confirmed 25-nt Tll bound sequence in kr enhancer (Ruth et al., 1994) and 27-nt PntP1 bound sequence (Janssens et al., 2017) in erm enhancer fragment R9D11 were chosen as positive probes, which were labeled with Cy5 at the 5'-end of both strands. Other Cy5 labelled probes with sequences in either ase enhancer or till enhancer were shown as indicated in Figs. II-5A and II-7C, respectively. The binding reactions were performed by incubating 0.05 pmol of Cy5-probes with 3µl TNT-T7 expressing product and/or cold competitors in an amount of 50, 200 or 500-fold over Cy5-probes as indicated. All probes and competitors were synthesized by Integrated DNA Technologies (Carolville, IA) and annealed to form dsDNAs. To verify the binding specificity of probe sequence, the competitors containing the same sequence as Cy5-probes were used as specific competitors or anti-Xpress antibody (Catalog #R910-25, Thermo Fisher Scientific, Waltham, MA) was added to the binding reactions for detecting a supershift. To test the bindings between two putative Tll binding sites (Tll_site_L and Tll_site_R) in ase enhancer region and Tll protein, a series of competitors (competitor 2-13) with sequences containing the wild type sites and/or mutated sites of Tll_site_L or Tll_site_R along with the flanking nucleotides were generated and added in the amount as indicated to the binding reactions. To test the bindings between putative PntP1 binding sites in till enhancer R31FD04 and PntP1 protein, a series of competitors with sequences containing the wild type PntP1 binding sites (competitors T1-T7) or mutated sites (competitors T1Mu-T7Mu) along with the flanking nucleotides were generated and added in the amount as indicated to the binding reactions. Two sites in till enhancer (competitors neg#1 and neg#2) and competitor 1 were also used as negative competitors. Proteins were preincubated with competitors for 15 min at room temperature.
followed by 25-min incubation with Cy5-probes. 10μl of EMSA binding reactions were analyzed on 5% nondenaturing polyacrylamide Mini-PROTEAN TBE gels (Catalog #4565015, Bio-Rad Laboratories, Hercules, CA), which were scanned and analyzed with ChemiDoc™ XRS. Sequences of the oligonucleotides in all competitors are summarized in Figs. II-5A and II-7C.

**Chromatin immunoprecipitation and qPCR**

ChIP experiments were performed as described before (Komori et al., 2014). Briefly, *Drosophila* larva with the genotype of UAS-mCD8-GFP, UAS-Dcr2/UAS-HA-PntP1; pntPA-GAL4/UAS-Brat RNAi were raised at 30°C to late 3rd instar stage for dissection and collecting intact brains. Around 100 brains were needed for accumulating around $2 \times 10^6$ supernumerary type II NBs for each single IP reaction. For each repeat of IPs, around 250 brains were collected and fixed in 1.8% formaldehyde solution for 20 min at room temperature. The fixation was ended by adding Glycine to a final concentration of 250mM at room temperature for 5 min. Fixed samples were washed with cold 1X DPBS (Catalog #14190144, Thermo Fisher Scientific) and homogenized in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH8) with proteinase inhibitors (Catalog #78430, Thermo Fisher Scientific) and 1mM AEBSF (Catalog #78431, Thermo Fisher Scientific). In order to fragmentize DNAs to a range of 200-700bps, around 30 brains in 110μl lysis buffer in one microtube-130 AFA Fiber Pre-Slit Snap-Cap (Catalog #520045, Covaris, Inc. Woburn, MA) were sonicated on a Covaris ME220 sonicator (Catalog #500295, Covaris, Inc.) using following settings: 50W Peak incident power, %10 Duty factor, 200 Cycles per burst (Cpb) for 90S and repeated for 3 times with 20S interval. Sonicated lysate was pooled and centrifuged to remove debris, and diluted with ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl pH8, 167 mM NaCl) containing proteinase inhibitors and 1mM AEBSF to 1 brain per 10μl. 1ml diluted lysate were used for each IP and 200ul (20%) of diluted lysate was saved for Input. Samples were pre-cleared with Dynabeads Protein A and Dynabeads Protein G
(Catalog #10001D and #Catalog #10003D, Thermo Fisher Scientific) for 1h at 4°C first and then incubated with rabbit anti-PntP1 antibody (a gift of J. B. Skeath; 1:200) (Alvarez, 2003) or 3µg/µl isotypic rabbit IgG (Catalog #10500C, Thermo Fisher Scientific) overnight at 4°C.

Overnight pre-blocked Dynabeads Protein A and G by 2mg/ml BSA, 0.5mg/ml salmon test DNA (Catalog #D7656, Sigma-Aldrich Co, St. Louis, MO) in ChIP dilution buffer were added for extra 4hrs incubation at 4°C. Beads were then washed three times with low salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris–HCl pH8, 150 mM NaCl), twice with high salt wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris–HCl pH8, 500 mM NaCl), three times with LiCl wash buffer (1% NP40, 1% sodium deoxycholate, 1mM EDTA, 10mM Tris–HCl pH8, 0.25M LiCl), twice with TE buffer (10mM Tris-HCl pH8, 1mM EDTA) and eluted with freshly made elution buffer (1% SDS, 0.1M NaHCO3). Samples were incubated at 65°C for reverting cross-linking of chromatin–protein complex, for degrading RNAs with 100µg/ml RNase A (Catalog # 12091021, Thermo Fisher Scientific) overnight and then treated with 100µg/ml proteinase K at 45°C for 1hr. DNA samples were purified with GeneJET PCR Purification Kit (Catalog #K0701, Thermo Fisher Scientific) following manufacture’s manuals and used for qPCR.

Primer sets targeting PntP1 binding loci in R31F04 fragment were:

- **Pnt_13F**: CCTACCATGGGCGATGAGGT
- **Pnt_13R**: ACTCTTACCGAATTCGCC
- **Pnt_1F**: TGGAAATGGAAGCAGCGACT
- **Pnt_1R**: CCTCCAAGATTTGGGCCACT
- **Pnt_3F**: CCCGCCGTGTAATTATTGGG
- **Pnt_3R**: CCGAGAACGAGATCCACAGG
- **Pnt_4F**: CAAGCCTCTTTGAGTTGACT
- **Pnt_4R**: CCTCCATGGGCGATGAGGT
Primer sets targeting negative loci in R31D09 fragment were:

Neg#3F: CAGAGAGCTGTCCCCACGTT
Neg#3R: TTCTGTCTTGCGGATCAGCG
Neg#5F: GGTATTCAACCCCTGCTGCT
Neg#5R: GCTGCCATTATTCGCGGCTTT

Primer sets targeting negative loci in ase enhancer were:

AseF: CTTGCAGTGCAACGAAAGGC
AseR: CAACGCTTGCTCGTACTGAG

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Competing interests

The authors declare no competing or financial interests
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Figure II-S1. Overexpressing Tll in type I NBs using ase(F:2.0)-GAL4 increases the number of type I NBs but does not suppress Ase expression.

(A-A') All wild type type I NBs are Ase+ in VNCs.

(B-B') Expressing UAS-Tll in type I NBs driven by ase(F:2.0)-GAL4 barely represses Ase expression (B-B'), although supernumerary type I NBs are produced.

(C-D) Quantifications of total number of type I NBs per VNC (C) and percentage of type I NBs which are still Ase positive (D). ***, P < 0.001.
Figure II-S2. Knockdown of PntP1 in Tll overexpressing type II NBs results in generation of supernumerary type II NBs and loss of INPs.

In all images, type II NB lineages are labeled with mCD8-GFP (in green) driven by pntP1-GAL4 and counterstained for Ase (in red) and Dpn (in blue). Arrows or arrowheads point to some NBs or their progenies as examples. Scale bars equal 50µm.

(A) A wild type brain lobe contains eight type II NBs (white arrows) and their associated mINPs (Ase+ Dpn+, white arrowheads) and GMCs (Ase+ Dpn-, yellow arrowheads).

(B) Knockdown of PntP1 by expressing UAS-pnt RNAi results in ectopic Ase expression in type II NBs (white arrows with purple outlines), reduction or depletion of INPs (white arrowheads) and a slight increase in the number of total GFP labeled type II NBs. Yellow arrowheads point to GMCs.

(C) Overexpressing Tll alone promotes the generation of supernumerary type II NBs (white arrows) while mINPs (white arrowheads) are still generated.

(D) Knockdown of PntP1 in type II NBs enhances the generation of supernumerary type II NBs (white arrows) and depletes mINPs when UAS-Tll is simultaneously expressed.

(E) Quantifications of total number of mINPs per brain lobe with indicated genotypes. ***, P < 0.001.
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CHAPTER III. Homeodomain protein Six4 prevents the generation of supernumerary Drosophila type II neuroblasts and regulates the development of intermediate neural progenitors

Running title: Six4 regulating development of type II neuroblasts and intermediate neural progenitors

Key words: intermediate neural progenitors/homeostasis/neuroblasts/Six4/Drosophila


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Contribution Statement

R.C. and S.Z. conceived the idea, designed the project and approaches. R.C. carried out the experiments, collected and analyzed the data, generated figures and wrote and revised the manuscript. Y.H. performed transcription factor screen and identified Six4 gene in regulating type II NB lineage development. S.Z, R.C. and M.C revised the manuscript.
Abstract

Intermediate neural progenitors (INPs) play critical roles in boosting the number and diversity of neurons generated from neural stem cells (NSCs) during development but also could be the origin of brain tumors when their cell fate cannot be specified properly or their proliferation is deregulated. Elucidating how INPs are specified and how their proliferation is controlled are thus critical for understanding the generation of brain complexity and brain tumor formation, but molecular and cellular mechanisms regulating the specification and proliferation of INPs are not fully understood. In this study, we investigated the role of the homeodomain transcription factor Six4 in INP development in *Drosophila* type II neuroblast (NB) lineages, which produce neurons by generating INPs like in mammals. We find that Six4 is specifically expressed in type II NB lineages and knockdown of Six4 leads to supernumerary type II NBs, whereas overexpression of Six4 results in transformation of type II NBs to type I-like NBs and loss of INPs. Six4 may act partial redundantly with or functionally replace Earmuff (Erm) to prevent dedifferentiation of imINPs, by antagonizing type II NB master regulator, Pointed P1 (PntP1). Our data also reveal that Six4 antagonizes PntP1 by not only compromising its activity but also suppressing its expression, likely by forming a trimeric complex with Erm and PntP1. Furthermore, knockdown of Six4 exacerbates the loss of INPs resulting from the loss of PntP1 by enhancing ectopic Prospero expression in imINPs, suggesting that Six4 is also required for preventing premature differentiation of INPs. Taken together, our work identified a novel transcription factor that likely plays important roles in maintaining INP homeostasis.
Introduction

The delicate network in metazoan CNS builds the foundation of their high-level intellelctive activity. The origin underlying the delicacy and complexity during development is neural stem cells (NSCs), which differentiate to directly or indirectly generate varieties of postmitotic neurons and glia (Kriegstein & Alvarez-Buylla, 2009). In order to establish intact neural circuits derived from NSCs, precise transcriptional regulations and coordination of different factors within specific cell types are required to regulate the identity, the polarity, the proliferation and the development potentials of NSCs and their progenies. Abnormal neurogenesis, which compromises above strategies, leads to a variety of neurogenetic disorders such as microcephaly, hemimegalencephaly, focal cortical dysplasia or tumorigenesis. Drosophila NBs have been demonstrated to mostly recapitulate above features in mammals to generate brain complexity, which makes them an ideal model for studying neural stem cell biology (Homem & Knoblich, 2012).

Two types of NBs, type I and type II NBs reside in Drosophila CNS. Type I NBs are specified in the embryonic stage and express neural precursor gene asense (ase) (Brand et al., 1993). These NBs divide asymmetrically to produce ganglion mother cells (GMCs) and GMCs divide once to produce neurons. The composition of one single NB, several GMCs and multiples neurons establishes one type I NB lineage in Drosophila embryonic and larval CNS (Homem & Knoblich, 2012). Type II NBs are distinguished from type I NBs by the lack of Ase expression (Bello et al., 2008; Boone & Doe, 2008; Bowman et al., 2008). An outstanding feature of type II NBs compared to their type I counterpart is that they directly generate immature INPs (imINPs) other than GMCs. ImINPs differentiate to become mature INPs (mINPs) which undergo multiple rounds of asymmetric divisions to self-renew and produce GMCs (Bello et al., 2008; Boone & Doe, 2008; Bowman et al., 2008). By this way, each type II NB lineage contains about 450
neurons on average, much more than an average number of 120 neurons in one type I NB lineage (Bello et al., 2008; Izergina et al., 2009; Wang et al., 2014). In addition to increasing the neuronal output, type II NBs along with INPs are responsible for further increasing the diversity of both neurons and glia, which finally incorporate into functional circuits (Bayraktar et al., 2010; Bayraktar & Doe, 2013).

Regulations on multiple levels engage in boosting neuronal output and diversity. First, type II NBs need to be specified and maintained properly, in which several key factors have been identified. One breakthrough is the identification of PntP1, which is a transcriptional factor (TF) belonging to E26 transformation-specific (Ets) family and is specifically expressed in type II NBs and their derived imINPs (Zhu et al., 2011). Functional analysis shows PntP1 specifies and maintains type II NBs by suppressing Ase expression and promotes generation of INPs (Zhu et al., 2011). The lack of Ase is essential to maintain type II NBs identify, as ectopic expression of Ase in type II NBs eliminates INPs and transforms type II NB lineages to type I NB lineages (Bowman et al., 2008). Self-renewal factor Deadpan (Dpn) promote the self-renewal of type II NBs (San-Juán & Baonza, 2011; Zacharioudaki et al., 2012; Zhu et al., 2012). Loss of Dpn results in a complete absence of type II NBs in 3rd instar larval brains, which is mediated by ectopically turning on Ase and Prospero (Pros) or Fez transcription factor Earmuff (Erm) expression in type II NBs (Li et al., 2017; Zacharioudaki et al., 2012; Zhu et al., 2012). Klumpfuss (Klu) is also essential and sufficient to maintain the identity or self-renewal of type II NBs (Xiao et al., 2012). Activation of Notch signaling in type II NBs is essential for maintenance of type II NBs but independent of Dpn regulation (Song & Lu, 2011; Zhu et al., 2012). Notch also functions to inhibit the PntP1-activated Erm expression, contributing to maintain type II NBs (Li et al., 2017; Li et al., 2016). Trithorax (Trx), a histone methylation related protein, shows its functions in
maintaining the type II NB identity by maintaining an active chromatin state in \textit{pntP1} and \textit{buttonhead (btd)} locus to regulate the transcription (Komori \textit{et al}, 2014a).

Second, INPs need to be generated and avoid premature differentiation or dedifferentiation. Btd functions cooperatively with PntP1 to promote generation of INPs (Xie \textit{et al}, 2014). Loss of PntP1 and/or Btd leads to dramatic reduction or even depletion of mINPs. Ectopic coexpression of PntP1 and Btd induces INP-like cells in almost all type I NB, although PntP1 or Btd alone has limited ability to induce the generation of INP-like cells in type I NB lineages (Komori \textit{et al}. , 2014a; Xie \textit{et al}. , 2014; Zhu \textit{et al}. , 2011). To prevent premature differentiation of INPs, differentiating cell fate determinant Prospero (Pros) needs to be suppressed in Ase- imINPs after asymmetric divisions of type II NBs (Bayraktar \textit{et al}. , 2010; Bello \textit{et al}. , 2008; Boone & Doe, 2008; Bowman \textit{et al}. , 2008). PntP1 and/or Btd is required in imINPs to inhibit ectopic Pros expression (Xie \textit{et al}. , 2016; Xie \textit{et al}. , 2014). Significant progress has been made to elucidate how INPs keep differentiation and avoid dedifferentiation. ImINPs need to turn off the expression of self-renewal factors including Dpn and the Notch downstream targets E(spl) family proteins right after they are born (San-Juán & Baonza, 2011; Song & Lu, 2011; Zacharioudaki \textit{et al}. , 2012; Zhu \textit{et al}. , 2012). The elimination of Dpn expression in imINPs involves Brain tumor (Brat)-mediated degradation of \textit{dpn} mRNA (Komori \textit{et al}. , 2018a; Reichardt \textit{et al}. , 2018) and degradation of Dpn protein by the ubiquitin-proteosome system (Komori \textit{et al}. , 2018a; Zacharioudaki \textit{et al}. , 2012), whereas the suppression of E(spl) proteins expression involves termination of Notch signaling by Numb-mediated degradation of Notch receptor (Haenfler \textit{et al}. , 2012) and prevention of aberrant Notch activation by retromer complex-mediated trafficking of Notch (Li \textit{et al}. , 2018). The underlying mechanism of Brat regulations also involves antagonizing Klu and attenuating β-catenin/Armadillo activity (Xiao \textit{et al}. , 2012), as well as inhibiting Zelda (Zld) through direct binding to their transcripts to decrease the stability of mRNAs (Reichardt \textit{et al}. , 2012).
Numb, another component of the basal complex, has similar functions as Brat to promote the maturation of INPs and prevent them reverting to NBs, via inhibiting Notch signaling (Bowman et al., 2008). Erm likely maintains the developmental potential of imINPs by activating Pros-dependent cell cycle exit (Weng et al., 2010) and prevents INPs from dedifferentiating to NBs by antagonizing Notch signaling or by forming suppressor complex with Brahma (Brm) and Histone deacetylase 3 (HDAC3) (Koe et al., 2014). In addition, Erm restricts imINPs fate commitment by attenuating their competence to respond to self-renewal factors, through a BAP chromatin-remodeling complex dependent way (Janssens et al., 2014).

Components of integrator, including IntS5 and IntS8, prevent dedifferentiation of INPs to NBs, by promoting Erm and Brm expression (Zhang et al., 2019).

*Drosophila* Six4 (D-Six4, or short for Six4 if without specification in this study) is a transcriptional factor belonging to sine oculis homeobox (SIX) family (Seo et al., 1999). Two conserved domains are identified in Six4 proteins: a SIX domain (SD) and a homeodomain (HD) to the C-terminus of SD. Based on phylogenetic analysis of SD and HD in SIX class family proteins, D-Six4 with its murine/human homolog Six4/AREC3 and murine/human Six5 are designated into one subgroup (Kawakami et al., 2000; Kumar, 2009; Seimiya & Gehring, 2000; Seo et al., 1999). The HD and its adjacent extension usually engage in DNA binding (Hu et al., 2008; Kumar, 2009), while the SD engages more in protein-protein interactions (Kumar, 2009; Pignoni et al., 1997). An activation domain of murine Six4/AREC3 is characterized in the C-terminus (Kawakami et al., 1996a; Ohto et al., 1999), while some studies also show zebrafish Six4 with a repressor activity (Kobayashi et al., 2001). Six4 is required for *Drosophila* muscle and gonad development, patterning of the *Drosophila* embryonic mesoderm, and promoting differentiation of early-stage follicle stem cell lineages (Clark et al., 2006; Clark et al., 2007; Johnston et al., 2016; Kirby et al., 2001). Though Six4/AREC3 expression is detected in mouse
retina, the genuine function of Six4 in eye development is not known (Kawakami et al., 1996b; Kawakami et al., 2000; Kumar, 2009; NIIYA et al., 1998; Ohto et al., 2002). Unlike other two SIX family proteins in Drosophila, Optix and Sine oculis (So), which are required in eye development (Pignoni et al., 1997; Seimiya & Gehring, 2000), Six4 is not crucially involved in Drosophila eye or retina formation (Johnston et al., 2016; Kumar, 2009). Though it has been shown that in Drosophila embryogenesis Six4 is expressed in procephalic neurectoderm, from which brain lobes and ventral nerve cords (VNCs) originate (Hwang & Rulifson, 2011; Kirby et al., 2001; Seo et al., 1999), little is known about roles of Six4 in regulating the development of Drosophila NSCs. In this study, we characterized the expression pattern of Six4 in Drosophila larval CNS for the first time and examined its roles in regulating the development of type II NBs and INPs. We also tested the regulatory relationship of Six4 with Erm, PntP1 or Pros, which are key regulators in type II NB lineage progression. Thus, our study reveals novel functions of the SIX family/homeodomain protein Six4 in determining the proper differentiation and proliferation of INPs to maintain their homeostasis.

**Results**

**Six4 is specifically expressed in Drosophila larval type II NB lineages**

Previous studies have shown the expression of Six4 in procephalic neurectoderm during Drosophila embryogenesis, by detecting six4 mRNA using in-situ hybridization or Six4 protein using anti-Six4 antibody (Hwang & Rulifson, 2011; Kirby et al., 2001; Seo et al., 1999). Both mRNAs and proteins of Six4 were detected in the head midline dorsomedial procephalic (Pdm) neurectoderm placode, which generates diverse NB identities, including both type I and type II NBs (Hwang & Rulifson, 2011; Seo et al., 1999). It generates the first question that if the
embryonic expression of Six4 in Pdm placode, the origin of larval NBs, persists to larval NBs. However, using the available antibody against Six4 did not generate valid signal in *Drosophila* larval brains. We examined a Six4 reporter line from model organism Encyclopedia of Regulatory Networks (modERN) project (Kudron *et al.*, 2018). The Six4 reporter (*six4-GFP*) line was generated by fusing a superfolder GFP (sfGFP) tag in-frame to the C-terminus of *six4* in *P[acman]CH322-192H07* BAC clone and integrating into genome through φC31 integrase system (Kudron *et al.*, 2018) (Fig. III-1A). We examined the GFP tagged Six4 protein expression through immunohistochemistry to detect GFP expression from 1st through late 3rd instar larval stages. We found that a consistent Six4-GFP expression pattern in type II NB lineages through all three larval developmental stages (data not shown). In 3rd instar larval brain lobes, all eight type II NB lineages have specific Six4-GFP expression, with the expression in only one lateral type II NB weaker than others (Fig. III-1B-B”, Fig. III-2C and C”). The expression pattern of Six4-GFP is consistent with recent RNAseq data showing that Six4 is enriched in type II NBs (Rives-Quinto *et al.*, 2020; Yang *et al.*, 2016). In each type II NB lineage, Six4-GFP is expressed in type II NB, imINPs and early mINPs, as well as a few early GMCs. The expression of Six4-GFP is the strongest in the NB but becomes progressively weaker in the daughter cells with the increase of their distance from the NB (Fig. III-1B-B” and insets). We also found Six4-GFP proteins were robustly expressed in two type I NB in VNC regions right next to the brain lobe. These two Six4+ type I NBs seems symmetrical to midline. Six4-GFP proteins were also weakly expressed in around another 3~4 type I NB lineages in VNCs. In each brain lobe, around 10~12 type I NBs were Six4-GFP positive, which is a small subset of total type I NBs. All GMCs associated with these GFP+ NBs were also Six4-GFP positive (Fig. III-2B-B”). Although the BAC clone used to generate *six4-GFP* transgenic line contains a ~9.4kb fragment upstream to *six4* transcription start site (TSS), it is still possible that the expression pattern of *Six4-GFP* would not reflect the endogenous Six4 expression in type II NB lineages. To exclude this possibility, we knocked
down Six4 by expressing $UAS$-$Six4\ RNAi^{HM05254}$ construct (corresponding to blooming stock line BL#30510) driven by type II NB lineage-specific $pntP1$-$GAL4$ (Zhu et al., 2011). The knockdown led to almost a complete depletion of Six4-GFP expression specifically in all type II NB lineages, but left the Six4-GFP expression outside type II NB lineages unaffected (Fig. III-1C-C’’). Our results indicate that the Six4-GFP expression mimics the endogenous Six4 expression in type II NB lineages and the $UAS$-$Six4\ RNAi^{HM05254}$ reagent is specific and efficient to use for loss-of-function (LOF) analysis. Unless indicated otherwise, $UAS$-$Six4\ RNAi^{HM05254}$ was used for Six4 knockdown in the rest of the study.

**Knockdown of Six4 results in supernumerary type II NB lineages**

We then knocked down Six4 to investigate the function of Six4 in regulating type II NB lineage progression. In each wild type *Drosophila* brain lobe, there are always 8 type II NB lineages. Each type II NB lineage contains one $\text{Dpn}^+/\text{E(spl)mγ}^+$ (Song & Lu, 2011) but $\text{Ase}^-$ NB, 3-4 $\text{Dpn}^-/\text{E(spl)mγ}^- \text{imINPs}$, 20-30 $\text{Dpn}^+/\text{E(spl)mγ}^+/\text{Ase}^+$ mINPs, several $\text{Dpn}^-/\text{E(spl)mγ}^-/\text{Ase}^+$ GMCs and postmitotic neurons (Bello et al., 2008; Boone & Doe, 2008; Bowman et al., 2008; Song & Lu, 2011; Zhu et al., 2011; Zhu et al., 2012) (Fig. III-1D and N). When Six4 was specifically knocked down by expressing $UAS$-$Six4\ RNAi^{HM05254}$ in type II NBs as well as associated imINPs driven by $pntP1$-$GAL4$, it resulted in an average of 16 type II NBs per brain lobe (Fig. III-1E and N). When we restored Six4 levels by expressing one copy of $\text{six4}$ coding region ($UAS$-$\text{Six4ORF}$) (Bischof et al., 2013), it fully inhibited the generation of supernumerary type II NBs. In order to exclude the possibility that the generation of supernumerary type II NBs was due to off-target effects, we generated and expressed an RNAi-resistant version of Six4 ($UAS$-$\text{Six4HM05254Resis}$) and examined its rescue ability. Indeed, expressing $UAS$-$\text{Six4HM05254Resis}$ repressed supernumerary type II NB generation caused by Six4 knockdown. We also demonstrated that the rescue was not due
to the dilution of *UAS-Six4RNAi* construct, since introducing extra one copy of *UAS-mCD8-RFP* did not affect the generation of supernumerary type II NBs resulting from Six4 knockdown (Fig. III-1F-H and N). In addition, we further verified that the supernumerary type II NB lineages were due to Six4 knockdown, since expressing three other independent *UAS-Six4 RNAi* constructs produced a consistent generation of supernumerary type II NB lineages (Fig. III-1I-K and N). These results indicate Six4 functions in maintaining the homeostasis of type II NB pool by preventing generation of supernumerary type II NB.

![Figure III-1](image)

**Figure III-1.** Six4 is specifically expressed in type II NB lineages and knockdown of Six4 in type II NBs results in supernumerary type II NB lineages.
(A) A schematic diagram shows GFP is in-frame fused to C-terminus of six4, which is controlled by its endogenous promoter/enhancer in BAC clone P[acman]CH322-192H07.

(B-C'') *Drosophila* larval type I and type II NB lineages are labeled with Phalloidin (in green) or mCD8-RFP (in green), and counterstained with anti-Dpn (in red), anti-Ase (in blue), or anti-GFP (in white) antibodies. Six4 expression pattern in type II NB lineages (highlighted by dashed lines) is characterized by examining the GFP tag expression in *Drosophila* late 3rd instar larval brain lobes (B-B''). ( Insets) Six4-GFP is expressed in type II NBs (white arrows), imINPs (yellow arrows) and mINPs (white arrowheads), as well as a few GMCs (yellow arrowheads), with expression levels gradually decreased from NBs to daughter INPs. Expression of UAS-Six4 RNAiHM05254 in type II NBs driven by pntP1-GAL4 (C-C'') exclusively abolishes GFP expression in all type II NB lineages, while GFP expression outside type II NB lineages is not affected.

(D) A wild type brain lobe has 8 type II NB lineages, which are labeled by mCD8-RFP driven by pntP1-GAL4. Each lineage has an E(spl)mγ-GFP+ (in red) but Ase- NB. White arrows point to some type II NBs as examples.

(E-H) Specific knockdown of Six4 by expressing UAS-Six4 RNAiHM05254 driven by pntP1-GAL4 leads to an increase in the number of total type II NBs and their associated lineages (E). Restoring Six4 levels by expressing either UAS-Six4 ORF (F) or RNAi resistant UAS-Six4HM05254Resis (G) in type II NBs suppresses supernumerary type II NBs resulting from Six4 knockdown, and the suppression is not due to the dilution of UAS-Six4 RNAi construct when an extra copy of UAS-mCD8-RFP is included (H).

(I-K) Expressing three independent UAS-Six4 RNAi constructs from VDRC produces similar, though relative weaker phenotypes as expressing UAS-Six4HM05254Resis. Insets in (E), (I) and (K) show an enlarged view of a single isolated type II NB lineage (highlighted with white dashed lines) containing two NBs (pointed by white arrows) from the area highlighted with a dashed square.

(L-M) erm2/+ heterozygous mutant brain lobes (K) develop normally to produce type II NB lineages, whereas supernumerary type II NB lineages are generated when Six4 is knocked down under erm2/+ heterozygous background (L).

(N) Quantifications of type II NBs per brain lobe with indicated genotypes. ***, P < 0.001, **, P < 0.01. The number on top of, or within each bar in graphs of this figure and all following figures indicates the number of samples examined.

Scale bars equal 50μm.
Figure III-2. Expression patterns of Six4-GFP in Drosophila 3rd instar larval CNS.

(A-A’) Two type I NB lineages symmetric to midline of VNC have Six4-GFP expression in NBs and GMCs.

(B-B’) Several type I NB lineages in ventral lobes have Six4-GFP expression in NBs and GMCs.

(C-C’) In dorsal brain lobes, several type I NB lineages have Six4-GFP expression in NBs and GMCs. One lateral type II NB lineage has weaker Six4-GFP expression (green arrows) only in type II NBs compared to other type II NB lineages.

White dashed lines, type I NB lineages. Yellow dashed lines, type II NB lineages. Scale bars equal 50μm.

Six4 in imINPs prevents dedifferentiation of imINPs to ectopic type II NBs.

Next, we examined how extra type II NB lineages are generated when Six4 was knocked down. Previous studies have shown that supernumerary type II NBs or their derived lineages may be induced due to several regulatory deficiencies. These deficiencies include, but are not limit to, dedifferentiation of INPs to commit NB fate (Bowman et al., 2008; Komori et al., 2014b; Li et al., 2017; Li et al., 2016; Weng et al., 2010; Xie et al., 2016), asymmetric division or cellular polarity deficits of type II NBs leading to produce two equalized NB-fate daughters (Carmena, 2018),
hyperactive proliferation of type II NBs or imINPs (Li et al., 2017; San-Juán & Baonza, 2011; Xiao et al., 2012; Zacharioudaki et al., 2012; Zhu et al., 2012), aberrant specification of NBs from the neuroectoderm during embryonic stages (Hwang & Rulifson, 2011), or mixed reasons of above. We hypothesized that extra type II NBs in Six4 knockdown brains were resulting from dedifferentiation of INPs (as illustrated in Fig. III-3M) due to following clues. First, since we drove the expression of \textit{UAS-Six4 RNAi} transgenes with \textit{pntP1-GAL4}, which like endogenous PntP1 proteins is only expressed in type II NBs at embryonic stages but not in type I NBs or the neuroectoderm before type II NBs are specified (Alvarez & Diaz-Benjumea, 2018; Walsh & Doe, 2017), it is unlikely that Six4 knockdown would affect the specification of type II NBs from the neuroectoderm during the embryonic stages. Second, the coexistence of multiple type II NBs in a single Six4 knockdown lineage at the 3\textsuperscript{rd} instar larval stages (Fig. III-1E, I, K and insets), although at a low rate (around 4%), also suggests that the supernumerary type II NBs could be generated during the lineage development after they are specified, since these extra type II NBs could be just generated during the lineage development at late larval stages and have not established their own derived lineages yet. Third, Erm is shown to be specifically expressed in imINPs (except newly born imINPs) (Janssens et al., 2014; Li et al., 2016; Pfeiffer et al, 2008). Reduction or loss of Erm leads to expansion of supernumerary type II NB lineages (Janssens et al., 2014; Koe et al., 2014; Li et al., 2017; Li et al., 2016; Weng et al., 2010; Xie et al., 2016).

When we knocked down Six4 specifically in type II NBs and imINPs by using \textit{pntP1-GAL4}, the phenotype of supernumerary type II NBs was enhanced under \textit{erm^{2/+}} heterozygous background, while \textit{erm^{2/+}} heterozygous mutant brains did not have any ectopic type II NBs (Fig. III-1L- N), suggesting that Six4 may function like Erm in imINPs to prevent dedifferentiation of INPs. To test this hypothesis, we used \textit{erm-GAL4(II)}, which is specifically expressed in Ase\textsuperscript{−} and Ase\textsuperscript{+} imINPs (except newly born imINPs) (Li et al., 2017; Pfeiffer et al., 2008; Weng et al., 2010; Xie et al., 2016) to knock down Six4 in imINPs but not NBs. However, it did not generate extra type
II NBs when one copy of *UAS-Six4 RNAi* was expressed driven by one copy (data not shown) or two copies of *erm-GAL4(II)* driver (Fig. III-3A and B, D). We suspected it was because that the knockdown of Six4 by *erm-GAL4(II)* was not efficient enough given that Six4 is robustly expressed in type II NBs which are earlier than imINPs and Six4 proteins may be inherited from NBs to imINPs. Nevertheless, expression of two copies of *UAS-Six4 RNAi* constructs driven by two copies of *erm-GAL4(II)* consistently led to generation of 1–2 extra type II NBs per brain lobe with one lineage containing more than one type II NBs (Fig. III-3C and D). We next examined if the Six4 knockdown phenotype when using *erm-GAL4(II)* can be boosted by further reducing Erm levels, as was shown when using *pntP1-GAL4*. Similarly, the phenotypes of supernumerary type II NB also depended on the dose of *UAS-Six4 RNAi* construct. 20-30% of *erm2/+* brains generated only one extra type II NBs when using *erm-GAL4(II)* to label type INP lineages. Knockdown of Six4 by expressing one copy of *UAS-Six4 RNAi* under *erm2/+* background generated one or two extra type II NBs in 50% brains (Fig. III-3E and F, H). Whereas, knockdown of Six4 by expressing two copies of *UAS-Six4 RNAi* under *erm2/+* background generated as many as 40 type II NBs per brain lobe in the most severe case, which was an average of 1–2 extra type II NBs per brain lobe with one lineage containing more than one type II NBs (Fig. III-3G-H). Accordingly, the average number of mINPs per NB was reduced by 32% (Fig. III-3I). All these results indicate that Six4 in imINPs prevents dedifferentiation of imINPs to become supernumerary type II NBs (Fig. III-3L-M).

Furthermore, we tested if supernumerary type II NBs resulting from Six4 knockdown were caused by deficits in asymmetric divisions to produce two equalized NBs. Wild type *Drosophila* NBs, including both type I NBs and type II NBs, commit asymmetric divisions to produce one self-renewed NB and one small daughter cell through establishing cell polarity by segregating different proteins properly to their corresponding sub-regions in cells during mitosis. These
proteins are recruited to form basal complex or apical complex separately (Bello et al., 2006; Betschinger et al., 2006; Boone & Doe, 2008; Chia et al., 2008; Li et al., 2014; Wodarz & Huttner, 2003). We checked the cell polarity of type II NBs during mitosis by staining Miranda (Mira) and apical protein kinase C (aPKC), which are in basal complex and apical complex respectively (Atwood & Prehoda, 2009; Barros et al., 2003; Chia et al., 2008; Hannaford et al., 2018; Li et al., 2014; Wodarz & Huttner, 2003). Our results showed either Mira or aPKC still exhibited the same distribution patterns when Six4 was knocked down in type II NBs compared to wild type. Mira was expressed weakly and evenly in cytoplasm during interphase, while there was no aPKC expression detected during this stage (Fig. III-3J1 and J1’, III-3K1 and K1’). When NBs progressed into mitosis, chromosome segregation and condensation were coupled with phosphorylation of Histone H3 (PH3), which was recognized by anti-PH3 antibody and used to indicate cell cycle stages (Hendzel et al., 1997). In prophase, Mira expression levels were elevated and localized into cell cortex, with higher concentration on centrosome-like structures, while aPKC expression levels were still undetectable (Fig. III-3J2 and J2’, III-3K2 and K2’). At metaphase, Mira was first enriched in mitotic spindles and then segregated into basal cell cortex. However, aPKC expression was enriched and segregated oppositely into apical cell cortex (Fig. III-3J3 and J3’, III-J4 and J4’, III-3K3 and K3’, III-3K4 and K4’). At late stages of cell cycles, i.e., anaphase and telophase, Mira was enriched only in basal imINPs but not NBs, while aPKC persisted weakly only in apical NB. Furthermore, two future daughter cells displayed unequal sizes (Fig. III-3J5 and 3J5’, III-3K5 and 3K5’). These results suggest that Six4 is unlikely involved in regulating type II NB asymmetric divisions and supernumerary type II NBs in Six4 knockdown brains are not due to asymmetric division deficits of type II NBs.
Figure III-3. Six4 prevents dedifferentiation of imINPs to ectopic type II NBs with asymmetric divisions unaffected.

(A-B) There are eight type II NBs (white arrows) in a wild type brain lobe (A) or in a brain lobe with Six4 knockdown specifically in Ase\(^{-}\) and Ase\(^{+}\) imINPs by expressing one copy of UAS-Six4 RNAi driven by two copies of erm-GAL4(II) (B) (Only Six type II NBs are shown as examples).

(C) Knockdown of Six4 by expressing two copies of UAS-Six4 RNAi driven by two copies of erm-GAL4(II) results in extra type II NBs. Insets show two additional type II NB lineages at different focal plans. Dashed lines highlight a single lineage containing two type II NBs.

(D) Quantifications of type II NBs per brain lobe corresponding to (A-C). ***, \(P < 0.001\).

(E-G) There are eight type II NBs in an \(erm^{+/+}\) brain lobe (E). Extra type II NBs are generated in a low penetration when Six4 is knocked down by expressing one copy of UAS-Six4 RNAi driven by one copy of erm-GAL4(II) under \(erm^{+/+}\) background (F). Dramatic supernumerary type II NBs are generated when two copies of UAS-Six4 RNAi are expressed in \(erm^{+/+}\) brain lobes (G). Insets in (F) and (G) show additional type II NBs at different focal plans.

(H-I) Quantifications of number of type II NBs per brain lobe (H), or number of mINPs per lineage (I) with indicated genotypes. ***, \(P < 0.001\). **, \(P < 0.01\).

(J1-J5') Expression patterns of asymmetric division markers Mira (in red), aPKC (in blue) and mitotic marker PH3 (in blue) throughout a cell cycle in wild type type II NBs labelled by mCD8-RFP (in green). Mira is expressed weakly in cytoplasm and aPKC is not detected at interphase (J1 and J1'). At prophase (J2 and J2'), Mira levels are elevated, localized into cell cortex, and enriched in centrosome-like structures. aPKC levels are still undetectable. At metaphase, Mira is enriched into mitotic spindle first (J3 and J3') and then segregated to basal cortex only (J4 and J4'). aPKC is segregated into apical cortex weakly first and then robustly, at metaphase (J3 and J3', J4 and J4'). At anaphase or telophase (J5 and J5'), Mira is localized on basal cortex only and aPKC is oppositely localized at apical cortex of type II NBs only.

(K1-K5') Mira and aPKC expression patterns in Six4 knockdown type II NBs are as normal as in wild type type II NBs in indicated cell cycle stages.

(L-M) Schematic diagrams of wild type (L) and Six4 knockdown (M) type II NB lineages with expression patterns of Six4 (in green) and other key regulators characterized.

Scale bars equal 50\(\mu\)m in A-C and E-G, or 10\(\mu\)m in J1-K5'.

Six4 has synergistic effect with Erm to prevent dedifferentiation of imINPs

Since Erm also functions in imINPs to prevent the dedifferentiation of imINPs like Six4, then we asked do they function synergistically. Given that reducing either Six4 or Erm levels leads to
dedifferentiation of imINPs to type II NBs (Janssens et al., 2014; Koe et al., 2014; Li et al., 2017; Li et al., 2016; Weng et al., 2010; Xie et al., 2016), we tested if knocking down Six4 and Erm simultaneously in type II NBs generates more extra type II NBs than knocking down either one alone. Although knockdown of Six4 by using pntP1-GAL4 in E(spl)mγ-GFP transgenic line led to an average of 16 type II NBs, it is to our surprising that the phenotype was much weaker when Six4 was knocked down without E(spl)mγ-GFP (i.e., under wild type background). However, the weaker phenotype under wild type background makes it more sensitive to test potential synergistic effects between Six4 and Erm. First, we performed knockdown of Six4 and/or Erm at 25°C, which generated milder phenotypes due to lower RNAi knockdown efficiency. When animals were raised at 25°C, knockdown of Six4 did not generate any ectopic type II NBs (Fig. III-4A, B and R). Knockdown of Erm alone at 25°C led to an average of 15 type II NBs per brain lobe (Fig. III-4C and R). However, knockdown of both Six4 and Erm at the same time resulted in an average of 26 type II NBs (Fig. III-4D and R), which was increased by 73.3% than knockdown of Erm alone. We then knocked down Six4 and/or Erm but at 29°C to enhance the efficiency. When Six4 was knocked down without E(spl)mγ-GFP, only 1/7 brains generated one extra type II NB lineage compared to wild type (Fig. III-4E, F and R). Knockdown of Erm alone led to more than 100 type II NBs per brain lobe, which is consistent with previous studies (Li et al., 2017; Li et al., 2016; Xie et al., 2016) (Fig. III-4G and R). Knockdown of Six4 at 29°C enhanced Erm knockdown phenotypes by 36.4% to an average of 190 type II NBs per brain lobe (Fig. III-4H and R). These results suggest that Six4 functions synergistically with Erm to prevent dedifferentiation of imINPs. Then we asked if gain of each one’s function also led to a detectable synergy, considering Six4 and Erm function in the same direction. Because misexpressing Erm alone in type II NBs depletes almost every Ase+ type II NBs at late 3rd instar larval stage (data not shown) (Li et al., 2016; Weng et al., 2010), we switched to check phenotypes at 2nd larval instar stage when a subset of type II NBs still remain. Compared with wild type brains, which always
contain eight Ase$^{-}$ type II NBs per lobe, overexpressing $UAS$-$Six4ORF$ did not deplete any GFP$^{+}$ type II NBs. However, Ase was ectopically expressed in 4% of type II NBs on average when Six4 was overexpressed (Fig. III-4I, J, R and S). On the other hand, Erm misexpression resulted in an average of 4 GFP$^{+}$ type II NBs remained per brain lobe and 67% of these GFP$^{+}$ type II NBs became Ase positive (Fig. III-4K, R and S). When Six4 was overexpressed and Erm was misexpressed simultaneously in type II NBs, only 1 GFP$^{-}$Ase$^{+}$ type II NBs per brain lobe on average remained and in a subset of brain lobes all type II NBs were depleted (Fig. III-4L, R and S).

Next, we examined if Six4 and Erm function in a linear pathway, in which one is regulated by the other. To this end, we knocked down one and checked the expression levels of the other. In wild type type II NB lineages, Erm is specifically expressed in Ase$^{-}$ (except newly born) and Ase$^{+}$ imINPs (Fig. III-4M and M’, T). We found that Erm expression was not affected when Six4 was specifically knocked down in type II NBs and their associated progenies by using $pntP1$-$GAL4$ (Fig. III-4N and N’ and T). Neither we detected any changes on Six4-GFP levels in either type II NBs or Ase$^{-}$ imINPs when Erm or its activator, PntP1 was knocked down (Fig. III-4O-Q’ and U).

All these results together indicate that Six4 and Erm function synergistically in imINPs to prevent them dedifferentiating to NBs. However, Six4 is more likely to assist Erm, which is the dominant factor over Six4. In addition, there is no mutual regulation between Six4 and Erm on the expression of each other.
Figure III-4. Six4 functions synergistically with Erm to prevent dedifferentiation of imINPs.

In all images, type II NB lineages are labeled with mCD8-GFP or mCD8-RFP and counterstained with anti-Dpn, anti-Ase, or anti-Erm antibodies. White arrows point to some of wild type Dpn⁺ Ase⁻ type II NBs appearing red as examples. White arrows with purple outlines point to Ase⁺ type II NBs appearing magenta. Scale bars equal 50μm in A-L, or 10μm in M-Q’.

(A-D) Eight type II NB lineages labeled with mCD8-GFP reside in one wild type brain lobe (A). Simultaneous knockdown of Six4 and Erm (D) at 25°C in type II NBs significantly boosts the generation of supernumerary type II NBs compared with knockdown of Six4 (B) or Erm (C) alone.
(E-H) There are eight type II NBs in a wild type brain lobe (E). Expressing \textit{UAS-Six4 RNAi} alone at 29°C under wild type background barely generates supernumerary type II NBs (F). Expressing \textit{UAS-Erm RNAi} at 29°C leads to the generation of supernumerary type II NBs (G). Simultaneous expression of \textit{UAS-Six4 RNAi} and \textit{UAS-Erm RNAi} at 29°C leads to a synergistic increase in the number of type II NBs (H).

(I-L) All eight type II NBs are Ase⁻ in wild type brains (I). Overexpression of Six4 by expressing \textit{UAS-Six4ORF} in type II NBs at 29°C leads to ectopic Ase expression (white arrows with purple outlines) in a small subset of type II NBs at 2\textsuperscript{nd} instar stage (J). Misexpressing Erm in type II NBs at 29°C leads to ectopic Ase expression (white arrows with purple outlines) in a larger portion of type II NBs and a reduction in the number of type II NBs (K). Simultaneously overexpressing Six4 and misexpressing Erm (L) leads to a synergistic reduction in the number of type II NBs and ectopic Ase expression in all remaining type II NBs.

(M-M') Erm is expressed in Ase⁻ (open white arrows) and Ase⁺ (yellow arrows) imINPs in wild type type II NB lineages (dashed lines).

(N-N') Erm expression is not affected in Six4 knockdown type II NB lineages.

(O-Q') Six4 expression patterns and levels, reflected by GFP immunostaining, are the same in wild type (O-O'), Erm knockdown (P-P') and \textit{pnt} knockdown (Q-Q') type II NB lineages. White arrows, type II NBs. Yellow arrows, imINPs. White arrowheads, mINPs. Yellow arrowheads, GMCs.

(R-U) Quantifications of number of total GFP⁺ type II NBs (R), percentage of Ase⁺/GFP⁺ type II NBs over total GFP⁺ type II NBs (S), Erm intensity in imINPs (T), and Six4-GFP intensity in type II NBs or Ase⁻ imINPs (U) with indicated genotypes. ***, \(P < 0.001\). NS, no significance.

\textbf{Overexpressing Six4 in imINPs substitutes Erm to prevent dedifferentiation of imINPs}

We have demonstrated Six4 in imINPs functions like Erm to prevent the dedifferentiation of INPs to become type II NBs. However, knocking down Six4 by using either \textit{pntP1-GAL4} or \textit{erm-GAL4(II)} did not result in as many supernumerary type II NBs as knocking down Erm did. We hypothesized it is due to either less efficient activity or less abundance of Six4. To demonstrate this, we tested if the phenotypes caused by Erm knockdown would be rescued by increasing Six4 levels specifically in imINPs. We artificially elevated Six4 levels by overexpressing \textit{UAS-Six4ORF} in Ase⁻ and Ase⁺ imINPs driven by \textit{erm-GAL4(II)} at 29°C and found it did not affect the
number of type II NBs or the generation of INPs (Fig. III-5A and B, E). Knocking down Erm alone in imINPs by using *erm-GAL4* (*II*) at 29˚C led to an average of 111 type II NBs/lobe with 100% of penetrance of the phenotype (Fig. III-5C and E). Interestingly, the supernumerary type II NB phenotype resulting from Erm knockdown was largely rescued by overexpressing Six4 in imINPs. There were only 12 type II NBs/brain lobe on average and about 10% of brain lobes had no extra type II NBs when Six4 was overexpressed in Erm knockdown imINPs (Fig. III-5D-E). These results demonstrate that Six4 can functionally substitute Erm to prevent dedifferentiation of imINPs if its expression level is elevated, suggesting that Six4 functions with partial redundancy with Erm to prevent dedifferentiation of imINPs.

**Figure III-5. Overexpressing Six4 in imINPs rescues Erm knockdown phenotypes.**

(A-B) Eight type II NBs reside in one wild type brain lobe (A) or in one lobe with *UAS-Six4ORF* expressed driven by imINP-specific *erm-GAL4* (*II*) (B).

(C) Supernumerary type II NBs are generated when Erm is knocked down specifically in imINPs.

(D) Specific overexpression of Six4 by expressing *UAS-Six4ORF* in imINPs largely inhibits the generation of supernumerary type II NBs resulting from Erm knockdown.

(E) Quantifications of total type II NBs per brain lobe with indicated genotypes. 

\[
\text{***, } P < 0.001.
\]

Scale bars equal 50μm.
Six4 prevents dedifferentiation of imINPs likely by antagonizing PntP1 function

Previous studies have shown that, in order to avoid the dedifferentiation, imINPs need to gradually restrict their development potential and become mature properly, in which the transcription levels or activity of genes important for a type II NB functional identity need to be properly repressed or timely terminated (Janssens et al., 2017; Li et al., 2017), including the most important one, PntP1, which suppresses Ase expression in type II NBs and promotes INP generation (Li et al., 2017; Li et al., 2016; Xie et al., 2016; Zhu et al., 2011). Thus, we investigated why knockdown of Six4 led to dedifferentiation of imINPs, by checking if there are enhanced PntP1 expression in Six4 knockdown type II NB lineages. In wild type type II NB lineages, PntP1 was strongly expressed in type II NBs, Dpn\(^{-}\) E(spI)m\(\gamma\) imINPs, but was terminated in Dpn\(^{+}\) E(spI)m\(\gamma\) mINPs (Fig. III-6A-A”, C, and (Zhu et al., 2011)). In Six4 knockdown type II NBs lineages, PntP1 expression levels were not obviously increased. Instead, PntP1 expression was not timely terminated in those newly generated E(spI)m\(\gamma\) mINPs right next to imINPs (Fig. III-6B-B”, and C). Since Erm also restricts the developmental potential in imINPs by repressing or antagonizing PntP1 (Janssens et al., 2017; Li et al., 2017), we also examined if the persistence of PntP1 in mINPs in Six4 knockdown type II NB lineages was mediated by decreasing Erm expression. Our results did not detect any changes on Erm expression when Six4 was knocked down (Fig. III-4M-N’, and T). Thus, all the results together suggest that late during imINP development Six4 promotes maturation of imINP by compromising PntP1 function to prevent their dedifferentiation, which will be further examined in bellowing 1) -3).
1) Overexpressing Six4 in type II NBs reduces the generation of INPs and de-suppresses Ase expression by antagonizing PntP1 activity

In general, there are two ways to compromise the function of one protein, i.e., either by disrupting its endogenous activity or by decreasing its expression levels. Thus, we next determined if Six4 compromises PntP1 function through repressing its activity or expression. Since our results showed that overexpression of Six4 by using pntP1-GAL4 led to ectopic Ase expression and loss of mINPs at 2nd instar stage in a small subset of type II NBs (Fig. III-3J), it suggests that type II NB identity was transformed to type I NB identity and Six4 overexpression would be an ideal approach to examine how Six4 compromises PntP1 function. To achieve this, we first verified the phenotypes at late 3rd instar larval brains by overexpressing UAS-Six4ORF in type II NBs (and imINPs) by using pntP1-GAL4 at 29 °C. Our results showed that overexpression of Six4 resulted in an average of three (i.e., 37.5%) GFP+ type II NBs losing all of their associated mINPs (Fig. III-7C and M). The average number of INPs per type II NB lineage was reduced to 10 when Six4 was overexpressed compared to an average of 23 INPs in a wild type type II NB lineage. In addition to reduced INP genesis, 25% of GFP+ type II NBs on average had ectopic Ase expression. These Ase+ type II NBs recapitulated type I NB lineage morphology and directly produce 4-6 Ase+ GMC-like cells (Fig. III-7A, C, N and O). The reduced INP generation and/or ectopic Ase expression in Six4 overexpressing type II NBs were consistent with the phenotypes due to compromised PntP1 function, as shown in previous studies (Xie et al., 2016; Xie et al., 2014; Zhu et al., 2011). Because our previous study showed the presence of INPs is essential to maintain PntP1 expression levels in type II NBs (Xie et al., 2014), which makes Six4 not the only factor to be examined, we did not check PntP1 expression levels first in Six4 overexpressing type II NBs. Instead, we examined if Six4 disrupted PntP1 activity first. In order to test this, we need to maintain, or even elevate, PntP1 levels anyway by expressing UAS-PntP1 driven by type II NB lineage-specific pntP1-GAL4. Type II NB lineages with PntP1 overexpressed develop as
normally as wild type II NB lineages do (Fig. III-7A and B). To our surprising, even when PntP1 levels were maintained overexpressing Six4 still reduced INP numbers to the same extent (Fig. III-7D, M and N), although Ase was suppressed in all type II NBs when PntP1 was maintained (Fig. III-7D and O). It suggests that Six4 may antagonize PntP1 activity. Because both Six4 and PntP1 were expressed natively in type II NBs and imINPs, we then switched to type I NB lineages which provide clear background to test the antagonization of PntP1 by Six4. In wild type Drosophila larvae, ventral brain lobes and ventral nerve cord (VNC) accommodate only type I, but not type II NB lineages and each type I NB lineage contains one Ase+ type I NB and 4~6 Ase+ GMCs (Fig. III-7E, only VNCs were shown). Misexpressing UAS-PntP1 in type I NBs by using pan-NB driver insc-GAL4, resulted in 15% of type I NBs on average produced Ase+ Dpn+ mINP-like cells and 90% of type I NBs on average were Ase− in VNCs (Fig. III-6F, P and Q), which was consistent with previous studies (Li et al., 2016; Xie et al., 2014; Zhu et al., 2011). Type I NB lineages developed normally when Six4 was misexpressed (Fig. III-7G, P and Q). However, when Six4 and PntP1 were misexpressed simultaneously in type I NBs, nearly no type I NBs produced ectopic mINP-like cells and only 15% of type I NBs on average were Ase− in VNCs (Fig. III-7H, P and Q), suggesting that PntP1 could no longer function effectively to suppress Ase or induce the generation of INP-like cells when Six4 was simultaneously expressed. Consistently, we found that Erm expression in imINPs was abolished when Six4 was overexpressed alone or together with PntP1 overexpression in type II NBs (Fig. III-7I-L’ and R), indicating that even if PntP1 expression was maintained in type II NBs, it could no longer activate Erm expression when Six4 was overexpressed. Taken together, the inhibition of PntP1-induced transformation of type I NB lineages to type II-like NB lineages and the suppression of PntP1-activated Erm expression in imINPs indicate that Six4 antagonizes the activity of PntP1.
2) Ectopic Pros in Ase⁻ imINPs was responsible for INP reduction in Six4 overexpressing type II NB lineages

Since we have demonstrated that overexpressing Six4 in type II NBs antagonized PntP1 activity, leading to INP reduction, ectopic Ase expression in type II NBs and Erm abolishment in imINPs. Then we asked why Six4 overexpression in type II NBs reduced INP number. Because PntP1 suppresses Pros expression in Ase⁻ newly born imINPs to prevent the premature differentiation of INPs to GMCs (Xie et al., 2016; Xie et al., 2014), we investigated if ectopic Pros, a cell cycle exit determinant, was the factor to reduce INP number. First, we checked Pros expression when UAS-Six4ORF was overexpressed in type II NBs driven by pntP1-GAL4. In contrast to wild type type II NB lineages, in which Pros was absent in all imINPs but weakly expressed in mINPs cytoplasm, nuclear Pros was clearly detected from Ase⁻ imINPs-like cells to GMCs in Six4 overexpressing type II NB lineages with reduced INPs (Fig. III-8A-B”). Next, we reduced Pros expression levels by utilizing pros¹⁷/+ heterozygous mutant to test if INPs could be restored in Six4 overexpressing type II NB lineages. In one pros¹⁷/+ brain lobe, all 8 type II NB lineages have as many mINPs on average as wild type type II NB lineages do (Fig. III-8C, C’, I and J). Reducing Pros levels in Six4 overexpressing type II NB lineage under pros¹⁷/+ background indeed completely restored mINP number to a comparable value as that in wild type type II NB lineages (Fig. III-8D, D’, I and J), even though 25% of type II NBs still had ectopic Ase expression (Fig. III-8D-D’ and K). These results indicate that ectopic expression of Pros in Ase⁻ imINPs is the cause for INP reduction or depletion in Six4 overexpressing type II NB lineages (Fig. III-8N).

3) Six4 inhibits PntP1 expression

As stated above, there are two ways to compromise the function of PntP1, either by disrupting its endogenous activity or by decreasing its expression levels. Though we have demonstrated that
Six4 antagonized PntP1 activity (Fig. III-7), it is still possible that Six4 also inhibits PntP1 expression. Since INPs are completely restored when ectopic Pros are reduced in pros$^{17/+}$ brains, which restored the potential feedback signals by INPs to maintain PntP1 levels in type II NBs, it is ideal to check PntP1 expression levels in type II NBs when Six4 was overexpressed in pros$^{17/+}$ background. In wild type type II NB lineages, PntP1 was clearly detected by anti-PntP1 antibody in type II NBs and imINPs (Fig. III-8E-E” and L). When Six4 was overexpressed, PntP1 levels were decreased by 30% in Ase- type II NBs and by 75% in Ase+ type II NBs (Fig. III-8F-F” and L). When Six4 was overexpressed in pros$^{17/+}$ brains where INPs are restored, PntP1 levels were still reduced by 30% in Ase- type II NBs or by 42% in Ase+ type II NBs. This result showing that recovering INPs restored the PntP1 expression levels (from 25% to 58% of wild type levels) in Ase+ type II NBs, was consistent with previous study and supported the notion that INPs may provide potential signals to maintain PntP1 in type II NBs (Xie et al., 2014). However, our above results show that even in the restoration of INPs, PntP1 expression levels are still significantly reduced in Six4 overexpressing type II NBs. Consistently, PntP1 levels in Ase- imINPs in Six4 overexpressing lineages were reduced by 50% whenever mINPs are reduced or restored (Fig. III-8E-H”, and M). Therefore, our results demonstrate that, in addition to antagonizing PntP1 activity, Six4 also suppresses PntP1 expression (Fig. III-8N).

Taken together, the above results demonstrate that like Erm, Six4 can not only suppress PntP1 expression but also antagonize PntP1’s activity. The inhibition of PntP1’s expression and activity in imINPs by Six4 ensures imINPs differentiate properly to become fate committed mINPs.
Figure III-6. Six4 knockdown delays the termination of PntP1 in type II NB lineages.

Type II NB lineages are labeled with mCD8-GFP driven by pntP1-GAL4 and counterstained with a combination of various antibodies as indicated. Some type II NB lineages are highlighted with dashed lines as examples. Scale bars equal 10μm in (A-B').

(A-A') In wild type type II NB lineages, PntP1 is expressed in type II NBs (white arrows) and imlINPs (E(spl)mγ-, yellow arrows), but absent in newly generated mlINPs (E(spl)mγ+, white arrowheads).

(B-B') PntP1 is expressed in newly generated E(spl)mγ+ mlINPs (white arrowheads) in addition to type II NBs (white arrows) and imlINPs (yellow arrows) in Six4 knockdown type II NB lineages.

(C) Quantifications of PntP1 intensity in type II NBs, imlINPs or newly generated mlINPs in wild type or Six4 knockdown type II NB lineages. ***P < 0.001. NS, no significance.
**Figure III-7. Six4 antagonizes PntP1 activity.**

In all images, White arrows point to type II NBs. Yellow arrows point to imINPs. White arrowheads point to mINPs or mINP-like cells. Yellow arrowheads point to GMCs.

Scale bars equal 50μm in A-H or 10μm in I-L.

(A-D) Wild type (A and inset) and PntP1 overexpressing type II NBs (B and inset) are Ase- and have associated mINPs. Expressing UAS-Six4ORF in type II NBs leads to ectopic Ase expression in NBs and loss of INPs, resulting in only GMCs produced (C and inset). Even in the presence of overexpressed PntP1, overexpressing Six4 in type II NBs still leads to loss of INPs, though Ase is not activated anymore (D and inset).
(E-H) All wild type type I NBs are Ase+ and have Ase+ GMCs (E and inset). Misexpressing PntP1 in type I NBs by using insc-Gal4 suppresses Ase expression in type I NBs and results in the generation of Ase+ Dpn+ mINP-like cells (F and inset). Type I NBs with Six4 misexpression keep Ase positive and produce normal GMCs (G and inset). Simultaneously expressing UAS-Six4ORF in type I NBs blocks the generation of INP-like cell and inhibits ectopic Ase expression caused by PntP1 misexpression (H).

(I-L') Erm is specifically expressed in wild type (I and I') or PntP1 overexpressing imINPs (J and J'). Erm expression is abolished in imINPs when Six4 is overexpressed (K and K'), even though PntP1 is simultaneously overexpressed (L and L').

(M-R) Quantifications of number of type II NB lineages with mINPs (M), number of mINPs per lineage (N), number of Ase- type II NBs (O), percentage of type I NB lineages with ectopic mINP-like cells in VNCs (P), percentage of Ase- type I NBs in VNCs (Q), Erm protein intensity in imINPs (R) with indicated genotypes. ***, P < 0.001.
Figure III-8. Overexpressing Six4 in type II NB lineages suppresses PntP1 expression and leads to ectopic Pros in imINPs which is responsible for INP reduction.

(A-A") In wild type type II NB lineages (highlighted by dashed lines), Pros is expressed in mlINPs (white arrowheads) and GMCs (yellow arrowheads), but not in any imlINPs (open white arrows and yellow arrows) or type II NBs (white arrows).

(B-B") Pros is ectopically expressed in Ase- imINPs (open white arrows) and Ase+ progeny cells (yellow arrows) when Six4 is overexpressed in type II NBs.

(C-D") pros17/+ heterozygous mutant type II NBs have normal mlINPs associated with Ase- type II NBs (C-C'). Reduced Pros in pros17/+ mutant fully rescues INPs in all type II NB lineages when UAS-Six4ORF is expressed (D-D'), including those lineages which contain Ase+ type II NBs (white arrows with purple outlines). Note that even though mlINPs are restored by reducing Pros level in pros17/+ heterozygous mutant, in those Ase+ type II NB lineages all imlINPs (white arrowheads with purple outlines, Dpn-) which are next to Ase+ type II NBs are still Ase+.

(E-E") PntP1 is expressed in type II NBs (white arrows) and imlINPs (open white arrows and yellow arrows) in wild type type II NB lineages.

(F-F") When Six4 is overexpressed in type II NBs, PntP1 expression levels are reduced in Ase- type II NBs (white arrows), Ase- and Ase+ imlINPs (open white arrows and yellow arrows). And PntP1 is almost abolished in Ase+ type II NBs (white arrows with purple outlines) or their associated Ase+ progeny cells (white arrowheads with purple outlines).

(G-G") pros17/+ heterozygous mutant type II NBs (white arrows) and imlINPs (open white arrows and yellow arrows) have normal PntP1 expression. Note that imlINPs, particularly the Ase- imlINPs, usually have much higher expression of PntP1 than type II NBs.

(H-H") When Six4 is overexpressed in pros17/+ mutant, PntP1 is still decreased in Ase- type II NBs (white arrows), Ase- and Ase+ imlINPs (open white arrows and yellow arrows). Note that, the expression of PntP1 in Ase+ type II NB (white arrows with purple outlines) lineages are partially restored in both type II NBs and Ase+ imlINPs (white arrowheads with purple outlines) when Six4 is overexpressed in pros17/+ mutant compared to that in Ase+ type II NBs and imlINPs when Six4 is overexpressed in wild type background. The expression of PntP1 in Ase- imlINPs (open white arrows) and Ase+ imlINPs (yellow arrows) is also reduced to levels that are comparable to or even lower than that in the type II NBs in the same lineages.

(I-M) Quantifications of number of lineages with mlINPs (I), number of mlINPs per lineage (J), and number of Ase- type II NBs (K), PntP1 intensity in type II NBs (L) and Ase- imlINPs (M) with indicated genotypes. ***, P < 0.001. The number on top of each bar in graph (L) and (M) indicates the number of brains examined and three type II NBs (L) or imlINPs (M) in each brain are randomly chosen for PntP1 intensity measurement.

(N) Schematic diagrams of Six4 overexpressing type II NB lineages with either reduction/elimination of INPs (left) or transformation to type I NB-like lineage phenotypes (right).
Six4, Erm, and PntP1 likely form a trimeric complex to inhibit PntP1 activity

Since our results show Six4 antagonizes PntP1 activity, we further wondered how the antagonization is generated. Previous studies demonstrated that PntP1 protein functions as a transcription factor (O'Neill et al., 1994), and our study further demonstrate it works as a transcriptional activator in type II NBs (CHAPTER II) (Zhu et al., 2011). An E26 transformation-specific (Ets) family domain (called Ets domain), which has a conserved ~85 amino acid sequence and binds to a purine-rich 5′-GGAA/T-3′ core DNA sequence (Karim et al., 1990), is identified in C-terminus of PntP1 (Klambt, 1993). The precise activity of PntP1 would be achieved only if PntP1 proteins (in the presence of other cofactors or not) accurately access, bind and efficiently activate their downstream targets. Thus, there are two potential mechanisms underlying the inhibition of PntP1’s function by Six4 or Erm. One is that Six4 and/or Erm binds to PntP1 proteins, which could prevent PntP1 from binding to its target genes or inhibit its transcriptional activity. Alternatively, Six4 and Erm function as transcriptional repressors to suppress the expression of PntP1 target genes in imINPs by binding to the same target genes without physically interacting with PntP1. To distinguish between these two possibilities, we performed biochemical interaction tests. We coexpressed HA tagged Six4 (UAS-Six4-HA) and Flag tagged PntP1 (Act5C-Flag-PntP1) in Drosophila S2 cells and examined their direct interactions by co-immunoprecipitation (Co-IP). Our co-IP results showed that there was indeed a direct binding between Six4 and PntP1 proteins when they were coexpressed (Fig. III-9A). Since, Erm also antagonizes PntP1 function (Li et al., 2016), we also demonstrated that Erm proteins weakly bound to PntP1 proteins by performing similar co-IP (Fig. III-9B). We also excluded the possibility that the binding to PntP1 in S2 cells was artificial due to high levels of exogenous
proteins when they are coexpressed, since a negative control protein, Myc tagged EGFP (Myc-EGFP) was not shown to bind Flag-PntP1 when co-IPs were performed under the same conditions (Fig. III-9D).

Since Six4 and Erm likely function in the same pathway based on our genetic interaction results but they do not regulate each other’s expression, one possibility could be that Six4 and Erm function in the same complex together with PntP1. The presence of all these three proteins, Six4, Erm, and PntP1, may enhance their interactions and increase the stability of the complex, which could lead to stronger inhibition of PntP1’s activity. To test this possibility, we first tested if Six4 and Erm could also bind to each other and co-exist with PntP1 in the same complex. Indeed, our co-IP results showed that Six4-HA and Myc-Erm could be co-IPed when they were coexpressed in S2 cells (Fig. III-9C). When Six4-HA, Myc-Erm, and Flag-PntP1 were coexpressed, we could also pull down all three proteins in the same complex (Fig. III-10F, lanes #2, #6, #10, and #14), indicating that Six4, Erm, and PntP1 form a complex. Since there is no detectable expression of endogenous PntP1 in S2 cells (Fig. III-10A-B), the interaction between Six4 and Erm is likely direct, but we do not completely rule out the possibility that other unknown proteins mediate their interaction.

Next, we tested whether co-existence of Six4, Erm, and PntP1 would make the complex more stable than a complex that contains only two of them. To this end, we first performed co-IP for a combination of any two of these three proteins with graded concentrations (150mM to 1200mM) of NaCl. Our results showed that with the increase of the salt concentration, the co-IP became less efficient. However, even at 1200mM of NaCl, we did not see complete disruption of the interactions between any two of these proteins (Fig. III-10C-E). Then we compared the co-IP efficiency between the complex that contains all these three proteins and a complex that contains
only two of them with 150mM or 1200mM of salt. We found that with 1200mM of salt, coexpression of Flag-PntP1 significantly enhanced the binding between Six4-HA and Myc-Erm. The % co-IPed Myc-Erm with the anti-HA antibody was increased from 6.7% to 13.6% in the presence of Flag-PntP1 (Fig. III-10F, lanes #6 and #8, and Fig. III-10F”). However, with 150mM of NaCl, no significant enhancement was observed probably because at the low concentration of salt, the binding between Myc-Erm and Six-HA was more stable. In addition to the enhancement of the binding between Myc-Erm and Six4-HA by the presence of Flag-PntP1, we found that the presence of Six4-HA probably also enhanced the binding between Flag-PntP1 and Myc-Erm. The % co-IPed Myc-Erm with the anti-Flag antibody was increased by about 35% with 150mM of NaCl and 50% with 1200mM of NaCl when Six4-HA was coexpressed (Fig. III-10F, lanes #9, #10, #13, and #14, and Fig. III-10F”). Although the enhancement was not statistically significant when the statistical analyses were done separately for the co-IPs carried out with 150mM and 1200mM of NaCl probably because of the small sample size (n=3 for each condition), we did see statistically significant difference of the % co-IPed Myc-Erm when all the data from two different salt concentrations were pooled together for a paired t-test (Fig. III-10F””).

Taking together, all these co-IP data suggest that 1) Six4, Erm, and PntP1 can form a trimeric complex; and 2) the presence of all these three proteins makes the complex more stable, which may in turn confers stronger inhibition of PntP1’s activity.
Figure III-9. Six4 functions together with Erm to prevent dedifferentiation of imINPs by forming a complex with PntP1.

Biochemical interactions between Flag-PntP1 and Six4-HA (A), between Flag-PntP1 and Myc-Erm (B), between Six4-HA and Myc-Erm (C), are detected by transfecting S2 cells with their corresponding expression constructs followed by co-IP and western blotting analyses, but not between Flag-PntP1 and Myc-EGFP under the same condition (D).
Figure III-10. Six4, PntP1 and Erm likely form a trimeric complex.

(A-B) Transfected Flag-PntP1 but not endogenous PntP1 are detected by western blot (A) or immunostaining (B) in S2 cells using the anti-PntP1 antibody or anti-Flag antibody. S2 cells are labeled with transfected Act5c-Myc-EGFP and scale bars equal 10μm in (B).

(C-E) Co-IP of Six4-HA and Flag-PntP1 (C), Myc-Erm and Flag-PntP1 (D), and Six4-HA and Myc-Erm (E) from S2 cells with graded concentrations (150mM–1200mM) of NaCl. Note that interactions between these protein pairs can still be observed but are compromised at higher
concentrations of salt with the interaction between Six4-HA and Myc-Erm being compromised most.

(F) Co-IP of Six4-HA, Flag-PntP1, or Myc-Erm by the anti-HA antibody (lanes #1–8) or anti-Flag antibody (lanes #9–16) with 150mM or 1200mM of NaCl from S2 cells that express a combination of any two or all three of these proteins. Note that when all these three proteins are expressed together in S2 cells, they can be pulled down in the same complex (lanes #2, #6, #10, and #14). Because of unusually high background due to unknown reasons, the bands for co-IPed Six4-HA with the anti-Flag antibody were not shown, but the 2% input shows that Six4-HA was indeed expressed when its expression DNA construct was transfected into S2 cells.

(F’-F”) Quantifications of normalized % co-IPed Flag-PntP1 or % co-IPed Myc-Erm by the anti-HA (F’) or anti-Flag (F”) antibody in the indicated lanes in panel (F). The % co-IP was calculated by dividing the co-IPed protein by the 100% input of the co-IPed protein, then normalized by the IPed protein to correct the differences in immunoprecipitation. Note that the % co-IPed Myc-Erm by the anti-HA antibody with 1200mM of NaCl is significantly increased when Flag-PntP1 is coexpressed in S2 cells (compare lane #6 with lane #8). The % co-IPed Erm by the anti-Flag antibody with both 150mM and 1200 mM of NaCl is also consistently increased although not statistically significant when Six4-HA is coexpressed (compare lane #10 with lane #9, and lane #14 with lane #13). Values are mean ± SEM (N = 3). NS, no significance. *, $P < 0.05$, paired t-test.

(F””) Spot graphs of normalized % co-IPed Myc-Erm of individual replicates included in plotting the bar graph in (F”). *, $P < 0.05$ when all replicates under both salt conditions are pooled together for a paired t-test.

**Endogenous Six4 suppresses Pros expression in newly born Ase⁻ imINPs**

In Six4 overexpressing type II NB lineages, Pros was ectopically expressed in Ase⁻ imINPs to promote imINP cell cycle exiting, resulting in a dramatic reduction of mINPs. Since our results have demonstrated that Six4 compromises the function of PntP1 on both its activity and expression (Fig. III-7 and 8) and previous studies have demonstrated PntP1 also suppresses Pros expression (Xie et al., 2016; Xie et al., 2014), the ectopic Pros expression is most likely due to the compromised PntP1 function resulting from overexpressing Six4. However, in order to prove this, we need to exclude one possibility that Six4 itself, especially overexpressing Six4, may
activate Pros expression independently of PntP1, considering Six4 function oppositely to PntP1. In the presence of PntP1, it is difficult to examine Six4’s potential activation on Pros by simply knocking down Six4 because Pros is still suppressed by PntP1 (Fig. III-11A-B”). Nevertheless, because knocking down PntP1 released the suppression of Pros in imINPs (Xie et al., 2016; Xie et al., 2014), it is feasible to test if Six4 activates Pros when the PntP1 is knocked down.

Therefore, we performed double knockdown of PntP1 and Six4 to check Pros expression pattern. If Pros was activated by Six4, additional knockdown of Six4 would revert Pros⁺ Ase⁻ imINPs in PntP1 knockdown type II NB lineages to Pros negative.

**Figure III-11. Six4 knockdown alone does not affect Pros expression in type II NB lineages.** (A-B”). Pros is absent in Ase⁻ (open white arrows) and Ase⁺ (yellow arrows) imINPs in both wild type (A-A”) and Six4 knockdown type II NB lineages (B-B”). Scale bars equal 10μm. (C-D”). At 100hrs AEL, knockdown of PntP1 alone (C-C”) or together with Six4 knockdown (D-D”) leads to ectopic Pros expression in Ase⁻ imINPs (open white arrows).

Two RNAi constructs, *UAS-pnt RNAi (35038)* and *UAS-pnt RNAi (31396)* (corresponding to lines BL#35038 and BL#31396 respectively), targeting common regions shared by all pnt isoforms in *pnt* locus, were used to knock down PntP1, and the former one was shown more efficient to downregulate PntP1, reflected by loss of more INPs and generation of more
supernumerary type II NBs (Xie et al., 2016). Therefore, we chose to use \textit{UAS-pnt RNAi (35038)}
for knocking down PntP1 to examine Pros expression. We knocked down PntP1 and/or Six4
driven by \textit{pntP1-GAL4} and checked Pros expression in Ase$^\text{e}$ imINPs at 48hrs after egg lying
(AEL), 60hrs AEL and 84hrs AEL separately. We chose these three early developmental stages
because that at later stages the majority of type II NBs would lose their associated INPs and
supernumerary type II NBs would form big clusters in PntP1 knockdown or mutant brains (Xie et
al., 2016), making identifying imINPs difficult (Fig. III-12H-I, and L-M). In \textit{pnt} knockdown type
II NB lineages, though almost all imINPs eventually became Pros positive at 100hrs AEL (Fig.
III-11C-C”), we did find that there were quite a few Ase$^\text{e}$ imINPs which were Pros$^\text{e}$ per brain lobe
at 48hrs, 60hrs and even 84hrs AEL (Fig. III-12A-C” and G). When Six4 and PntP1 were
knocked down simultaneously, to our surprising, at 48hrs AEL and onward, as long as Ase$^\text{e}$
imINPs (identified by Ase$^\text{e}$ next to GFP$^\text{+}$ NBs) were produced, none of them (less than one per
lobe on average) was Pros$^\text{e}$ (Fig. III-11D-D” and Fig. III-12D-F”, G). Thus, our results do not
support the hypothesis that Six4 activates Pros. In contrast, double knockdown of Six4 and PntP1
expanded the ectopic Pros expression to all imINPs compared to PntP1 knockdown alone,
suggesting that native Six4 also suppresses Pros expression in newly born Ase$^\text{e}$ imINPs.
Furthermore, the expanded Pros expression was not likely due to the exacerbation on reducing
PntP1 levels, because Six4 knockdown did not decrease PntP1 expression levels (Fig. III-6A-C).
Taken together, these results indicate that although Six4 antagonizes PntP1’s function, Six4 also
suppresses Pros expression, but as a supplementary factor in addition to PntP1, in newly born
Ase$^\text{e}$ imINPs of type II NB lineages.

\textbf{Simultaneous reduction of Six4 and PntP1 results in depletion of mINPs but not an increase
of type II NBs}
It is known that PntP1 knockdown or mutant results in not only reduction/loss of INPs but also generation of supernumerary type II NBs. The reduction/loss of INPs is due to imINPs with ectopic Pros (Pros) prematurely differentiating to GMCs, while the generation of supernumerary type II NBs is resulting from imINPs with Erm reduction/abolishment (Erm) dedifferentiating to NBs (Xie et al., 2016). Thus, imINPs would develop abnormally but competitively to become either NBs in response to Erm reduction or GMCs in response to ectopic Pros, which is supported by the variance in the extent of supernumerary type II NBs and the percentage of type II NBs with mINPs when PntP1 was knocked down by expressing two UAS-pnt RNAi constructs. Knockdown of PntP1 using UAS-pnt RNAi (35038) led to an average of 20 total GFP+ type II NBs and an average of 2% of them with associated mINPs at late 3rd instar larval stage. Knockdown of PntP1 using UAS-pnt RNAi (31396) led to an average of 16 total GFP+ type II NBs and an average of 7% of them with associated mINPs. All those Ase+ type II NB lineages with no INPs are transformed to type I NB-like lineages (Fig. III-12H, I, L, M and U). Given that Six4 also suppresses Pros expression and double knockdown of Six4 and PntP1 leads to all imINPs become Pros positive, we asked if these Pros+ imINPs prefer prematurely differentiating to GMCs other than reverting to NBs. To this end, we knocked down Six4 and PntP1 simultaneously and examined phenotypes at late 3rd instar larval stage. Our results showed that coexpressing UAS-Six4ORF with either of the two UAS-pnt RNAi constructs led to depletion of supernumerary type II NBs and only eight type II NBs with no mINPs (Fig. III-12J, K, L, M and V). Given that PntP1 knockdown resulted in ectopic Ase expression in type II NBs (Fig. III-12H-I, N, and previous studies (Li et al., 2016; Xie et al., 2016; Xie et al., 2014; Zhu et al., 2011)) and ectopic Ase expression itself in type II NBs lead to INP loss (Bayraktar et al., 2010; Bowman et al., 2008), it is possible that the depletion of mINPs was due to, at least partially, ectopic Ase expression in more type II NBs in Six4 and Pnt double knockdown type II NB lineages. However, we excluded this possibility by quantifying the percentage of Ase+ GFP+ type II NBs over total
GFP⁺ type II NBs, which was the same value of 80% on average in pnt knockdown versus pnt and Six4 double knockdown type II NBs (Fig. III-12H-K, N, U and V). These results demonstrate that all Pros⁺/Erm⁻ imINPs in Six4 and PntP1 double knockdown type II NB lineages prematurely differentiate to GMCs other than reverting to NBs.

Nevertheless, because both two UAS-pnt RNAi constructs we used target not only PntP1 but also other Pnt isoforms or maybe other unknown off-targets, which may be expressed in type II NBs and genetically interacts with Six4 to regulate imINP development, it is possible that knockdown of Six4 also disrupts such interactions to affect imINP preference on differentiation versus dedifferentiation. It is necessary to validate the above results by using PntP1-specific loss-of-function reagents. One PntP1-specific mutant line, pntP190, was generated previously by using CRISPR/Cas9 technology, which produces only truncated C-terminal 180aa of wild type PntP1. This mutant is ideal for our purpose because homozygous animals survive through late 3rd instar stage and homozygous brains generate extra Ase⁺ type II NBs with mINP reduction or depletion (Xie et al., 2016). Because type II NB-specific pntP1-GAL4 is localized in the locus very close to pntP190, it is difficult to recombine them to specifically label pntP190 mutant type II NBs. We used insc-GAL4 to label all type II and type I NBs. Compared with wild type brains, in homozygous pntP190 brains total number of Ase⁺ type II NBs was increased to an average of 20 per lobe, which are identified by Ase⁺ and GFP⁺ (Fig. III-12O, Q, S and U). Due to extra type II NBs forming clusters rather than individual discrete lineages, it is difficult to determine the number of lineages with INPs. Instead, we quantified how many mINPs on average were associated with one Ase⁺ NB. Compared with an average of 23 mINPs in wild type type II NB lineages, the average number of mINPs per Ase⁺ type II NB in homozygous pntP190 brains was dramatically reduced to less than 5 (Fig. III-12O, Q, Q’, T and U). When two copies of UAS-Six4 RNAi were expressed driven by insc-GAL4, total 9 type II NBs per brain lobe on average were
produced but with INP number unaffected (Fig. III-12P, S and T). However, when Six4 was knocked down in homozygous pntP190 mutant brains, only 8 Ase− type II NBs were observed and all mINPs were depleted (Fig. III-12R-T and V). Furthermore, the restoration to 8 Ase− type II NBs in homozygous pntP190 mutant brains with Six4 knockdown was not likely due to the transformation of Ase− type II NBs to Ase+ type I-like NBs, because the total number of Ase+ NBs in Six4 knockdown homozygous pntP190 mutant brain lobes was not increased than homozygous pntP190 mutant (Fig. III-12S).

Thus, our results indicate that, due to the complete de-suppression of Pros resulting from simultaneously reducing/abolishing Six4 and PntP1, all imINPs become Pros positive and choose to prematurely differentiate to GMCs (Fig. III-12U and V), making it impossible for imINPs to dedifferentiate back to type II NBs as demonstrated in btd brat double mutant type II NB lineages (Komori et al., 2014a). These results further support that Six4 in newly born imINPs at its physiological expression levels may also contribute to the suppression of Pros.
Figure III-12. Endogenous Six4 suppresses Pros expression in Ase- imINPs.

(A-C”) Type II NB lineages are labeled with mCD8-GFP (in green) driven by pntP1-GAL4 and counterstained for Ase (in red) and Pros (in blue). When UAS-pnt RNAi (35038) is expressed in type II NBs, a few Pros- imINPs (yellow arrows) are localized in type II NB lineages which are examined at 48hrs AEL (A-A”), 60hrs AEL (B-B”) and 84hrs AEL (C-C”).

(D-F”) In PntP1 and Six4 double knockdown type II NBs, all Ase- imINPs (yellow arrows) have ectopic Pros expression at 48hrs AEL (D-D”), 60hrs AEL (E-E”) and 84hrs AEL (F-F”).
(G) Quantifications of Ase Pros’ mlINPs per brain lobe with pnt knockdown Vs. pnt and Six4 double knockdown. ***, \( P < 0.001 \). **, \( P < 0.01 \).

(H-K) Type II NB lineages are labeled with mCD8-GFP (in green) driven by pntP1-GAL4 and counterstained for Dpn (in red) and Ase (in blue). As examples, white arrows point to some Ase+ type II NBs and white arrows with purple outlines point to some type II NBs with ectopic Ase expression. White arrowheads point to Ase+ Dpn+ mlINPs and yellow arrowheads point to Ase- Dpn- GMCs. Knockdown of PntP1 by expressing UAS-pnt RNAi (35038) (H) or UAS-pnt RNAi (31396) (I) leads to supernumerary GFP labeled type II NB lineage clusters (dashed lines), reduction or elimination of INPs (white arrows) or transformation of type II NB lineages to Ase+ type I NB (white arrows with purple outlines) lineages. Double knockdown of Six4 and PntP1 with either UAS-pnt RNAi (35038) (J) or UAS-pnt RNAi (31396) (K) completely blocks the increase in the number of type II NB lineages or the generation of INPs, resulting in the direct generation of GMCs (yellow arrowheads). Insets show enlarged views of type II NB lineages from the area which are highlighted with dashed squares in (H-K).

(L-N) Quantifications of number of GFP labeled type II NBs (L), percentage of GFP* type II NB lineages with mlINPs (M) and percentage of Ase+ type II NBs (N) corresponding to (H-K). ***, \( P < 0.001 \). **, \( P < 0.01 \). *, \( P < 0.05 \). NS, no significance.

(O-R) Type I NB lineages and type II NB lineages are labeled with mCD8-GFP (in green) driven by insc-GAL4 and counterstained for Dpn (in red) and Ase (in blue). One wild type brain lobe has eight Dpn+ Ase- type II NBs (white arrows) with associated mlINPs (white arrowheads) (O). Knockdown of Six4 by expressing two copies of UAS-Six4 RNAi driven by insc-GAL4 results in one ectopic type II NB lineage per lobe, while each type II NB lineage contains normal mlINPs (P). In pntP190 mutant brain lobes (Q), supernumerary type II NBs (outlined by dashed lines and pointed by white arrows) are produced and there are still abundant of Dpn+ Ase+ mlINPs (inset Q', from a different focal plan) are associated with type II NBs. When Six4 is knocked down in pntP190 mutant, there are eight type II NBs which are identified by Dpn+ Ase- (white arrows) and all type II NBs completely lose their associated mlINPs (R).

(S-T) Quantifications of number of Ase- type II NBs or Ase+ type I NBs (S) and number of mlINPs per Ase+ NB (T) with indicated genotypes. ***, \( P < 0.001 \). NS, no significance.

(U-V) Schematic diagrams of PntP1 knockdown or mutant type II NB lineages (U) vs. type II NB lineages with simultaneous reduction of PntP1 and Six4 (V).

Scale bars equal 10\( \mu \)m in A-F, and 50\( \mu \)m in H-K and O-R.
Discussion

Mechanisms underlying the maintenance of homeostasis of INP pool have been explored and decoded by a variety of previous studies. In this study, we identified a novel transcription factor, Six4, engaging into the regulatory network through interacting with other crucial factors during the development of type II NB lineages. We found Six4 is specifically expressed in type II NB lineages. Knockdown of Six4 results in supernumerary type II NBs, which arise from dedifferentiation of imINPs. We demonstrate that there is synergistic effect between Six and Erm and overexpressing Six4 in imINPs substitutes Erm’s function to prevent the dedifferentiation imINPs. Elevating Six4 levels in type II NBs antagonizes PntP1 activity and reduces PntP1 expression, leading to reduction/elimination of INPs and ectopic expression of Ase in type II NBs or Pros in imINPs. We also demonstrate that endogenous Six4 as a supplementary factor in addition to PntP1 in imINPs suppresses Pros to prevent premature differentiation of imINPs. Thus, our study reveals that Six4 maintains INP homeostasis in type II NB lineages (Fig. III-13).

Figure III-13. A schematic model of potential functions of Six4 in regulating INP development in type II NB lineages.

A schematic diagram shows potential functions of Six4 in type II NB lineage development. Late during imINP development, Six4 acts together with Erm to inhibit PntP1’s activity and expression, which ensures that imINPs become fatecommitted mINPs instead of dedifferentiating to type II NBs. In the newly generated imINPs, Six4 also contributes to the inhibition of nuclear Pros expression, thus preventing premature differentiation of INPs to GMCs.
**Expression of Six4 in cell types within type II NB lineages**

Although we have shown that Six4 is specifically expressed in cells from type II NBs to early mature GMCs in type II NB lineages by checking GFP tagged Six4 and the expression is specifically reduced/depleted by expressing UAS-Six4 RNAi, it is still worthwhile to test its protein expression patterns by using either anti-Six4 antibody or its mRNA expression patterns by using RNA probes. Because we noticed that the expression pattern of this particular six4-GFP reporter in brains younger than 2nd instar larval stage was more sensitive to genetic backgrounds and animal raising temperature, leading to GFP expression was detected in more type I NBs and persisted till neurons, although the specific expression within type II NBs or imINPs was similar (data not shown). In addition to genetic background-dependent effects, the superfolder GFP fusion may also affects the stability of Six4 proteins. Thus, the variance on GFP fusion protein expression makes characterizing Six4 expression in young type II NB lineages inaccurate. It is interesting and important to determine the expression of early Six4 because previous studies show that type II NBs originate from at least one origin in embryonic placode in Pdm neuroectoderm, which is demarcated as Six4 positive (Alvarez & Diaz-Benjumea, 2018; Hwang & Rulifson, 2011; Walsh & Doe, 2017), suggesting a possibility that Six4 may somehow involve in type II NB specification or early development. In addition, our study shows that there is a subset of Six4+ type I NBs. It is also interesting to digest do they share common features or common functions with Six4+ type II NBs, or does Six4 in these NBs show generic functions, given that some of type I NBs also originate from the same Six4+ Pdm placode (Alvarez & Diaz-Benjumea, 2018).

**Six4 prevents dedifferentiation of imINPs**

The main phenotype resulting from Six4 knockdown in type II NB lineages is the generation of supernumerary type II NBs. We provide several lines of evidence to demonstrate that the
generation of supernumerary type II NBs is likely due to dedifferentiation of imINPs. First, asymmetric divisions of type II NBs are not affected by Six4 knockdown as demonstrated by the normal asymmetric segregation of aPKC and Mira. Second, the expression pattern of Six4-GFP indicates that Six4 could function in imINPs. Third, the generation of supernumerary type II NBs resulting from the knockdown of Six4 in imINPs, although the phenotype is weak due to the persistence of Six4 expression in imINPs, provides evidence to support that Six4 could function in imINPs to prevent dedifferentiation of imINPs. The persistence of Six4 proteins after expressing UAS-Six4 RNAi in imINPs could be due to the following two reasons. One is that knockdown of Six4 in imINPs with erm-GAL4 may not be very efficient. The imINP state only lasts about 8-10hrs (Janssens et al., 2017). erm-GAL4 expression in imINPs is not turned on immediately and does not reach its peak at least until 2-3hrs after the birth of imINPs (Janssens et al., 2017). There may not be enough time for significant knockdown of Six4 because of the short duration of the imINP state and the delayed expression of erm-GAL4 in imINPs. Another reason could be that Six4 proteins expressed in type II NBs may be inherited by imINPs and continue to function in imINPs if these proteins cannot be degraded soon enough. These rationales were demonstrated by the results that knocking down Six4 driven by erm-GAL4 (II) and erm-GAL4 (III) (which is expressed in Ase+ imINPs) failed to produce any extra type II NBs (Fig. III-S1A-C) and expression of Six4 protein was still present at a comparable level as that in wild type imINPs (Fig. III-S1D-E”). Fourth, the genetic and biochemical interactions between Six4 and Erm suggest that Six4 functions in the same pathway as Erm, which has been well demonstrated for its role in preventing dedifferentiation of imINPs (Janssens et al., 2014; Koe et al., 2014; Weng et al., 2010). Finally, the rescue of the supernumerary type II NB phenotype resulting from Erm knockdown by overexpressing Six4 specifically in imINPs also supports that Six4 could functions in imINPs to prevent their dedifferentiation. However, to provide definitive evidence to demonstrate that that the supernumerary type II NBs resulting from Six4 knockdown are
generated from dedifferentiation of imINPs, it will be essential to perform a lineage tracing experiment to follow the fate of the progeny of imINPs. Unfortunately, we were not able to perform the lineage tracing experiment due to some technical difficulties.

We also demonstrate that the majority of supernumerary type II NBs resulting from Six4 knockdown are probably generated at embryonic stages and possible also early larval stages. First, the majority of the supernumerary type II NBs establish their own derived lineages and coexistence of multiple type II NBs, which are presumably generated from dedifferentiation of imINPs, is only observed in a small percentage of single isolated type II NB lineages at late larval stage. Second, knocking down Six4 specifically at larval stages with \textit{pntP1-GAL4} in combination with \textit{tub-GAL80ts} results in much fewer supernumerary type II NBs than knocking down Six4 as soon as type II NBs are specified at embryonic stage with \textit{pntP1-GAL4} alone (Fig. III-S2).

Although the exact underlying mechanisms remain to be investigated, the supernumerary type II NBs seems unlikely to be generated by aberrant specification of NBs from the neuroectoderm because the \textit{pntP1-GAL4} driver used for Six4 knockdown is only expressed in type II NB lineages but not in type I NBs or the neuroectoderm before type II NBs are specified (Alvarez & Diaz-Benjumea, 2018; Walsh & Doe, 2017; Zhu et al., 2011). A recent study has shown that GMCs generated from type I NBs and INPs from type II NBs during early development are more susceptible to malignant transformation because of high expression of the BTB-zinc finger protein Chinmo (Narbonne-Reveau \textit{et al}, 2016). Chinmo is expressed in young NBs and their progeny and its expression gradually decreases with the progression of the development. After puparium formation, Chinmo expression is suppressed. Chinmo was first identified as a neuronal temporal identity gene and is generally involved in specification of larval-born neurons, including those generated in type II NB lineages (Kao \textit{et al}, 2012; Narbonne-Reveau \textit{et al}., 2016; Ren \textit{et al}, 2017; Zhu \textit{et al}, 2006). Later studies suggest that Chinmo also promotes the self-renewal of stem
cells and tumor growth (Dillard et al., 2018; Flaherty et al., 2010). In the absence of Chinmo, the growth of GMC- or INP-derived tumors resulting from the loss of Pros or Brat, respectively, is significantly suppressed (Narbonne-Reveau et al., 2016). Therefore, one interesting possibility could be that the majority of the supernumerary type II NBs resulting from Six4 knockdown are generated early during development when Chinmo expression is high. At embryonic stages, type II NBs also divide to produce imINPs (Alvarez & Diaz-Benjumea, 2018; Walsh & Doe, 2017), which could dedifferentiate to become type II NBs when Six4 is knocked down. When Six4 is knocked down at later developmental stages, the chance of imINPs to initiate tumorigenic dedifferentiation might be much lower because of significantly reduced expression of Chinmo. It would be interesting to investigate in the future how manipulating Chinmo expression might affect the generation of supernumerary type II NBs resulting from Six4 knockdown.

Previous studies demonstrate that one main driving force for the dedifferentiation of imINPs is PntP1 (Janssens et al., 2017; Li et al., 2017). Other factors, such as Brat, Numb, and Erm, that are required for preventing dedifferentiation of imINPs, all function by terminating PntP1’s function/expression in imINPs either directly or indirectly (Janssens et al., 2017; Komori et al., 2018b; Li et al., 2017; Li et al., 2016; Reichardt et al., 2018). Our results suggest that Six4 prevents dedifferentiation of imINPs similarly by inhibiting PntP1’s function and expression in imINPs. From our loss-of-function studies, we show that knockdown of Six4 leads to delayed termination of PntP1 expression, which results in ectopic expression of PntP1 in mINPs. From our gain-of-function analyses in both type I and type II NB lineages, we demonstrate that Six4 can inhibit PntP1’s function. In type I NB lineages, misexpressing Six4 blocks the PntP1 misexpression-induced suppression of Ase and generation of INP-like cells. In type II NB lineages, overexpressing Six4 inhibits the activation of Erm by PntP1 even if PntP1 is overexpressed. Results from our biochemical studies suggest that Six4 inhibits PntP1’s activity
probably by forming a complex with PntP1. Six4 may function as a transcriptional repressor by
recruiting co-repressors such as Gro or Eya like in other tissues (Johnston et al., 2016; Kobayashi
et al., 2001). By recruiting co-repressors, Six4 may inhibit PntP1 transcriptional activity.
Alternatively, binding of Six4 may disrupt binding of PntP1 to DNAs or its tissue-specific
cofactor(s). However, it is not clear whether the suppression of PntP1 expression by Six4 has
anything to do with the inhibition of PntP1’s activity or involves a totally independent
mechanism. One possibility could be that PntP1 activates its own expression directly or indirectly
(such as through positive feedback mediated by Tll, see CHAPTER II) for maintaining the
expression and inhibition of PntP1’s activity by Six4 disrupts the positive self-regulatory loop of
PntP1, which eventually leads to termination of PntP1 expression in imINPs and maturation of
INPs. Such positive self-regulation is a common mechanism in regulating gene expression
(Crews & Pearson, 2009). In support of this notion, inhibition of PntP1’s activity seems to occur
before the termination of PntP1 expression in imINPs because PntP1 coexists with Ase in imINPs
late during imINP development, which indicates PntP1 is no longer functioning to suppress Ase
in these cells before PntP1 expression is lost. In order to elucidate exactly how Six4 inhibits
PntP1’s activity and expression, it will be essential to examine the interactions between Six4 and
PntP1 specifically in type II NB lineages and map the DNA binding profiles of Six4 and PntP1 in
the future.

Our Six4 knockdown results show a consistent increase of type II NBs, though the phenotype of
Six4 knockdown is not as dramatic as knockdown of Erm, another master transcription factor to
maintain the differentiation of INPs. The weaker effect of Six4 is also reflected by the fact that
overexpressing Six4 in imINPs largely, but still not fully, rescues the Erm knockdown
phenotypes. In addition to above reasons, including the low efficiency of UAS-RNAi constructs
due to short imINPs state, late erm-GAL4 expression, functional Six4 proteins inherited by
imINPs from type II NBs, and the absence of Chinmo during late larval stages, it is reasonable that there may be other cofactors functioning with Six4 in the same pathway or directly forming complex with Six4 to carry out its full activity to prevent dedifferentiation of imINPs, considering that the conserved SD domain in Six4 protein engages in protein-protein interactions (Kumar, 2009; Pignoni et al., 1997). In other tissues or organs, Six4 directly or indirectly interacts with multiple cofactors, such as Eya, Gro, So, or UTX (Bischof et al., 2018; Johnston et al., 2016; Kobayashi et al., 2001; Ohto et al., 1999; Seenundun et al, 2010). However, our screen of knocking down Six4 together with any of above candidates did not identify positive hit which would dramatically enhance the generation of supernumerary type II NBs. It will be significant if any cofactors of Six4 are identified, which will complete the regulatory network of INP development.

**Functional relationship between Six4 and Erm in preventing dedifferentiation of imINPs**

Findings from this study suggest that Six4 and Erm function similarly to prevent dedifferentiation of imINPs by antagonizing the activity and expression of PntP1. Six4 and Erm could act in the same pathway or in two independent pathways in parallel to perform their functions. We rule out the possibility of cross-regulation between Six4 and Erm by showing that knocking down the expression of either one of them does not affect the expression of the other. However, Six4 and Erm genetically interact in preventing dedifferentiation of imINPs. We show that the supernumerary type II NB phenotype resulting from Six4 knockdown is significantly enhanced in $erm^{+/}$ heterozygous mutant background and that double knockdown of Six4 and Erm leads to synergistic enhancement of the supernumerary type II NB phenotype. The genetic interaction suggests that Six4 and Erm function in the same pathway even though they do not regulate each other’s expression. In support of these genetic interaction data, our biochemical studies reveal
that Six4, Erm, and PntP1 could form a complex. Therefore, Six4 and Erm likely act together in
the same complex to inhibit PntP1 activity in imINPs and both Six4 and Erm are required for
complete inhibition of PntP1 activity. The presence of all three proteins, Six4, Erm, and PntP1, is
probably essential for the stability of the complex as our co-IP data demonstrate that the presence
of Flag-PntP1 or Six4-HA enhances the binding between Myc-Erm and Six4 or between PntP1
and Erm, respectively. In the absence of Six4, PntP1 and Erm may form a less stable complex and
some PntP1 proteins may remain free and active, which will lead to activation of its target genes
and subsequent dedifferentiation of imINPs. In addition to increasing the stability of the complex,
the presence of both Six4 and Erm in the complex may also help recruit different cofactors that
are essential for complete inhibition of PntP1’s activity in imINPs. For example, Six4 may recruit
unknown co-repressors that will confer transcriptional repression activity, whereas Erm may
recruit epigenetic modifiers SWI/SNF complex proteins and/or HDAC proteins to make the
genetic loci that contain PntP1 target genes inactive. However, it is unlikely that the presence of
both Six4 and Erm is just for recruiting different cofactors because it will be difficult to explain
why overexpressing Six4 can rescue the Erm knockdown phenotype if all PntP1 proteins have
already bound to Six4 in the absence of Erm. Excessive amounts of Six4 proteins would not help
to recruit the cofactor(s) that are normally only recruited by Erm to the complex because Six4 and
Erm belong to different protein families and do not share protein domains or structural
similarities. We did not address if the direct binding compromising PntP1 functions through
sequestering PntP1 proteins, facilitating PntP1 degradation, interfering the accessibility of PntP1
to downstream target gene regulatory regions, or disrupting the interactions between PntP1 and its
context dependent cofactors. It is important to determine which exact aspect would Six4 or Erm
interrupt in the future.
Our working model that a complete inhibition of PntP1’s function requires its formation of a complex with both Six4 and Erm probably also explains why PntP1 activity is normally not inhibited by Six4 in type II NBs and newly generated imINPs even though Six4 is expressed in these cells. It could be simply because Erm is not expressed in these cells. Therefore, Six4 can only inhibit PntP1 activity in type II NBs when it is overexpressed and even overexpressed Six4 can only partially inhibit PntP1’s function. In contrast, when Erm is misexpressed in type II NBs, it can fully inhibit PntP1’s function as indicated by the ectopic Ase expression and the loss of INPs in all type II NB lineages (Li et al., 2016; Weng et al., 2010) because endogenous Six4 is already expressed in type II NBs.

Our results demonstrate Six4 prevents imINPs reverting to type II NBs, though the effect is much weaker than Erm. We have shown that they are mutually independent on transcriptional levels, suggesting that they are not likely to function in the linear pathway. Given that our results show Six4 and Erm indeed have weak but synergistic effects and they both antagonize PntP1 function through direct binding, it is high likely that they could crosstalk at least by targeting both PntP1. However, it remains unknown if they crosstalk at other points, considering that Erm itself is shown in committing imINPs fate through impacting on various targets other than PntP1, including Notch, Tll, maybe Pros and other unknown pathway (Koe et al., 2014; Rives-Quinto et al., 2020; Weng et al., 2010). Thus, to elucidate the question, it is important and exciting if their downstream targets in imINPs are deciphered.

**Equilibrate the expression levels and activity of Six4 in type II NBs**

PntP1 is a master regulator to specify and maintain type II NB identity, promote INP genesis, and maintain INP pool (Xie et al., 2016; Xie et al., 2014; Zhu et al., 2011). Considering that
endogenous Six4 co-localizes with PntP1 in type II NBs and imINPs, it is surprising that overexpression of Six4 by using pntP1-GAL4 antagonizes the function of endogenous or even the overexpressed PntP1, resulting in loss of Ase suppression in type II NBs, loss of Pros suppression in imINPs, and reduction/elimination of INP numbers. All the deficits are detrimental for maintaining INP homeostasis or normal type II NB lineage development. It implies that in order to maintain normal type II NB lineage development, Six4 in type II NBs need to be precisely controlled at physiological levels to keep PntP1 function normally, in addition to the suppression of Erm. Erm also shows antagonization on PntP1. However, Erm is not expressed in type II NBs at all and the suppression of Erm is achieved by Notch pathway or some bHLH-O proteins to block its activation (Almeida & Bray, 2005; Janssens et al., 2017; Li et al., 2017; Li et al., 2016). Therefore, it is very likely that type II NBs or imINPs would utilize a specific negative feedback machinery to equilibrate Six4 expression levels. On the other hand, though the above results are based on Six4 gain-of-function analysis, it is possible that even endogenous Six4 itself has the potential to antagonize PntP1 function. Considering PntP1 still functions normally in the present of native Six4 in type II NBs and imINPs, we hypothesize that there are two possible mechanisms to ensure the function of PntP1 is not interrupted by Six4. 1) It is possible that the overall activity of Six4 at physiological levels does not antagonize PntP1 efficiently and PntP1 activity or expression levels are still above the limit to perform its normal function, probably due to the lack of other cofactors such as Erm which helps stabilize the complex of Six4-PntP1. Second, there may be additional independent mechanisms involved to inhibit the activity of Six4 in type II NBs to prevent it antagonizing PntP1. Thus, it is important in future to identify either the specific negative feedback regulatory pathway to restrict Six4 expression levels or the mechanisms involved to inhibit Six4 activity in type II NBs.

A potential role of Six4 in preventing premature differentiation of INPs
In addition to preventing dedifferentiation of imINPs by inhibiting the function and expression of PntP1, our results suggest that Six4 might also be involved in preventing premature differentiation of INPs. Although knockdown of Six4 alone in type II NB lineages does not lead to dramatic loss of mINPs, knockdown of Six4 completely eliminates mINPs in PntP1 knockdown or mutant type II NB lineages. The elimination of mINPs is not because of complete transformation of type II NBs to type I NBs because knockdown of Six4 does not affect the expression of Ase in the PntP1 knockdown or mutant type II NBs. Rather, it was because of enhanced ectopic expression of Pros in newly generated imINPs, which leads to premature differentiation of INPs to GMCs. Our results show that in Six4 and PntP1 double knockdown brains, nuclear Pros is ectopically expressed in all newly generated imINPs at 48hrs AEL, whereas in PntP1 knockdown brains, 2-5 newly generated imINPs in each brain lobe remain Pros-negative at the same stage. Although we did not examine the expression of Pros before 48hrs AEL in Six4 and PntP1 double knockdown larvae, the ectopic nuclear Pros is probably expressed in every single newly generated imINPs at earlier developmental stages as soon as they are produced from type II NBs because the generation of supernumerary type II NBs is completely inhibited in these animals. The ectopic expression of nuclear Pros inhibits the dedifferentiation of imINPs as demonstrated in btd brat double mutant type II NB lineages (Komori et al., 2014a). Thus, the significant enhancement of the ectopic Pros expression suggests that Six4 likely acts together with PntP1 to suppress Pros expression in newly generated imINP and prevent premature differentiation of INPs. It is important to decipher the molecular mechanisms how Six4 and PntP1 function together to suppress Pros expression in type II NBs and imINPs. One possibility is that Six4 may need to interact with other cofactors, other than Erm, to suppress Pros. Alternatively, considering PntP1 is the main factor to specify type II NBs and PntP1 binds Six4, the direct binding between Six4 and PntP1 may also be involved in suppressing Pros in not only imINPs but also type II NBs. We have not tested this possibility yet.
and it would be difficult to determine optimal Six4 expression levels, since the elevated levels of Six4 compromise PntP1 function when Six4 and PntP1 are coexpressed in type I or type II NBs. On the other hand, it is quite surprising that Six4 functions together with PntP1 to suppress Pros, considering that late during imINP development Six4 acts together with Erm to inhibit PntP1’s expression and function. The opposite effects of Six4 on PntP1’s function at different stages of imINP development indicate that Six4’s function might be context-dependent. It could be due to availability of different cofactor(s) that bind to Six4, such as Erm or other factors, or other unknown mechanisms. Six4 and PntP1 might have partial redundancy in suppressing Pros expression in newly generated imINPs, but PntP1 could have a more prominent role. Alternatively, Six4 may not be involved in the suppression of Pros in normal type II NB lineages, but only when PntP1’s expression is reduced and/or the development of type II NB lineages is impaired would Six4 somehow become a suppressor of Pros expression. Our previous studies have shown that Btd is also required to suppress Pros expression in newly generated imINPs and prevent premature differentiation of INPs to GMCs (Xie et al., 2014). It would be interesting to test the functional relationship between Six4 and Btd in suppressing Pros expression in newly generated imINPs.

Materials and Methods

Fly stocks

The Six4-GFP line (#67733, Bloomington Drosophila Stock Center [BDSC]) was used to detect Six4 expression. E(spl)m-GFP reporter line (Almeida & Bray, 2005) was used to label NBs and mINPs. GAL4 lines, including pntP1-GAL4 (also called GAL414-94) (Zhu et al., 2011), erm-GAL4(II) or erm-GAL4(III) (Pfeiffer et al., 2008; Xiao et al., 2012), insc-GAL4 (Luo et al., 1994), and ase-GAL4 (Brand et al., 1993; Jarman et al., 1993) were used for UAS-transgenes expression. UAS-Six4 RNAiHM05254 (#30510, BDSC) and UAS-Six4 RNAi lines (#30456, #48598, and
#104958, VDRC) were used for knocking down Six4. Knockdown of Erm was carried out with UAS-Erm RNAi (#26778, BDSC). Knockdown of pnt was carried out with UAS-pnt RNAi (#35038, BDSC) or pnt RNAi (#31396, BDSC). FlyORF #F000049 line (named after UAS-Six4ORF-HA or UAS-Six4ORF in this study) was used for mis-/over-expression of Six4. UAS-PntP1 (Zhu et al., 2011) was used for mis-/over-expression of PntP1. pros17 (Doe et al., 1991), erm2 (Weng et al., 2010) or pntP159 (Xie et al., 2016) alleles were used to reduce Pros, Erm or PntP1 expression. Tub-GAL80ts line (#7019 or #7017, BDSC) were used for temporal control of UAS-Six4 RNAi or UAS-PntP1 expression.

**UAS-transgene expression**

For RNAi knockdown, except in Figure III-4A-D, embryos were collected for 8–10hrs at 25°C and shifted to 29°C to boost the efficiency. In Figure 4A-D, embryos were collected for 8–10hrs and raised at 25°C. UAS-dcr2 was coexpressed with UAS-RNAi transgenes to enhance the RNAi knockdown efficiency. For mis-/over-expression of Six4, PntP1 in type II NB lineages, embryos were collected for 8–10hrs at 25°C and shifted to 29°C. When PntP1 is misexpressed by insc-Gal4, temperature sensitive tub-Gal80ts (#7019, BDSC) was used to avoid the lethality at embryonic stages: insc-GAL4 and tub-Gal80ts was recombined first and embryos were collected for 8–10hrs and raised to hatch at 18°C, then shifted to 29°C for 3–4 days to maximize the efficiency. When tub-GAL80ts (#7017, BDSC) was used to temporally control the expression of UAS-Six4 RNAi, embryos were collected for 8–10hrs and raised at 18°C for 2 days. Then hatched larvae were cultured at 29°C for additional 3–4 days before dissection.

**Immunostaining and confocal microscopy**

Larvae at desired stages were sacrificed and dissected. Brain lobes along with VNCs were immunostained as previously described (Lee & Luo, 1999) except that the fixation time was
increased to 35 mins. Immunocytochemistry for transfected S2 cells at log phase were performed as described previously (Neal et al., 2019). Primary antibodies used were chicken anti-GFP (Catalog #GFP-1020, Aves Labs, Tigard, Oregon; 1:500-1000), rabbit anti-DesRed (Catalog #632392, Takara Bio USA, Inc., Mountain View, California; 1:250), rat anti-mCD8 (Catalog #12-0088-42, Thermo Fisher Scientific, Waltham, Massachusetts, 1:100), rabbit anti-Dpn (1:500) (a gift from Dr. Y.N. Jan) (Bier et al., 1992), guinea pig anti-Ase (a gift from Dr. Y.N. Jan, 1:5000) (Brand et al., 1993), rat anti-Erm (a gift of Dr. C. Desplan; 1:300), rabbit anti-PntP1 (a gift of Dr. J. B. Skeath; 1:500) (Alvarez, 2003), mouse anti-Pros (Developmental Studies Hybridoma Bank; 1:20), rabbit anti-Miranda (a gift from Dr. Y.N. Jan, 1:1000), mouse anti-aPKC (Catalog #sc-17781, Santa Cruz Biotechnology, Inc., Dalla, Texas, 1:500), mouse anti-PH3 (Catalog # ab80612, Cambridge, Massachusetts, 1:500). Secondary antibodies conjugated to Daylight 405 (1:300-400), Daylight 488 (1:100), Cy3 (1:500), Rhodamine Red-X (1:500), Daylight 647 (1:500) or Cy5 (1:500) used for immunostaining are from Jackson ImmunoResearch (West Grove, Pennsylvania). Images were collected using a Carl Zeiss LSM780 confocal microscopy and processed with Adobe Photoshop. Two-tailed student’s T-test or Welch's T-Test, or Mann-Whitney test (for data not normally distributed) was used for statistical analyses.

**Plasmid Constructs and generation of transgenic lines**

For generating the UAS-Six4·HM05254Resis construct which is resistant to UAS-Six4 RNAi·HM05254:

A middle fragment containing the region targeted by the dsRNA-HM05254 (Ni et al., 2009) was synthesized with the introduction of silent mutations according to the optimal *Drosophila* codon usage. The sequence of the fragment (named dsRNA-HM05254Resis) is as follows:

\[
5'\text{gaagtggcggcgggattggggggcaatgccggcagtggtggccaccTgATcagcAAcCTgACgGCtGCgCAtAAtAT}\n\text{GagcGCtGTctcctcTTcCCeATtGAeGCgAAaATGCTeCAaTTfTcgcACeCAGgtaggatacttc}\n\text{cctatatctagactttagcagacataaatgatgaacttccttttagATtCAaTgtATGTGtGAAgCeCTGCAaCAaA}
\]
AaGGAGAtATgGAAaCTGACgACcTTtCTgTCcCTGcCcCCgCtecGAAaTTtTTCAaACaA
AtGAatccGTgCTGCgtGCTgTGCgATGGTcGCCTAtAACCTcGAsCAaTTtCAGGAaCTcTAC
AArCtgcggagacgcactgcttttcgat3’. The underlined parts are the regions that are targeted by the double-strand dsRNA-HM05254 and carry silent mutations indicated by lowercase. The lowercase region between the two underlined parts is the 2nd intron of Six4. The lowercase regions in both 5’ and 3’ terminus are genomic Six4 sequences that are flanking the dsRNA-HM05254.

A 5’-end fragment that includes a sequence from exon 1, intron 1, and 5’-part of exon 2 with a 40-bp overlap with the middle fragment; and a 3’-end fragment that includes a sequence from 3’-part of exon 3 to the last exon (exon 5) with a 45-bp overlap with the middle fragment were amplified with CloneAmp HiFi PCR Premix (Takara Bio., Mountain View, California) from Drosophila genomic DNA using the following primer sets. The underlined parts of these primers are the sequences that overlap either with the sequence of the middle fragment (primers Six4J1R and Six4J3F) or with the sequence of the pJET vector (primers Six4J1F and Six4J3R).

Six4J1F: 5’- agctgagaatattgtgaggagatcttcttaggaagaagattttcgctgctttgcagtcgagttttga-3’
Six4J1R: 5’-ccaccactgccggcattgcccccaatccgggcaactttctctgcaggccggccataga-3’
Six4J3F: 5’-attttacaactcaatactgtgagacgcactgcttttctcttctctgatcaagtacccca-3’
Six4J3R: 5’-aatccgagatatgtggagatcttcttaggaagaagattttcgctgctttgcagtcgagttttga-3’

The above three fragments along with a commercial linearized pJET1.2/blunt vector (Thermo Fisher Scientific, Waltham, Massachusetts) were assembled with NEBuilder® HiFi DNA Assembly Master Mix (Catalog # E2621S, New England Biolabs, Ipswich, Massachusetts). The assembled 5’-UTR- Six4H05254Resis (with introns) -3’UTR cassette (named as Six4H05254Resis) was excised with BglII/XhoI from the pJET vector and subcloned into pUAST. The construct was injected into Drosophila embryos by the Rainbow Transgenic Flies, Inc. (Camarillo, California) and the transformants were generated based on a standard P-element transposons protocol.
pAFW-PntP1 (i.e., Act5C-Flag-PntP1) and pAMW-Erm (i.e., Act5C-Myc-Erm) vectors (gifts from Dr. H. Y. Wang) (Koe et al., 2014) were used for expressing Flag-PntP1 and Myc-Erm in S2 cells under the control of the Act5C promoter. For generating the pUAST-Six4-3×HA (i.e., UAS-Six4-HA) construct, the Six4-3×HA cassette was amplified from FlyORF #F000049 line with CloneAmp HiFi PCR Premix (Takara Bio USA, Inc.) and inserted into pUAST vector between BglII/XhoI sites. For generating the pAMW-EGFP construct, the EGFP sequence was amplified from pH-stinger (Drosophila Genomics Resource Center, Bloomington, Indiana) and was used to replace the erm fragment between AgeI/NheI sites in pAMW-Erm. pAMW-EGFP (i.e., Act5C-Myc-EGFP) was used as a negative control. Copper inducible pMT-GAL4 (Klueg et al., 2002) was used to drive UAS-Six4-HA expression. Mock vectors pUAST and pAW (generated by deleting Flag-PntP1 cassette from pAFW-PntP1) were co-transfected as controls to balance the amounts of each vector transfected. Primers used for making these constructs are listed below.

Six4f: 5’-AAATAGATCTATGGTATTTGACAGAATTGGACGGCAA-3’
Six4-HAr: 5’-AAATCTCGAGACGCTTAGTGCTAGCGTCAA-3’
EGFPf: 5’-AAATACCGGTATATGGTGAGCAAGGGCGAGGA-3’
EGFPr: 5’-TTAAGCCTAGCTTACTTGTACAGCTCGTCCATGCCCAGA-3’

S2 cell culture and co-immunoprecipitation

Drosophila S2 cells were maintained and transfected in Schneider's Medium (Catalog #21720004, Thermo Fisher Scientific) containing 10% Heat-Inactivated Fetal Bovine Serum (Catalog #10082147, Thermo Fisher Scientific) following Drosophila Schneider 2 (S2) Cells USER GUIDE (Thermo Fisher Scientific). 5µg of each indicated vector were co-transfected by using Calcium Phosphate Transfection Kit (Catalog #K278001, Thermo Fisher Scientific). 500 µM CuSO4 (Catalog #451657, Sigma-Aldrich Co., St. Louis, Missouri) was used to induced
GAL4 expression 1 day after transfection. S2 cells were collected 72hrs after CuSO₄ induction for protein homogenization in 1% NP-40 lysis buffer (50 mM Tris-Cl, pH 8.0/1% NP-40/150mM NaCl) with Halt™ Protease Inhibitor Cocktail (Catalog #78430, Thermo Fisher Scientific). Immunoprecipitation was performed with 2μg rabbit anti-HA mAb (Catalog #MA5-27915, Thermo Fisher Scientific), mouse anti-Myc mAb (Catalog #MA1-21316, Thermo Fisher Scientific) or mouse ANTI-FLAG® M2 antibody (Catalog #F1804, Sigma-Aldrich Co, St. Louis, Missouri) for overnight incubation at 4°C, followed by incubation with protein A/G agarose beads (Catalog #sc-2003, Santa Cruz Biotechnology, Inc.) (pre-blocked with 5% BSA) at 4°C for 4~5hrs. Collected beads were washed 4~5 times with cold lysis buffer containing protease inhibitor cocktail and eluted for SDS-PAGE separation. Proteins transferred to PVDF were blotted with mouse anti-HA antibody (Catalog #sc-7392, Santa Cruz Biotechnology Inc.), rabbit anti-Myc antibody (Catalog #2272S, Cell Signaling Technology, Danvers, Massachusetts), or mouse ANTI-FLAG® M2 antibody (Catalog #F1804, Sigma-Aldrich Co, St. Louis, Missouri), or rabbit anti-PntP1 (a gift of J. B. Skeath; 1:500). Secondary antibodies used were HRP conjugated anti-mouse IgG (Catalog #7076S, Cell Signaling Technology) or anti-rabbit IgG (Catalog #7074S, Cell Signaling Technology). Chemiluminescent Assay was performed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Catalog #34577, Thermo Fisher Scientific) or Femto Maximum Sensitivity Substrate (Catalog #34095, Thermo Fisher Scientific). Images were collected with ChemiDoc Imaging Systems (Bio-Rad, Hercules, California). For quantifying the co-IP, the intensities of the related bands from the western blot were first determined by Image Lab Software (Bio-Rad, Hercules, California). The % co-IP was calculated by dividing the co-IPed protein by the 100% input of the co-IPed protein, then normalized by the IPed protein to correct the differences in immunoprecipitation. Three replicates were performed for each condition and the paired t test was used for statistical analyses.
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Competing interests

The authors declare no competing or financial interests

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Figure III-S1. Expression of UAS-Six4 RNAi driven by erm-GAL4 does not abolish Six4 expression.

(A-B) A larval brain expressing UAS-Six4 RNAi driven by erm-GAL4(II) together with erm-GAL4(III) (B) has the same number of type II NBs as a wild type brain does (A).

(C) Quantifications of number of type II NBs in wild type brains and brains expressing UAS-six4 RNAi driven by erm-GAL4 (II) and erm-GAL4(III). The number on top of each bar indicates the number of brains examined. NS, not significance.

(D-E”) Six4-GFP expression in wild type type II NB lineages (D-D”) and type II NB lineages that express two copies of UAS- Six4 RNAi driven by erm-GAL4(II) (E-E”). Open arrows point to Ase− Dpn− imINPs and yellow arrows point to Ase+ Dpn− imINPs.
Figure III-S2. Late knockdown of Six4 in larval stage leads to generation of extra type II NBs to a lesser extent.

(A) A wild type larval brain lobe contains only 8 type II NBs.
(B-C) Larval-specific expression of one (B-B2) or two copies (C) of UAS-Six4 RNAi driven by pntP1-GAL4 in combination with tub-GAL80ts to larval stages still leads to the generation of extra type II NBs, but the phenotype is much weaker than knocking down Six4 from embryonic stage. (B1) and (B2) are enlarged views of the areas highlighted with dashed squares in (C). Note that two type II NBs (arrows) co-exist in a single lineage in both (B1) and (B2).
(D) Quantifications of the number of type II NBs in brains expressing one or two copies of UAS-Six4 RNAi after larval hatching. The number on top of each bar indicates the number of brains examined. ***, p < 0.001.
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Brand M, Jarman AP, Jan LY, Jan YN (1993) asense is a Drosophila neural precursor gene and is capable of initiating sense organ formation. *Development* 119: 1-17


CHAPTER IV. Mediator complex subunits Kohtalo and Skuld interact with the Ets protein Pointed P1 to prevent dedifferentiation of intermediate neural progenitors

Running title: Skd and Kto regulating development of intermediate neural progenitors

Key words: intermediate neural progenitors/neuroblasts/Skd/Kto/Drosophila

The work of this chapter is under preparation for manuscript submission in the future.

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Contribution Statement

R.C. and S.Z. conceived the idea, designed the project and approaches. R.C. carried out the majority of experiments, collected and analyzed the data, generated figures and wrote the manuscript. X. L. performed transcription factor screen and identified Skd and Kto genes in regulating type II NB lineage development and partial experiments related to Figure IV-1A-H, Figure IV-2, Figure IV-4F-H”, and Figure IV-5A-L. S.Z. and R.C. revised the manuscript.
Abstract

In order to boost the number and diversity of neurons generated from neural stem cells, intermediate neural progenitors (INPs) in *Drosophila* type II neuroblast (NB) lineages need to avoid dedifferentiation, which otherwise will result in tumorigenic overproliferation of type II NBs. In the genetically unstable immature INPs (imINPs), the Ets protein PntP1 promotes differentiation or maturation of INPs by activating the expression of dfez zinc finger protein Earmuff (Erm), which in turns inhibits the activity and expression of PntP1. In this study, we identified additional components involved in preventing the dedifferentiation of imINPs and uncovered how they engage into the PntP1-Erm regulatory network. Our results from genetic and biochemical studies show that mediator complex kinase module subunits Skuld (Skd) and Kohtalo (Kto) specifically function as cofactors of PntP1 in imINPs to activate Erm expression. Loss of Skd and Kto leads to generation of supernumerary type II NBs due to the loss of Erm expression and subsequent dedifferentiation of imINPs. We demonstrate that Skd and Kto form a complex with PntP1 by co-immunoprecipitation (co-IP). By systematically testing genetic interactions with Six4 in preventing dedifferentiation of imINPs, we also found that only a subset of mediator complex core subunits appears to be involved in preventing dedifferentiation of imINPs, indicating that not all mediator core subunits may function together in the same complex. Therefore, our study reveals novel functions of Skd and Kto in regulating the development of *Drosophila* larval INPs.
**Introduction**

Intermediate neural progenitors (INPs) in *Drosophila* larval brains are analogues of intermediate progenitor cells (IPCs) in mammalian neocortex, serving as a secondary amplifier in addition to the primary neural stem cells (NSCs) to boost the neuronal output and diversity, leading to the enlarged brain complexity (Franco & Müller, 2013). To achieve this, INPs undergo several rounds of division, to maintain themselves in a manner of transcriptional heterogeneity and produce terminal dividing ganglion mother cells (GMCs), which generate neurons or glia (Bayraktar & Doe, 2013; Bello *et al.*, 2008; Boone & Doe, 2008; Bowman *et al.*, 2008; Wang *et al.*, 2014). Any dis-regulation on proliferation or differentiation of INPs would lead to developmental disorders of brain structure and functions. For example, loss of control on proliferation/differentiation of INPs results in brain tumor genesis, whereas, depletion of INP pool leads to reduction of brain size, i.e. microcephaly, or premature neurogenesis with intellectual disability (Bowman *et al.*, 2008; Emmenegger & Wechsler-Reya, 2008; Krieger *et al.*, 2019; Shimada *et al.*, 2016; Zhang *et al.*, 2019). Thus, properly maintaining the identity and pool of INPs is crucial to generate enough quantity with sufficient diversity of neurons, which finally are integrated into functional circuits in brains.

Before INPs commit to transit amplification, they are transcriptionally unstable and they are called immature INPs (mINPs). ImINPs are produced during a process of asymmetric divisions of type II neuroblasts (NBs, the NSCs of *Drosophila*), during which type II NBs are also self-renewed. Then imINPs differentiate to become genetically mature INPs (mINPs) which undergo transit-amplification by self-renewing and producing GMCs to generate neurons or glia (Bello *et al.*, 2008; Boone & Doe, 2008; Bowman *et al.*, 2008). Due to the transit-amplification and temporally transcriptional diversity of INPs, the number and diversity of neurons produced from type II NBs are significantly boosted (Bello *et al.*, 2008; Boyan & Reichert, 2011; Izergina *et al.*, 2018).
2009; Wang et al., 2014), compared to those from type I NBs, another type of Drosophila NSCs that directly generate GMCs (Truman & Bate, 1988). Type II NBs can be distinguished from type I NBs by the lack of proneural protein Asense (Ase) (Brand et al., 1993; Jarman et al., 1993) and the expression of Ets protein PntP1 which is a master regulator that specifies and maintains the identity of type II NBs and promotes generation of INPs (Zhu et al., 2011).

Genetically unstable ImINPs are susceptible to various dysregulation, for example, failure to remove NB self-renewal factors, such as the bHLH family transcription factor Deadpan (Dpn) or the Notch effector E(spl) family proteins, or Tailless (Tll), another type II NBs specific regulator. Studies have shown or suggested that one of the common mechanisms used by these factors is to directly or indirectly restrict the expression levels or activity of PntP1, the master regulator of type II NBs (Hakes & Brand, 2020; Quinto-Rives et al., 2020; San-Juán & Baonza, 2011; Song & Lu, 2011; Zacharioudaki et al., 2012; Zhu et al., 2012). Other specific factors are also required to restrict or antagonize PntP1 function in imINPs to facilitate their maturation and prevent them from reverting to NBs, including Earmuff (Erm) (Janssens et al., 2014; Weng et al., 2010), Six4 (CHAPTER III) or Hamlet (Ham) through distinct mechanisms (Quinto-Rives et al., 2020). Both Six4 (CHAPTER III) and Erm directly bind to PntP1 to antagonize PntP1 function in imINPs (Janssens et al., 2017; Li et al., 2017; Li et al., 2016). Erm and Ham may function through remodeling chromatin to silence NB identity genes, such as by interacting with Brm-Hdac3 or Hdac1/ Rpd3 and thus prevent INP from reverting to NBs (Janssens et al., 2017; Koe et al., 2014; Quinto-Rives et al., 2020). However, unlike the other two, Erm is activated by PntP1 in imINPs before it attenuates PntP1, (Janssens et al., 2017; Li et al., 2017; Li et al., 2016; Zhu et al., 2011). Therefore, the negative regulation loop between PntP1-Erm is critical to mINP commitment. In spite of significant discoveries about regulations of INP development, it is still an open question how the regulation loop is established and whether any other factors play important roles in INP development.
One of the most significant mechanisms to control appropriate developmental progression is to coordinate gene transcriptions across multiple factors, each expressed at right time in the right place. Of machineries to initiate and elongate the transcription on specific enhancer/promoter regions, mediator complex serves to recruit RNA polymerase II and the basal transcriptional machinery in the assistance of available master transcription factors and cofactors. Generally, The mediator Complex is known to bridge transcription activators bound at specific enhancers with the basal transcriptional machinery of the pre-initiation complex bound at a gene promoter. (Biddick & Young, 2005; Poss et al, 2013; Soutourina, 2018; Soutourina et al, 2011; Yin & Wang, 2014). However, by interacting with transcriptional repressors (which can be either certain subunits per se in the mediator complex, such as the Cyclin-dependent kinase 8 (CDK8) subcomplex (Akoulitchev et al, 2000; de Ayala Alonso et al, 2007; Knuesel et al, 2009), or other non-mediator repressors) in the enhancer region, mediator complex also exerts suppressive regulation on gene transcription (Beyer et al, 2007; Ding et al, 2008; Eyboulet et al, 2015). Table 1 (which is adapted from flybase: https://flybase.org/reports/FBgg0000359.html) provides a list of known Drosophila mediator complex components, including experimentally verified ones or computationally predicted orthologs and paralogs with a unified nomenclature (Bourbon et al, 2004), there is a compiled list. However, it is important to keep mind that there is compositional and functional heterogeneity of mediator complex across development or evolution (Park et al, 2001). For example, there are at least two classes of mediator complexes in Hela cell nuclear extract, one containing Cyclin C (CycC) and CDK8 and the other lacking this Cyclin-CDK pair (Wang et al, 2001). Similarly, subunits MED1 and MED26 are not present in all isolates (Malik & Roeder, 2010). The CDK8 submodule components Med12 (i.e. Kto in Drosophila) and Med13 (i.e. Skd in Drosophila) differentially regulate Notch target genes, including activating e(spl)m8 and cut (ct) in Drosophila wing margin, but repressing wingless (wg) and vgBE, a specific
enhancer fragment in neighboring cells (Janody & Treisman, 2011). Over decades of studies on its function and underlying mechanisms, it has been shown that mediator complex is not simply involved in initiation of transcription, but also participates in multiple steps of transcription, including recruiting epigenetic regulators (D'Urso et al., 2016; Ding et al., 2008; Malik & Roeder, 2008; Tsutsui et al., 2013), transcription elongation (Donner et al., 2010; Galbraith et al., 2013; Takahashi et al., 2011), transcription termination (Mukundan & Ansari, 2011; Takahashi et al., 2020) and RNA processing (Chen et al., 2012; Wang et al., 2017).

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Notwithstanding the general role of mediator complex in transcription, our focus is on its contribution to the development of specific cells or tissues. It has long been known that mediator complex may engage in regulating cell-type-specific gene activity to control proliferation (Homem et al., 2014) or interacting with master regulators of cell fate to control cell identity (Yin & Wang, 2014). Since Six4 (CHAPTER III) and PntP1 (Xie et al., 2014; Zhu et al., 2011), as well as Erm (Janssens et al., 2017; Janssens et al., 2014; Li et al., 2017; Weng et al., 2010) have been shown as specific regulators in type II NBs or INPs, we examined if components of the mediator complex interact with them to regulate INP development. Our results demonstrate that Skd and Kto are required to prevent the dedifferentiation of imINPs by functioning as cofactors of PntP1 in activating Erm, which in turn directly interacts with Six4 to ensure imINPs become committed.
mINPs rather than revert to type II NBs. Thus, our study provides new insights into how general transcriptional coregulators Kto/Skd specifically regulates INP development through interacting with type II NB or INP specific regulators.

Results

**Skd and Kto act synergistically to prevent generation of supernumerary type II NBs.**

In order to identify additional transcription factors or transcriptional coregulators that could regulate type II NB lineage development, we conducted an RNAi knockdown screen of transcription factors in type II NB lineages. In this screen, individual type II NB lineages are labelled with \textit{UAS-mCD8-RFP} driven by type II NB lineage-specific \textit{pntP1-GAL4} (Zhu \textit{et al.}, 2011), which is also used to drive the expression of \textit{UAS-RNAi} transgenes in type II NB lineages. In type II NB lineages, all NBs and mature INPs were labeled by \textit{E(spl)mγ-GFP} (Song & Lu, 2011). With these markers, we could identify the type II NB (the biggest \textit{E(spl)mγ-GFP} positive cell), imINPs (next to the type II NBs but \textit{E(spl)mγ-GFP} negative), and mINPs (\textit{E(spl)mγ-GFP} positive but smaller than the NB) in individual type II NB lineages and detect changes of these cell types in live larval brains. Inclusion of a copy of \textit{E(spl)mγ-GFP} transgene also makes animals more susceptible to the generation of supernumerary type II NBs. Thus, providing a sensitized background ideal to the identification of additional players. In wild type larvae, each brain lobe always has 8 type II NBs and an individual type II NB lineage contains a single NB, 3-4 imINPs and 20-30 mINPs (Bello \textit{et al.}, 2008; Boone & Doe, 2008; Bowman \textit{et al.}, 2008; Zhu \textit{et al.}, 2011). We found that knockdown of Skd in type II NBs resulted in an average of 30 type II NBs per brain lobe and knockdown of Kto led to an average of 60 type II NBs per lobe (Fig. IV-1A-C, H). The supernumerary type II NB phenotype resulting from Skd or Kto knockdown were largely rescued by expressing \textit{UAS-Skd} or \textit{UAS-Kto} (Fig. IV-1F-H), whereas expressing \textit{UAS-Skd} or
UAS-Kto alone driven by pntP1-GAL4 did not produce any obvious phenotypes in type II NB lineages (Fig. IV-1D-E, H), demonstrating that the Skd or Kto RNAi knockdown phenotypes were not caused by off-target effects. These results indicate that Skd or Kto normally prevents the generation of supernumerary type II NBs.

Since Skd and Kto function in the same submodule of the mediator complex to regulate specific developmental processes (Janody et al, 2003a; Lim et al, 2007; Treisman, 2001), we next examined if they also function together in type II NB lineages by testing their synergistic effects on preventing the generation of supernumerary type II NBs. Since E(spl)mγ-GFP transgenic lines are more susceptible to the generation of supernumerary type II NBs, we knocked down Skd and/or Kto in absence of E(spl)mγ-GFP for testing their genetic interactions. Our results showed that although knockdown of Skd alone barely produced extra type II NBs or knockdown of Kto alone only slightly increased the total number of type II NBs to 16 per lobe on average, double knockdown of Skd and Kto led to a dramatic increase of type II NBs to more than 80 per lobe an average (Fig. IV-1H-L). The synergistic effect of Skd and Kto double knockdown support that they function together to prevent the generation of supernumerary type II NBs.
**Figure IV-1. Skd or Kto is required to prevent supernumerary type II NB generation specifically.**

In panels which are images of *E(spl)mγ*-GFP transgenic *Drosophila* brain lobes (A-G), type II NB lineages are labelled with mCD8-RFP (in red) driven by *pntP1-GAL4* and counterstained with anti-GFP (in green) and anti-Ase (in blue) antibodies. In panels which are images of wild type brain lobes (I-L) or VNCs (M-N), type II NB lineages or type I NB lineages are labelled with mCD8-GFP (in green) driven by *pntP1-GAL4* or *insc-GAL4* respectively and counterstained with anti-Dpn (in red) and anti-Ase (in blue) antibodies. Examples of type II NBs are pointed with white arrows. Examples of type I NBs are pointed with yellow arrows. Scale bars equal 50µm.

(A) In *E(spl)mγ*-GFP transgenic *Drosophila* brains, there are eight type II NB lineages (only seven are shown) per lobe.

(B-C) In *E(spl)mγ*-GFP transgenic *Drosophila* brains, expressing UAS-Skd RNAi (B) or UAS-Kto RNAi (C) results in supernumerary type II NBs.

(D-E) Overexpressing UAS-Skd (D) or UAS-Kto (E) does not affect the number of type II NBs or the development of their lineages.

(F-G) Expressing UAS-Skd or UAS-Kto largely suppresses the generation of supernumerary type II NBs caused by knockdown of Skd (F) or Kto (G) respectively.

(H) Quantifications of number of type II NBs with indicated genotypes per brain lobe. ***, P < 0.001. The number on top of, or within each bar in graphs of this figure and all following figures indicates the number of samples examined.

(I-L) There are eight type II NB lineages per lobe in wild type brains (I), Skd knockdown (J) or Kto knockdown (K) brains under wild type background, whereas simultaneous knockdown of Skd and Kto results in a synergistic production of supernumerary type II NBs (L).

(M-N) Compared to wild type brains (M), concomitant knockdown of Skd and Kto in type I NBs using *insc-GAL4* reduces the number of type I NBs (N).

(O) Quantifications of number of type I NBs per VNC with indicated genotypes. ***, P < 0.001.

**Kto and Skd are ubiquitously expressed in cells of Drosophila central nervous system**

Then we examined if Skd and Kto are expressed specifically in type II NBs or ubiquitously expressed in fly larval brains. First, we examined Kto expression patterns using a Kto reporter that contains a superfolder GFP (sfGFP) fused to the C-terminus of Kto within a genomic DNA fragment that spanned the locus (Sarov *et al*, 2016). The GFP expression was ubiquitously
detected in both type II NB lineages and type I NB lineages, including NBs and all their progeny (Fig. IV-2A-A’). The same ubiquitous expression of endogenous Kto was also detected using an anti-Kto antibody and expression of UAS-Kto RNAi driven by pntP1-GAL4 nearly abolished endogenous Kto in type II NB lineages (Fig. IV-2B-C’), which further supports that the Kto knockdown phenotype is indeed caused by the loss of Kto. Next, we examined Skd expression by examining UAS-myr-Tdtom reporter expression driven by Skd-GAL4, in which GAL4 expression is under the control of a Skd enhancer sequence. The reporter showed that Skd is expressed in both type I and type II NB lineages in Drosophila larval CNS, with the strongest expression in the NBs and their immediate progeny (Fig. IV-2D-D”), suggesting that Skd expression may be enriched in the NBs and their immediate progeny. The Kto and Skd expression patterns are consistent with their roles as general transcription factors to regulate gene expressions.
Figure IV-2. Skd and Kto are expressed in *Drosophila* CNS, including both type I and type II NB lineages.

(A-B’) Kto-GFP (in green) is detected with anti-GFP antibodies (A), or Kto (in blue) is detected with anti-Kto (B) antibodies in brain lobes, including all cell types in both type II NB lineages (yellow dashed lines) and type I NB lineages (purple dashed lines). Separate channels of Kto-GFP (A’) or Kto (B’) are shown.

(C-C’) Kto expression is specifically abolished in type II NB lineages (yellow dashed lines) when *UAS-Kto RNAi* is expressed driven by *pntP1-GAL4*.

(D-D”) Myr-TdTom is expressed in all type II NB (Ase-) lineages (yellow dashed lines) and type I NB (Ase+) lineages when *skd-GAL4* is used to drive the expression of *UAS-myr-tdTom*. Scale bars equal 50µm.
Skd and Kto are required to maintain self-renewal of type I NBs

Since Skd and Kto are ubiquitously expressed in both type I and type II NB lineages, we then asked if the generation of supernumerary NBs resulting from knockdown of Skd and/or Kto was specific to type II NBs. To this end, we simultaneously expressed UAS-Skd RNAi and UAS-Kto RNAi in type I NBs using a pan-NB driver insc-GAL4 (Luo et al., 1994) or type I NB-specific driver ase-GAL4 (Brand et al., 1993; Jarman et al., 1993). Our results showed that knockdown of Skd and Kto led to a decrease in the number of type I NBs by 20-30% at 4 days after egg laying (AEL) (Fig. IV-1M-O and Fig. IV-3A1-A4, B1-B4, C1-C4, F), which is opposite to the phenotypes observed in type II NB lineages. Further, the diameter of Skd/Kto double knockdown type I NBs and the number of their associated GMCs were also decreased (Fig. IV-3A5-A6, B5-B6, C5-C6, and G-H). The reduced number of GMCs was probably due to a decreased mitotic rate of type I NBs as the PH3 positive rate of type I NBs decreased by 50% (Fig. IV-3D-E’ and I). Taken together, these results indicate that Skd and Kto have a distinct role in type I NB lineages. However, for the rest of our studies, we focused on elucidating how Skd and Kto prevent the generation of supernumerary type II NBs.
Figure IV-3. Simultaneous knockdown of Skd and Kto in type I NBs decreases the number and size of type I NBs, and the number of GMCs.

In all panels, type I NB lineages in brain lobes or VNCs were labelled with mCD8-GFP (in green) and counterstained with anti-Ase (in red) and anti-Dpn (in blue) or anti-PH3 (in white) antibodies. White arrows point to type I NBs (Ase+ Dpn+, in magenta). Yellow arrowheads point to GMCs (Ase+ Dpn−, in red). Examples of type I NB lineages are highlighted with white dashed lines. Scale bars equal 50µm in A1-C4, D-E or 10µm in A5-C6.
(A1-B4) At 2d AEL and 3d AEL, compared to wild type brain lobes (A1, B1) or VNCs (A3, B3), the number of type I NBs does not change when Skd and Kto are simultaneously knocked down (A2, B2 or A4, B4).

(C1-C4) At 4 AEL, compared to wild type brain lobes (C1) or VNCs (C3), simultaneous knockdown of Skd and Kto leads to a decrease in the number of type I NBs in brain lobes (C2) or VNCs (C4).

(A5-B6) Examples of wild type type I NB lineages (A5) or Skd and Kto double knockdown type I NB lineages (A6) containing two GMCs at 2d AEL are shown. At 3d AEL, a wild type type I NB lineage (B5) contains five GMCs, and a Skd and Kto double knockdown type I NB lineage (B6) contains three GMCs.

(C5-C6) An example of wild type type I NB lineages containing six GMCs is shown (C5). When Skd and Kto are simultaneously knocked down, the cell size of NBs is reduced and the number of GMCs is decreased (C6).

(D-E') Mitotically active type I NBs are indicated as PH3 positive in wild type VNCs (D-D'). Fewer type I NBs are PH3 positive when Skd and Kto are knocked down (E-E').

(F-I) Quantifications of total type I NBs (F) in brain lobes or VNCs, number of GMCs per type I NB lineage (G), diameter of type I NBs (H), or mitotic rate of type I NBs (I) with indicated genotypes. *** , P < 0.001. NS, no significance.

Supernumerary type II NBs resulting from Skd/Kto knockdown arise from dedifferentiation of imINPs due to loss of Erm expression

We then tried to determine the origin of supernumerary type II NBs resulting from Skd and Kto knockdown. To this end, we coexpressed UAS-Skd RNAi and UAS-Kto RNAi specifically in imINPs under E(spl)mγ-GFP background driven by erm-GAL4(II), which starts expression in Ase- imINPs but not type II NBs (Janssens et al., 2014; Pfeiffer et al., 2008), to test if the supernumerary type II NBs could be generated from dedifferentiation of imINPs. Our results showed that knocking down Skd and Kto simultaneously in imINPs led to a consistent increase of type II NBs to up to 18 per lobe with an average of two NBs/lineage (Fig. IV-4A-B and I-J).

However, we did not observe an obvious increase in the total number of type II NB lineages based on the number of their RFP labeled axon bundles, suggesting that these ectopic type II NBs had not established their independent lineages yet by the time of examination and that these
ectopic type II NBs were not generated from defects in initial NB specification during embryonic stages. These results suggest that the supernumerary NBs resulting from Skd/Kto knockdown could be derived from dedifferentiation of imINPs.

We then investigated the underlying mechanism of the dedifferentiation of imINPs caused by Kto/Skd knockdown. Previous studies have shown that Erm (Janssens et al., 2014; Li et al., 2016; Weng et al., 2010) and Six4 (CHAPTER III) are required to prevent dedifferentiation of imINPs by forming a complex with PntP1 to inhibit PntP1 activity and expression (CHAPTER III, Fig. III-9A-D). Therefore, we examined Six4 and Erm expression after knocking down Skd/Kto. In wild type larval brains, Six4 is expressed in type II NBs and their progeny including imINPs and mINPs, whereas Erm is expressed in imINPs (except the newly born one) (Fig. IV-4C-C”, K and Fig. IV-S1A-A”, G). We found that simultaneous knockdown of Skd and Kto in type II NB lineages did not affect the expression levels of Six4-GFP (data not shown). However, when either Skd or Kto was knocked down, Erm levels were reduced by 70% (Fig. IV-4D-E”, K and Fig. IV-S1C-C”, E-E”, G). Consistently, the expression of PntP1 in INPs was de-repressed when Skd and Kto were simultaneously knocked down. In normal type II NB lineages, PntP1 is expressed in 3-4 imINPs but not in mINPs labeled with E(spl)my-GFP (Fig. IV-4F-F” and L), whereas in Skd or Kto knockdown lineages, the number of PntP1+ INPs almost doubled and PntP1 was also detected in mINPs (Fig. IV-4G-H” and L), indicating that the suppression of PntP1 expression in mINPs was delayed due to the loss of Erm expression. These results suggest that Skd and Kto are required to activate Erm expression in imINPs.

To further confirm that Skd/Kto is required for Erm expression, next we examined if the loss of type II NBs resulting from Notch knockdown could be rescued by Skd/Kto knockdown. Our
previous studies show that the loss of type II NBs resulting from the loss of Notch is due to precocious activation of Erm by PntP1 in type II NBs (Fig. IV-4M and P) (Janssens et al., 2017; Li et al., 2016). Indeed, our results showed that expressing either UAS-Skd RNAi or UAS-Kto RNAi in type II NBs fully rescued the phenotype of loss of type II NB lineages resulting from Notch knockdown (Fig. IV-4N-P), suggesting that the ectopic activation of Erm in Notch knockdown type II NBs also requires Skd and Kto.

After demonstrating the Skd and Kto are required for Erm activation in imINPs, we next investigated if the loss of Erm expression and the subsequent de-repression of PntP1 in imINPs is responsible for the generation of supernumerary type II NBs resulting from Skd/Kto knockdown. We first tested if Erm and Skd/Kto genetically interact by examining if the supernumerary type II NB phenotype resulting from Skd/Kto knockdown would be enhanced in the \(erm^{+/+}\) heterozygous mutant background. Indeed, while our results showed that \(erm^{+/+}\) heterozygotes alone or knockdown of Skd and Kto driven by \(erm\)-GAL4(II) in wild type background barely produced supernumerary type II NBs, knockdown of Skd and Kto in imINPs under the \(erm^{+/+}\) background using \(erm\)-GAL(II) as a driver resulted in an average of 70 type II NBs per lobe (Fig. IV-5A-C and S). Similar enhancement was also observed when \(pntP1\)-GAL4 was used for knocking down Skd or Kto (Fig. IV-5D-L and T). Therefore, Skd and Kto genetically interact with Erm in preventing dedifferentiation of imINPs.

Second, we examined if restoring Erm expression by expressing UAS-Erm or knocking down PntP1 by expressing UAS-pnt RNAi specifically in imINPs would rescue the supernumerary type II NB phenotype resulting from Skd/Kto knockdown. As expected, we found that the generation of supernumerary type II NBs was fully inhibited by restoring Erm (Fig. IV-5M-O and U). Consistently, simultaneously knocking down PntP1 largely reduced the total number of type II
NBs from 110 per lobe to 50 per lobe on average in Skd and Kto double knockdown brains (Fig. IV-5P-R and V).

Taken together, these results demonstrated that Skd and Kto are required to activate Erm expression in imINPs and loss of Erm expression is responsible for the dedifferentiation of imINPs and subsequent generation of supernumerary type II NBs resulting from Skd/Kto knockdown.
Figure IV-4. Knockdown of Skd and/or Kto leads to abolishment of Erm and restores the loss of type II NBs caused by Notch knockdown.

Type II NB lineages are labeled with mCD8-RFP or mCD8-GFP driven by *erm-GAL4(II)* (A-B) or *pntP1-GAL4* (C-H and M-O) and counterstained with anti-RFP or anti-GFP, anti-Dpn, anti-Erm, anti-Ase, or anti-PntP1 antibodies. White solid arrows point to some of the type II NBs as examples. Open arrows point to some imINPs as examples. White arrowheads point to some E(spl)mγ+ mINPs as examples. Scale bars equal 50µm in A-B and M-O, or 10µm in C-H.

(A-B) A E(spl)mγ-GFP brain lobe (A) contains eight type II NB lineages labeled with mCD8-RFP (in green) which is expressed from imINPs when *erm-GAL4(II)* is used as a driver. Specific
knockdown of Skd and Kto in imlNPs by using *erm-GAL4*(II) under *E(spl)mγ-GFP* background (B) leads to the generation of supernumerary type II NBs.

(C-C”) In a wild type type II NB lineage, Erm is expressed in Ase- and Ase+ imINPs.

(D-E”) Knockdown of Skd (D-D”) or Kto (E-E”) alone in type II NB lineages almost abolishes Erm expression in imlNPs.

(F-F”) PntP1 is expressed in type II NBs (the largest cell with *E(spl)mγ*), imlNPs (the smaller cells with *E(spl)mγ* next to NBs), but terminated in mINPs (smaller cells with *E(spl)mγ*).

(G-H”) Expressing *UAS-Skd RNAi* (G-G”) or *UAS-Kto RNAi* (H-H”) driven by *pntP1-GAL4* results in PntP1 expression persisting in *E(spl)mγ* mINPs.

(I-J) Quantifications of number of type II NBs per lobe (I) or number of type II NBs per lineage (J) with indicated genotypes corresponding to (A-B). ***, *P* < 0.001.

(K-L) Quantifications of Erm intensity in Ase- imINPs (K) or number of PntP1+ INPs per lineage (L) with indicated constructs driven by *pntP1-GAL4*. ***, *P* < 0.001.
Figure IV-5. The supernumerary type II NBs caused by knockdown of Skd and Kto arise from dedifferentiation of imINPs due to loss of Erm expression.
Type II NB lineages are labeled with mCD8-RFP or mCD8-GFP driven by *pntP1-GAL4* (D-L and P-R) or *erm-GAL4*/*(II)* (A-C and M-O) and counterstained with indicated antibodies. White arrows point to some type II NBs as examples. White arrows with purple outline point to E(spl)mγ-GFP+ Ase+ type II NBs. Scale bars equal 50µm.

(A-C) There are no ectopic type II NBs in an *erm2/*heterozygous mutant brain lobe (A). When Skd and Kto are knocked down in imINP by expressing *UAS-Skd RNAi* and *UAS-Kto RNAi* driven by *erm-GAL4*/*(II)*, no ectopic type II NBs are produced (B). Double knockdown of Skd and Kto specifically in imINPs under *erm2/* background drastically produces supernumerary type II NBs (C).

(D-G) There are still eight type II NBs per brain lobe when Skd is knocked down in type II NBs by using *pntp1-GAL4* under wild type background (D), while supernumerary type II NBs are generated when Skd is knocked down in type II NBs under *erm2/* heterozygous background (E). Overexpressing Skd in type II NBs under *erm2/* background does not affect type II NB lineage development (F) and restoring Skd levels by expressing *UAS-Skd* suppresses supernumerary type II NBs generation caused by Skd knockdown (G).

(H-K) There is a slight increase in the number of type II NBs when Kto is knocked down in type II NBs by using *pntp1-GAL4* under wild type background (H), while *erm2/* heterozygous mutant background dramatically enhances the generation of supernumerary type II NBs resulting from Kto knockdown (I). Overexpressing Kto in type II NBs under *erm2/* background does not affect type II NB development (J) and restoring Kto levels by expressing *UAS-Kto* largely suppresses supernumerary type II NB generation caused by Kto knockdown (K).

(L) Total eight type II NBs and their derived lineages develop normally in *erm2/* brains.

(M-O) The supernumerary type II NBs resulting from knockdown of Skd and Kto (M) in imINPs driven by *erm-GAL4*/*(II)* are repressed by expressing *UAS-Erm* (N), while expressing *UAS-Erm* alone in imINPs does not reduce the number of type II NBs (O).

(P-R) Simultaneous knockdown of Skd and Kto in type II NBs leads to supernumerary type II NBs (P), while triple knockdown of Skd, Kto and PntP1 partially suppresses the generation of supernumerary type II NBs to a lesser extent (Q), which is similar to that when PntP1 is knocked down alone (R).

(S-V) Quantifications of number of type II NBs per lobe with indicated genotypes driven by *erm-GAL4*/*(II)* (S, U) or by *pntp1-GAL4* (T, V). ***, *P* < 0.001. NS, no significance.
**Skd and Kto are essential cofactors of PntP1**

The molecular mechanisms by which the mediator complex regulates target genes expression has been shown to involve direct or indirect protein-protein interactions with cell-type-specific transcription factors. (Yin & Wang, 2014). Since Erm expression in imINPs is activated by PntP1 (Janssens *et al.*, 2017; Li *et al.*, 2016; Zhu *et al.*, 2011), we next investigated whether Skd and Kto are essential cofactors for PntP1’s function. Because Skd and Kto are also expressed in type I NBs, we tested this hypothesis by simultaneously misexpressing PntP1 and knocking down Skd and Kto in type I NBs to check if PntP1 still functions to suppress Ase or induce INP-like cells (Xie *et al.*, 2014; Zhu *et al.*, 2011). We focused on larval VNCs, which contain only type I NB lineages, for phenotypic analysis. Our results showed that more than 90% of type I NBs lost Ase expression and 15% of them generated Dpn⁺ Ase⁺ mINP-like cells when PntP1 was misexpressed in type I NBs (Fig. IV-6B, and E-F). Knockdown of Skd and Kto did not affect the cell type composition in type I NB lineages (Fig. IV-6C, and E-F). However, when Skd and Kto were simultaneously knocked down, misexpressing PntP1 led to suppression of Ase in only 10% of type I NBs and failed to induce any INPs (Fig. IV-6D-F).

To further determine if Skd and Kto act as cofactors of PntP1, next we examined if PntP1 physically interacts with Skd or Kto by co-IP and western blotting. Our results showed that when Flag-tagged PntP1 was coexpressed with either Myc-tagged Skd or Myc-tagged Kto in *Drosophila* S2 cells, Skd or Kto could be precipitated together with PntP1 (Fig. IV-6G). We were also able to co-IP Skd and Kto when Myc-tagged Skd and HA-tagged Kto were co-expressed in S2 cells (data not shown) as shown in previous studies (Janody *et al.*, 2003b). Thus, the biochemical data demonstrate that PntP1 forms a complex with Skd and Kto and that Skd/Kto are indispensable for PntP1 to activate its target gene expression in type II NB lineages.
Figure IV-6. Skd and Kto are essential cofactors of PntP1.

(A-D) Type I NB lineages are labeled with mCD8-GFP (in green) driven by insc-GAL4 and counterstained with anti-Dpn (in red) and anti-Ase (in blue) antibodies. All wild type type I NBs (white arrows) are Ase+ Dpn+ (appearing magenta) and directly produce Ase+ GMCs (yellow arrowheads) (A). Misexpressing PntP1 in type I NBs results in repression of Ase in the majority of type I NBs (open arrows) and induces Ase+ Dpn+ mINP-like cells in a subset of lineages (dashed lines) (B). Insets show enlarged views of a lineage with mINP-like cells from the area which is highlighted with a dotted square in (B). Knockdown of Skd and Kto in type I NBs does not affect Ase expression or promote INP generation (C). Misexpressing PntP1 fails to repress Ase expression in type I NBs (white arrows) or induce INP-like cells, when Skd and Kto are simultaneously knocked down in type I NB lineages. Instead, GMCs (yellow arrowheads) are directly produced (D).

(E-F) Quantifications of percentage of type I NB lineage with mINP-like cells (E) and percentage of Ase- type I NBs (F) in VNCs with indicated genotypes. ***, $P < 0.001$.

Scale bars equal 50µm.

(G) Biochemical interactions between Flag-PntP1 and Myc-Kto or between Flag-PntP1 and Myc-Skd are detected by transfecting S2 cells with their corresponding expression constructs followed by co-IP and western blotting analyses.
Six4 genetically interacts with mediator complex to prevent supernumerary type II NBs

Since our studies in CHAPTER III show that, like Erm, Six4 functions in imINPs to prevent their dedifferentiation but Six4 knockdown alone only generated moderate supernumerary type II NB phenotypes, we hypothesized that other factors may function with partial redundancy with Six4 in preventing imINP dedifferentiation. Thus, we performed a double knockdown screen of Six4 together with other candidate factors that might function redundantly with Six4. Since the SD domain in Six4 mediates protein-protein interactions with other cofactors, we first focused on the candidates that directly or indirectly interact with Six4 based on previous publications or predicted protein-protein interaction in databases (such as STRING). These candidates include Eye absent (Eya), Groucho (Gro), Sine oculis (So), Optix, Twin of eyeless (Toy), Nautilus (Nau), Dachshund (Dac), Eyeless (Ey), Distal-less (Dll), or histone demethylase UTX (Bischof et al, 2018; Johnston et al, 2016; Kobayashi et al, 2001; Ohto et al, 1999; Pignoni et al, 1997; Seenundun et al, 2010). However, knockdown of these factors alone did not result in similar phenotypes as Six4 knockdown did, nor did it enhance the supernumerary type II NB phenotype resulting from Six4 knockdown. Therefore, we went on to test if Six4 genetically interacts with Kto/Skd in preventing the generation of supernumerary type II NBs. We tested this in the wild type background instead of the sensitized E(spl)mγ-GFP background in order to reduce the severity of Skd/Kto knockdown phenotypes so that synergistic enhancement of the phenotypes could be easier to detect if there is any. Our results showed that knockdown of Skd alone in the wild type background barely increase the total number of type II NBs and knockdown of Kto alone led to a slight increase in the total number of type II NBs (Fig. IV-7C1, D1 and L). When Six4 was simultaneously knocked down together with either Skd or Kto, it consistently resulted in a dramatic increase in the total number of type II NBs to more than 70 per lobe on average (Fig. IV-7C2, D2 and L), indicating that Six4 and Skd/Kto genetically interact in preventing the dedifferentiation of imINPs. Since Skd and Kto may or may not form a subcomplex with CDK8
and CycC (Mao et al., 2014) to regulate target gene transcription, we also tested if Six4 genetically interacts with CDK8 or CycC by knocking down CDK8 or CycC alone, or together with Six4 in type II NB lineages. However, no extra type II NBs were generated when either CDK8 or CycC was knocked down either alone or together with Six4 in type II NBs (data not shown), suggesting that Skd/Kto may not function together with CDK8 or CycC to prevent the generation of supernumerary type II NBs.

Because Skd and Kto may just function as a submodule independently of other mediator complex components (Janody et al., 2003b), we wondered if Six4 interacts with Skd/Kto exclusively or actually it generally interacts with other mediator complex subunits. Thus, we performed similar genetic interaction tests between Six4 and other 28 known subunits of the mediator complex. Our results showed that, when these subunits were knocked down alone, only MED14 or MED21 gave obvious supernumerary type II NB phenotypes (about total 20 type II NBs per lobe on average) (Fig. IV-7H1, K1 and L), others generate no or less than 1 extra type II NBs per brain lobe on average (Fig. IV-7E1, F1, G1, I1, J1 and L). However, when Six4 was knocked down simultaneously, knockdown of MED4, MED7, MED8, MED9, MED11, MED14, MED15, MED19, MED21, MED22, MED24, MED25, MED28 or MED29 led to synergistic enhancement of the supernumerary type II NB phenotype, with a total number of type II NBs ranging from 9 to 148 per lobe on average (Fig. IV-7B and E2, F2, G2, H2, I2, J2, K2 and L). Among these mediator proteins, knockdown of MED14 gave the strongest enhancement of the phenotype, which is consistent with its critical role as a central backbone that links all three main submodules (head, middle and tail) of the mediator complex and maintains the proper architecture and function of the complex (Soutourina, 2018), Thus, half of the mediator complex subunits, including Skd and Kto, genetically interact with Six4 to prevent the generation of supernumerary
type II NBs, which also suggests that the mediator complex components may not all function in the same complex but rather they may act in two functionally distinct complexes.

**Figure IV-7. Six4 genetically interacts with mediator complex components to prevent the generation of supernumerary type II NBs.**

In all panels, type II NB lineages are labeled with mCD8-GFP (in green) driven by pntP1-GAL4 and counterstained with anti-Dpn (in red) or anti-Ase (in blue) antibodies. Scale bars equal 50µm.

(A) A wild type *Drosophila* brain lobe consistently contains eight type II NBs.

(B) Specifically knocking down Six4 alone in type II NBs under wild type background barely produces ectopic type II NBs.

(C1-C2) Specifically knocking down Skd alone in type II NBs under wild type background barely produces ectopic type II NBs (C1), while simultaneous knockdown of Six4 and Skd resulting in a dramatic increase in the number of type II NBs (C2).

(D1-K2) Specifically knocking down indicated subunits of mediator complex alone in type II NBs under wild type background barely produces ectopic type II NBs (D1, E1, F1, G1, I1, J1) or results in mild generation of ectopic type II NBs (H1, K1), while simultaneous knockdown of Six4 with any one of these subunits leads to variable increase in the number of type II NBs (D2, E2, F2, G2, H2, I2, J2, K2).

(L) Quantifications of number of type II NBs per brain lobe with indicated genotypes. ***, $P < 0.001$. **, $P < 0.01$. *, $P < 0.05$. **
Discussion

Previous studies have demonstrated that *Drosophila* Skd and Kto bind each other to form a submodule, probably not fully participating in all of the activities of the mediator complex, to regulate eye-antennal disc development and wing disc development (Janody *et al*., 2003b; Treisman, 2001), through regulating target genes of EGFR signaling, Notch signaling, Wnt signaling or Hedgehog Signaling Pathway (Carrera *et al*., 2008; Lim *et al*., 2007; Mao *et al*., 2014). However, our study here is the first to report the novel roles of Skd and Kto in regulating INP development. We provide evidence to show that two of the mediator components, Skd and Kto, act together likely by forming a complex with PntP1 that regulates downstream targets in type II NBs to maintain type II NB identity by suppressing Ase, or in imINPs to prevent their dedifferentiation by activating Erm. The subsequent interaction between Erm and Six4 largely promotes the maturation of imINPs and prevents imINPs from reverting to type II NBs. We also discovered that the Six4 genetically and synergistically interacts with multiple components of mediator complex to regulate type II NB population (Fig. IV-8).

![Figure IV-8. A schematic model of potential functions of Skd/Kto in type II NB lineage development.](image)

In type II NBs, Skd and Kto likely assist PntP1 to repress Ase to maintain the identity of type II NBs. In early and late imINPs, except the newly born imINPs, Skd and Kto function as cofactors of PntP1 to activate the expression of Erm, which in turn directly binds Six4 to antagonize PntP1’s function to promotes the maturation of imINPs and prevent their dedifferentiation.
Skd and Kto function as cofactors of PntP1 in both type II NBs and imINPs

Skd and Kto have been reported that they function as either repressors or activators, which is context or cofactor dependent (Knuesel et al., 2009; Lim et al., 2007; Mao et al., 2014). Our study reveals that PntP1 is the one directly binding to Skd/Kto to perform its function in type II NB lineages. We also show Skd and Kto are upstream activators of Erm, as indicated by the results of knockdown of Skd or Kto experiment. These are consistent with the findings that Erm is activated by PntP1 (Janssens et al., 2017; Li et al., 2016; Zhu et al., 2011) and that PntP1 contains an activation domain and functions indeed as an transcriptional activator in type II NBs (see CHAPTER II). The co-requirement of PntP1 and Skd/Kto to activate Erm is also well supported by the rescue experiment showing that knockdown of Skd or Kto fully inhibits type II NB depletion resulting from blocking Notch signaling (and the subsequent inhibition of PntP1-activated Erm expression). The results also indicate that PntP1 functions together with Skd/Kto to activate Erm transcription not only in Notch negative imINPs but also in type II NBs, as along as Notch is compromised, suggesting the potential of PntP1 interacting with Skd/Kto to activate downstream genes, including Erm. In addition, PntP1 knockdown dominates the phenotypes of PntP1/Skd/Kto triple knockdown, including de-suppression of Ase in type II NBs, premature differentiation or dedifferentiation of imINPs, further supporting that Skd and Kto are cofactors of PntP1. Our biochemical experiments show direct bindings between Skd and PntP1 proteins or between PntP1 and Kto proteins in S2 cell, supporting the model that Skd and Kto are cofactors of PntP1 to activate Erm.

In addition to activating Erm, when PntP1 is misexpressed but Skd and Kto are knocked down in type I NBs, PntP1 is not able to suppress Ase efficiently or induce any mINPs, suggesting that
Skd and Kto are likely required for PntP1 to suppress Ase and promote INP generation in these NBs. Previous studies showed that PntP1 misexpression in type I lineages results in their transformation into type II. These transformed lineages respond to the absence of Brat similarly as de novo type II NBs do, producing a dramatic overproliferation of type II NB-like cells or lineages from dedifferentiation of imINPs (Zhu et al., 2011). However, no overproliferation is observed in type I NB lineages with misexpression of PntP1 and knockdown of Skd and Kto, although Erm would not be activated anymore due to Skd/Kto knockdown. Two considerations explain this phenomenon: 1) Ase was still expressed in most of PntP1 misexpressing and Skd and Kto knockdown type I NBs and 2) the suppression of Ase in NBs is a prerequisite for the generation of PntP1-induced ectopic INP-like Cells (Zhu et al., 2011). Indeed, with Ase still being expressed, we observed that there were very few, if any, imINPs generated, and therefore the subsequent chance for them to dedifferentiate to form ectopic type II NB-like cells would be low. On the contrary, we did not observe ectopic Ase expression in any Skd and Kto double knockdown type II NBs, suggesting that redundant cofactors other than Skd/Kto may exist that assist PntP1 in suppressing Ase in type II NB context. Alternatively, we did not exclude the possibility that Skd or Kto may interacts with certain downstream targets of PntP1 to exert a corresponding regulation. For example, they may interact with Tll (see CHAPTER II) to promote the suppression of Ase in type II NBs.

Triple knockdown of PntP1, Skd and Kto did not overcome the generation of supernumerary type II NBs resulting from PntP1 knockdown, which is opposite to the case of Six4 and PntP1 double knockdown or brat btd double mutants which de-repress Pros expression in imINPs (and blocks the dedifferentiation of imINPs). Therefore, based on these results, we did not determine whether Skd or Kto plays any role in repressing Pros in imINPs, where they are all coexpressed along with PntP1.
Erm mediates indirect interactions between Six4 and Skd or Kto in preventing dedifferentiation of imINPs

When either Skd or Kto was knocked down, Erm levels were reduced by 70% (Fig. IV-5B-B”, C-C”, M and Fig. IV-S1C-C”, E-E”, G). We did not detect a further reduction in Erm levels in imINPs when Six4 was knocked down together with Skd or Kto (Fig. IV-S1D-D”, F-F” and G), supporting that Six4 does not regulate Erm expression (CHAPTER III, Fig. III-4M-N”). Consistent with Erm reduction, we observed an increased number of PntP1+ INPs (10 per lineage on average) in Six4 and Skd, or Six4 and Kto double knockdown type II NB lineages (Fig. IV-S1D-D”, F-F” and H), indicating a subsequent loss of PntP1 inhibition in INPs. However, we did not detect an obvious increase in the number of PntP1+ INPs or type II NBs when Six4, Skd or Kto were knocked down alone in type II NB lineages in an otherwise wild type background (Fig. IV-S1B-B””, C-C””, E-E”” and H). This may be due to unaltered Erm expression or sufficient residual Erm levels to interact with Six4 and suppress PntP1 expression/activity in INPs with Six4, Skd or Kto knockdown alone in wild type background. To test this possibility, we assessed if further reduction in Erm expression or knockdown of Six4 in imINPs would synergistically enhance the supernumerary type II NB phenotypes resulting from Skd or Kto knockdown in imINPs. Our results showed that single knockdown of Six4 or Skd in the \(\text{erm}^2+/\) heterozygous background or Six4 and Skd/Kto double knockdown in the wild type background with \(\text{erm-GAL4 (II)}\) only led to generation of 1 extra type II NBs in less than 30% of brains (Fig. IV-S2A-D, F, I and J). Knockdown of Kto in imINPs in the \(\text{erm}^2+/\) heterozygous background led to an average of 15 type II NBs per brain lobe (Fig. IV-S2G and J). However, when Six4 and Skd/Kto double knockdown was performed in the \(\text{erm}^2+/\) heterozygous background, the total number of type II NBs was dramatically increased to more than 25/lobe (Fig. IV-S2E, H and J).
These data support the idea that Skd and Kto are required for Erm activation in imINPs and Erm could further act together with Six4 in these cells to prevent dedifferentiation of imINPs.

Although we demonstrated that the key role of Skd/Kto is functioning as cofactors of PntP1 to activate Erm (which in turn interacts with Six4 to inhibit the activity/expression of PntP1 in imINPs to prevent them from dedifferentiating to NBs), we cannot exclude the possibility that Six4 may directly interact with Skd or Kto to prevent ectopic type II NB production through other mechanisms. In addition, the possibility is supported by the fact that Six4 genetically interacts with multiple components of mediator complex to prevent the generation of supernumerary type II NBs. To test this, we simply examined their potential bindings in S2 cell culture via co-IP and western blotting. Our results showed that Six4 indeed physically binds to either Skd or Kto (Fig. IV-S2K-L). However, considering Skd and Kto facilitates PntP1’s endogenous function but Six4 antagonizes PntP1’s expression and activity, it is unlikely that the direct interactions between Six4 and Skd (or Kto) would act directly to suppress PntP1’s function. Given that Six4 is also a transcription factor that is specifically expressed in type II NB lineages, the binding may mediate some unknown pathways that may involve further interactions with other mediator complex components during regulation of transcription of Six4 downstream targets.

**Prevention of dedifferentiation of imINPs by the mediator complex and Six4**

We have also decoded at some levels the functions of mediator complex subunits, particularly Skd and Kto, in maintaining type II NBs lineages development in *Drosophila* larval brains. Our results showed that the type II NB lineage-specific homeodomain transcription factor, Six4, largely and synergistically interacts with multiple subunits of the mediator complex to prevent the expansion of tumor-like supernumerary type II NBs. Results based on knocking down Six4 and
subunits of mediator complex are consistent with the known structural composition or function of mediator complex. For example, MED14 is the key component that links all three parts of mediator complex, the head, middle and tail, and the simultaneous knockdown of MED14 with Six4 enhanced the supernumerary type II NB phenotype most, compared to other subunits. Knockdown of CycC or CDK8 either individually or together with Six4 does not result in production of any supernumerary type II NBs, suggesting that CycC or CDK8 may not be involved in the canonical mediator complex in regulating type II NB or INP development, which is consistent with previous studies showing that CycC or CDK8 can form a submodule that is dispensable from the entire mediator complex (Wang et al., 2001) or independent of the Skd-Kto submodule. Nevertheless, it would be helpful to further dissect if CycC or CDK8 involves in regulating type II NB or imINPs development, or if they function through a distinct pathway independent of Skd-Kto or Six4, considering this kinase module generally has a repressor activity (Akoulitchev et al., 2000; de Ayala Alonso et al., 2007; Knuesel et al., 2009). Our results show that Six4 can bind to Skd and Kto in S2 cells, suggesting in vivo it is likely that Six4 may bind to Skd and Kto to mediate the further interactions with other mediator complex subunits to form a larger complex to restrict imINPs maturation and differentiation. However, we did not examine the direct interaction of Six4 proteins with any of other mediator complex subunits. The conventional approaches, including yeast two-hybrid, Co-IPs, or fluorescence resonance energy transfer (FRET) microscopy, may not be powerful to analyze the subunit interaction within a multimer complex. Additional approaches in structural biology are needed, such as mass spectroscopy or cryo-electron microscopy to determine the structure or topology of the possible Six4-mediator complex.

Our genetic experimental results show that Six4 interacts with Skd and/or Kto to prevent generation of supernumerary type II NBs by inhibiting the reversion of imINPs to NB fate. However, we suspect the interaction, is likely indirect and mediated by targeting to both the Erm
but at different regulatory levels due to following reason. In imINPs (except the newly born imINPs), Skd and Kto mainly function as co-factors recruited by PntP1 to activate Erm transcription and regulate its subsequent protein expression, while Six4 functions by directly binding to Erm and PntP1 at the protein level to inhibit both the expression and activity of PntP1. Thus, PntP1, Skd or Kto, Erm, Six4 form a sequential regulatory cascade and a safeguard mechanism for diminishing neural stem cell genes to commit imINPs to mINPs. Nonetheless, we did detect a binding between Six4 and Skd or between Six4 and Kto but did not determine what the bindings exactly do. Given that Six4 mainly functions to suppress PntP1 whereas Skd and Kto assist PntP1 to activate its downstream target Erm, the binding between Six4 and Skd or Kto may not function to directly antagonize PntP1 to prevent dedifferentiation of imINPs. Considering that Six4 itself is also a transcription factor containing the SD domain often engaging in protein-protein interactions (Kumar, 2009; Pignoni et al, 1997) and is colocalized with Skd and Kto in both imINPs and type II NBs, the bindings to Skd, Kto or maybe other mediator complex components likely are crucial to regulate some key unknown factors, other than Erm, to regulate the development of imINPs or type II NBs.

**Potential function of Skd and Kto in mediating the cell cycle exit in type I NBs versus type II NBs**

Our results also demonstrate that Skd and Kto are required to maintain the self-renewal of type I NBs. The premature loss of type I NBs and decrease in cell-size at mid/late larval stages resulting from Skd/Kto knockdown is similar to that resulting from the loss of self-renewal factors like Dpn, Notch, Klu, et. However, we did not observe any obvious genetic interactions between Skd/Kto and these self-renewal factors. Thus, Skd or Kto may function through other pathways to prevent the precocious termination of the self-renewal of type I NBs. One potential mechanism
could be that Skd/Kto antagonizes the role of the steroid hormone ecdysone in promoting cell cycle exit and termination of NB self-renewal at early pupal stages. Mediator complex subunits Med4, Med6, Med9, Med10, Med11, Med22, Med27, and Med31 have been shown to promote timely termination of NB self-renewal at early pupal stages by directly binding to ecdysone receptor (EcR) and mediating its transcriptional activation activity, whereas Kto functions as an inhibitory subunit to repress the activity of these mediator complex subunits (Homem et al., 2014). Another study (Yang et al., 2017) also shows that in type I NBs or mushroom body NBs, Kto (maybe with Skd) levels are maintained post-transcriptionally, but indirectly, by IGF-II RNA-binding protein (Imp) to suppress the above mediator complex subunits and the subsequent NB decommission. Therefore, findings from our studies suggest that not all mediator proteins function in the same complex, but rather, the mediator proteins could be divided into two functionally distinct groups at least in fly NBs and may function in two separate complexes, which interact with distinct transcription factors (e.g. Ecdysone receptors and PntP1) to regulate NB self-renewal and INP maturation, respectively. However, given that Skd/Kto knockdown leads to the generation of supernumerary type II NBs which is different from the phenotypes of type I NBs, we do not tested if Skd and Kto are similarly evolved in maintaining the self-renewal of type II NBs, but it could be the case given that the termination of type II NBs is regulated by the ecdysone signaling and the subunits of mediator complex, including Kto, which have been shown in previous studies (Homem et al., 2014; Li & Hidalgo, 2020). One thing could be done potentially to test this possibility in the future is by examining whether knockdown of ecdysone receptors would enhance the supernumerary type II NB phenotypes resulting from Skd/Kto sliencing at larval stages before the Ecdysone receptor-mediated termination of NB self-renewal occurs at early pupal stage.
Materials and Methods

Fly stocks

The E(spl)my-GFP reporter line (Almeida & Bray, 2005) was used to label NBs and mINPs. The Kto-GFP line (#318680, Vienna Drosophila Resource Center [VDRC], Vienna, Austria) was used to detect Kto expression. The Six4-GFP line (#67733, Bloomington Drosophila Stock Center [BDSC]) was used to detect Six4 expression. GAL4 lines, including pntP1-GAL4 (also called GAL4<sup>14-94</sup>) (Zhu et al., 2011), erm-GAL4(II) (Pfeiffer et al., 2008; Xiao et al., 2012), insc-GAL4 (Luo et al., 1994), Skd-GAL4 (#76783, BDSC) and ase-GAL4 (Brand et al., 1993; Jarman et al., 1993) were used for UAS-transgenes expression. UAS-Six4 RNAi (#30510, BDSC) was used for knocking down Six4. Knockdown of Erm or Notch was carried out with UAS-Erm RNAi (#26778, BDSC) or UAS-N RNAi (#7078, BDSC). UAS-Skd RNAi (#34630, BDSC) and UAS-Kto RNAi (#34588, BDSC; #23142, VDRC; #23143, VDRC) were used for knocking down Skd or Kto respectively. UAS-Skd.J (#63800, BDSC) and UAS-Kto.J (#63801, BDSC) were used to rescue Skd or Kto knockdown phenotypes. Knockdown of pnt was carried out with UAS-pnt RNAi (#35038, BDSC) or UAS-pnt RNAi (#31396, BDSC). UAS-PntP1 (Zhu et al., 2011) was used for mis-/over-expression of PntP1.  erm<sup>2</sup> (Weng et al., 2010) allele was used to reduce Erm expression. Other UAS-RNAi lines for knocking down mediator complex subunits are listed in table 2.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>BL#</th>
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<td>34630</td>
<td>MED11</td>
<td>34083</td>
<td>MED25</td>
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Table 2. UAS-mediator complex subunit RNAi lines used, from BDSC (BL#) or VDRC (V#)
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**UAS-transgene expression**

For *RNAi* knockdown, except in Figure IV-4A-B or Figure IV-5M-O, embryos were collected for 8~10hrs at 25°C and shifted to 30°C to boost the efficiency. In Figure IV-4A-B or Figure IV-5M-O, embryos were collected for 4-6hrs at 25°C and right away shifted to 30°C to boost the efficiency. *UAS-dcr2* was coexpressed with *UAS-RNAi* transgenes to enhance the RNAi knockdown efficiency. For mis-/over-expression of PntP1, Skd or Kto, misexpression of PntP1 and knockdown of Skd and Kto in type II NB lineages, embryos were collected for 8~10hrs at 25°C and shifted to 30°C. When PntP1 is misexpressed by *insc-Gal4*, temperature sensitive *tub-Gal80* (#7019, BDSC) was used to avoid the lethality at embryonic stages: *insc-GAL4* and *tub-
Gal80ts was recombined first and embryos were collected for 8~10hrs and raised to hatch at 18°C, then shifted to 30°C for 3~4 days to maximize the efficiency.

**Immunostaining and confocal microscopy**

Larvae at desired stages were sacrificed and dissected. Brain lobes along with VNCs were immunostained as previously described (Lee & Luo, 1999) except that the fixation time was increased to 35 mins. Primary antibodies used are chicken anti-GFP (Catalog #GFP-1020, Aves Labs, Tigard, Oregon; 1:500-1000), rabbit anti-DesRed (Catalog #632392, Takara Bio USA, Inc., Mountain View, California; 1:250), rat anti-mCD8 (Catalog #12-0088-42, Thermo Fisher Scientific, Waltham, Massachusetts, 1:100), rabbit anti-Dpn (1:500) (a gift from Dr. Y.N. Jan) (Bier et al., 1992), guinea pig anti-Ase (a gift from Dr. Y.N. Jan, 1:5000) (Brand et al., 1993), rat anti-Erm (a gift of Dr. C. Desplan; 1:300), rabbit anti-PntP1 (a gift of Dr. J. B. Skeath; 1:500) (Alvarez, 2003), guinea pig anti-Kto (Janody et al., 2003b), mouse anti-PH3 (Catalog # ab80612, Cambridge, Massachusetts, 1:500). Secondary antibodies conjugated to Daylight 405 (1:300-400), Daylight 488 (1:100), Cy3 (1:500), Rhodamine Red-X (1:500), Daylight 647 (1:500) or Cy5 (1:500) used for immunostaining are from Jackson ImmunoResearch (West Grove, Pennsylvania). Images were collected using a Carl Zeiss LSM780 confocal microscopy and processed with Adobe Photoshop. Two-tailed student’s T-test was used for statistical analyses.

**S2 cell culture and co-immunoprecipitation**

pAFW-PntP1 (i.e., Act5C-Flag-PntP1) vector (gifts from Dr. H. Y. Wang) (Koe et al., 2014) was used for expressing Flag-PntP1 in S2 cells under the control of the Act5C promoter. pUAST-Myc-Skd (i.e., UAS-Myc-Skd), pUAST-Myc-Kto (i.e., UAS-Myc-Kto) and pUAST-HA-Kto (i.e., UAS-HA-Kto) (gifts from Dr. Y. Zhao) (Mao et al., 2014) were used to express Skd or Kto in S2 cells. Mock vectors pUAST and pAW (generated by deleting Flag-PntP1 cassette from pAFW-
PntP1) were co-transfected as controls to balance the amounts of each vector transfected. For cell culture and co-IPs flowed by western blotting, refer to corresponding methods in CHAPTER III.

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Competing interests

The authors declare no competing or financial interests

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Figure IV-S1. Skd or Kto, but not Six4, is required to activate Erm expression in imINPs.

In all panels, type II NB lineages are labeled with mCD8-GFP (in green) driven by pntP1-GAL4 and counterstained with anti-PntP1 (in red), anti-Ase (in blue) and anti-Erm (in white) antibodies. Scale bars equal 10µm. White arrows point to type II NBs. Yellow arrows point to imINPs. White arrowheads point to mINPs.

(A-A''') In a wild type type II NB lineage, Erm is expressed in Ase- and Ase+ imINPs and PntP1 is expressed in type II NBs, Ase- and Ase+ imINPs but terminated in mINPs.
(B-B’’) Knockdown of Six4 alone under wild type background did not affect Erm expression in imINPs or PntP1 expression in type II NBs and imINPs. PntP1 levels in Six4 knockdown mINPs are slightly upregulated than wild type mINP, but still do not reach the levels that are comparable to those in NBs (B’’).

(C-D’’) Knockdown of Skd alone (C-C’’) or simultaneous knockdown of Six4 and Skd (D-D’’) in type II NB lineages abolishes Erm expression in imINPs. Though PntP1 is not obviously turned on in mINPs when Skd is knocked down alone (C’’), the expression of PntP1 in mINPs is consistently upregulated to a comparable level as that in NBs, which is counted as PntP1 positive (PntP1+), leading to a drastic increase in the number of total PntP1+ INPs to 10 per lineage when Six4 and Skd are simultaneously knocked down (D’’).

(E-F’’) Knockdown of Kto alone (E-E’’) or simultaneous knockdown of Six4 and Kto (F-F’’) in type II NB lineages abolishes Erm expression in imINPs. Though PntP1 is not obviously turned on in mINPs when Kto is knocked down alone (E’’), the expression of PntP1 in mINPs is consistently upregulated to a comparable level as that in NBs, which is counted as PntP1 positive (PntP1+), leading to an increase in the number of total PntP1+ INPs per lineage when Six4 and Kto are simultaneously knocked down (F’’).

(G-H) Quantifications of Erm intensity in imINPs (G) and number of PnP1+ INPs (H) per type II NB lineage with indicated genotypes. ***, \( P < 0.001 \). NS, no significance.
Figure IV-S2. Six4 genetically interacts with Skd or Kto to prevent the generation of supernumerary type II NBs from dedifferentiation of imINPs.

In all images, type II NB lineages were labeled with mCD8-GFP driven by either *erm-GAL4(II)* and counterstained with anti-Dpn (in red) and anti-Ase (in blue) antibodies. White arrows point to some type II NBs as examples. Scale bars equal 50µm.

(A) Each wild type brain lobe contains eight type II NB lineages. Type II NBs in the head of the lineage was not labeled by mCD8-GFP when it is driven by *erm-GAL4(II)*.

(B) An *erm2/+* mutant brain lobe contains eight type II NB lineages which develop normally.

(C-D) Knockdown of Six4 (C) or Skd (D) alone in imINPs under *erm2/+* background barely produces ectopic type II NBs.

(E-F) Double knockdown of Six4 and Skd in imINPs under *erm2/+* mutant background induces supernumerary type II NBs (E), while double knockdown of Six4 and Skd in imINPs under wild type background does not (F).

(G) Knockdown of Kto in imINPs under *erm2/+* background weakly produces ectopic type II NBs.
(H-I) Double knockdown of Six4 and Kto in imINPs under erm\textsuperscript{3/+} background induces a dramatic generation of supernumerary type II NBs (H), while double knockdown of Six4 and Kto in imINPs under wild type background does not (I).

(J) Quantifications of number of type II NBs per brain lobe with indicated genotypes driven by \textit{erm-GAL4(II)}. ***P < 0.001. **P < 0.01. *P < 0.05.

(K-L) Biochemical interactions between Six4-HA and Myc-Skd (K) or between Six4-HA and Myc-Kto (L) are detected by transfecting S2 cells with their corresponding expression constructs followed by co-IP and western blotting analyses.
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CHAPTER V. General Discussion

Type II NB lineage determination during *Drosophila* embryogenesis and post-embryogenesis

The study in CHAPTER II of this dissertation addressed the important question of how PntP1 specifies type II NB identity by suppressing the type I NB identity marker Ase. Suppression of Ase is a prerequisite for producing transit-amplifying INPs and boosting both output and diversity of neurons/glia. Our results for the first time demonstrate a novel pathway, in which PntP1 acts as a transcriptional activator to induce the expression of the transcriptional repressor Tll which in turn directly suppresses Ase transcription in type II NBs. Our results not only show that PntP1 acts as a transcriptional activator in suppressing Ase to determine the type II NB identity, but also indicate that this transactivation activity is also generally involved late in the process of establishing type II NB lineages. PntP1 promotes the generation of imINPs and specification of mINPs through activating *erm*. This is demonstrated by the observation that the chimeric suppressor containing Ets domain of PntP1 suppresses the generation of imINPs and mINPs when it is expressed in type II NBs and the chimeric activator containing VP16 activation domain fused with C-terminal subfragment containing Ets domain of PntP1 is sufficient to induce imINP-like cells and mINP-like cells when it is expressed in type I NBs. In addition, three lines of evidence also strongly suggest that suppression of Pros in imINPs by PntP1 likely also requires the transactivation activity of PntP1 to activate its downstream target Tll. First, mINPs are induced when the above chimeric activator is misexpressed in type I NBs, suggesting that Pros also needs to be suppressed in those imINPs like cells, since its nuclear localization otherwise would lead to the premature differentiation of imINPs and completely block the generation of mINPs. Second, when Tll is misexpressed in type I NBs and the induced imINP-like cells, or overexpressed in native imINPs or in mushroom NBs, it promotes the generation of turmeric supernumerary NBs.
correlated with the suppression of Pros (Hakes & Brand, 2020; Kurusu et al, 2009; Rives-Quinto et al, 2020). Third, pros enhancer region is also direct bound by repressor Tll (Hakes & Brand, 2020).

Results of this dissertation demonstrate important roles of PntP1 in suppressing Ase mainly after type II NB are specified. It yet does not address the question that if PntP1’s function, including activating Tll or suppressing Ase, or the feedback activation of PntP1 by Tll, is actively required during the initial specification of type II NBs which are delaminated from neuroectoderm or passively induced by unknown upstream pathway during or after type II NB specification. It has been shown that maintaining Ase in PntP1 misexpression type I NBs by the pan-NB driver insc-GAL4 (which is expressed at around stage 9-10 (Kraut et al, 1996; Schaefer et al, 2000), earlier than type II NBs specification) blocks the transformation of these type I NB lineages into type II NB lineages and that EGFR pathway which is the upstream of PntP1 (which is consistently expressed since type II NB specification) or PntP1 itself is required for type II NB specification (Alvarez & Diaz-Benjumea, 2018; Walsh & Doe, 2017; Zhu et al., 2011). Thus, it suggests that PntP1 is essential for specifying type II NBs identity by suppressing Ase during embryogenesis. A recent study has shown that Tll is also expressed at embryonic stages (~stage 13) in type II NBs and that Tll mutant leads to almost a complete loss of PntP1+ Dpn+ type II NBs (Curt et al, 2019). Therefore, either the activation of PntP1 by Tll or the activation of Tll by PntP1 is likely required for the embryonic specification of type II NBs; these are possible candidate mechanisms to be tested in the future.

A synthesis of previous findings (Alvarez & Diaz-Benjumea, 2018; Jussen et al, 2016; Shilo, 2014; Walsh & Doe, 2017; Zhu et al., 2011) and the discoveries presented here, lead to the
following model of type II NBs. The specification of type II NBs occurs following a serial signaling pathway in a narrow time window. Spitz (Spi) as the Epidermal growth factor receptor (EGFR) ligand is secreted by protease rhomboid (rho) and binds to the EGFR, resulting in a serial activation of Sos/Ras/Raf/MEK and MAPK, which shows its expression at ventral-most neuroectoderm where type II NBs are formed and finally leads to the expression of PntP1 (a constitutively active form) mediated by yet unknown intermediate factor(s). Then PntP1 functions as a transcriptional activator, likely with its yet unidentified cofactors to activate Tll expression which in turn suppresses Ase and finally specifies the type II NBs to be distinguished from Ase+ NB identity. Independent of EGFR, Sp1 and Btd are also required for type II NB specification, through unknown pathways. After specifying type II NB identity, Tll and PntP1 form a mutually dependent pair by controlling each other’s expression levels in type II NBs and thus maintain the identity of type II NBs. In progeny of an individual type II NB lineage, the expression of PntP1 and Tll are silenced in a cell-type dependent manner (see below section of “Consecutive inactivation of neural stem cell genes and sequentially turning on differentiation factors in neural progenitors”). Therefore, it would be interesting and important to further identify unknown factors acting between MAPK and PntP1 to complete the determination of type II NB identity during embryogenesis. There are evidence showing the early expression and function of Tll (stage 5 and onwards) in patterning the embryonic Drosophila brain, including by activating EGFR pathway (Jussen et al., 2016). We did not exclude the possibility that Tll is upstream to activate PntP1 during type II NB specification in embryonic stages, instead of functioning as a downstream effector of PntP1 to activate PntP1 in positive feedback as shown in larval stages. Therefore, Tll is a worthy candidate to be tested (also see the discussion section in CHAPTER II).

Our results show removing Tll alone in type II NBs by using pntP1-GAL4 efficiently abolishes PntP1 and misexpressing Tll alone in type I NBs by using insc-GAL4 sufficiently induces PntP1
expression. This suggests that Tll alone is sufficient to maintain PntP1 expression after type II NBs are specified. However, it remains unclear if EGFR signaling is still required redundantly with Tll to maintain type II NB identity after type II NBs are specified. We did not exclude the possibility that Tll may positively regulate EGFR signaling in type II NBs, considering that Tll is reported to both antagonize EGFR (Daniel et al., 1999; Das & Bhadra, 2020) and induce EGFR ligand Spi (Jussen et al., 2016) during Drosophila early brain development. In addition, although we could not exclude the possibility that the loss of PntP1 expression in Tll knockdown type II NBs could be due to the loss of some unknown feedback signal from INPs, we don’t believe the potential feedback provided by Tll is similarly mediated through mINPs based on following findings. First, PntP1 is ectopically activated in just a very small subset (< 5%) of Tll misexpressing type I NBs which fail to produce any mINPs, since imINP-like cells are induced by Tll misexpression in type I NBs but fail to become mINPs due to the reversion to NB fate (Rives-Quinto et al., 2020). Second, overexpressing Tll in type II NB lineages leads to supernumerary type II NBs which are reverted from imINPs and these NB lineages still contain mINPs, while simultaneous knockdown of PntP1 and overexpression of Tll almost deplete mINPs (Fig. II-S2), suggesting that Tll does not promote imINPs mature to become mINPs. We also don’t believe that ectopic Ase alone will suppress PntP1 in Tll knockdown type II NBs either, since the majority of Ase’ Tll misexpressing type I NBs are still PntP1 negative and other evidence shows that removing Ase never transforms the identity of type I NBs to type II NBs (Bayraktar et al., 2010; Bowman et al., 2008).

**Determining functional specificity of the master regulator in type II NB lineage progression**

Studies in CHAPTER II-IV have demonstrated underlying mechanisms of regulating the development of type II NB lineage at different levels, including specifying/maintaining type NB identity and balancing dedifferentiation or differentiation of imINPs, which are mediated by
specific factors in distinct cell types. Results have demonstrated that these factors either depend on or target the master regulator PntP1. In type II NBs, PnP1 needs to be activated and maintained to activate Tll expression for suppressing type I NBs identity marker Ase. The suppression of Ase by PntP1 likely also requires the presence of Skd/Kto, implying that Skd/Kto may involve in the activation of Tll. This will need further investigation in the future. In newly born imINPs, PntP1 suppresses Pros expression to prevent imINPs from prematurely differentiating into GMCs, through a process mediated by interacting with Six4 or Btd and possibly the activation of Tll (see above). In late Ase` and Ase` imINPs, PntP1 directly interacts with cofactor Skd and Kto to activate Erm expression, likely by being directly recruited to the \(erm\) enhancer (Janssens et al., 2017). Erm then directly binds to Six4 and PntP1 to antagonize the activity of PntP1 and/or suppress the expression of PntP1, preventing imINPs from re-acquiring NB identity (Figure. IV-1). Given an updated definition of master regulator genes (MRGs) which “are expressed at the inception of a developmental lineage or a specific cell type, participate in the specification of that lineage by regulating multiple downstream genes’ expression either directly or via interacting with other master regulator genes or signaling pathways to form super-enhancers, and critically, when misexpressed, will lead to uncontrolled expression of downstream target genes and MRGs, and have the ability to respecify the fate of cells destined to form other lineages, causing more abnormal development of tissues and organs (Cai et al., 2020)”, our results support the concept that \(pntP1\) is indeed the master regulator gene for controlling type II NB lineage specification and development (Li et al., 2016; Xie et al., 2016; Xie et al., 2014; Zhu et al., 2011) and that its cell-type dependent function is mediated by interacting with or targeting distinct factors. However, the existing results including this current study do no fully address PntP1’s specific but disparate functions among type II NBs, newly born imINPs and late imINPs, For example, the transcriptional specificity of PntP1 to activate Tll in type II NBs, or Erm in imINPs. To address this question, the first point is to decipher the downstream transcriptional
profile of PntP1 in either type II NB context or imINP context, by high-throughput sequencing approaches such as DamID-Seq or ChIP-Seq. A second putative mechanism is that the cell-type or cell-fate specific cofactors confer PntP1 with enhanced transcriptional specificity to target its downstream enhancers/promoters. This mechanism has been broadly shown to enhance the transcriptional specificity for TFs, including Ets proteins, or even for those cofactors intrinsically lacking of DNA-binding (Haberle et al., 2019; Siggers et al., 2011). Thus it is important to follow up to study how PntP1 achieves the desired transcriptional specificity in type II NBs or imINPs, by using either binding affinity assay (Siggers et al., 2011) to check if they could facilitate the DNA-binding of PntP1 ETs domain to specific downstream target promoter or enhancer, or by using high-throughput self-transcribing active regulatory region sequencing (STAP) based approaches on genome scale (Haberle et al., 2019; Siegenfeld et al., 2019) to identify potential downstream promoters/enhancers with high compatibilities to PntP1. Nevertheless, identifying additional cofactors of PntP1 is also important to fully understand the transcriptional specificity of PntP1, for example by performing proteomic analysis of PntP1-cofactor binding complex (Rivera-Reyes et al., 2018).
Figure V-1. Interaction network centered on PntP1 involved in regulating type II NB lineage development.

The regulatory network (Left) depicts that transcription factors which are studied in this current dissertation are regulated transcriptionally by or interact with PntP1, and in turn regulate PntP1 to engage in governing type II NB or imINP development, which are summarized in the text to the right.

Regulations and contributions of intermediate neuronal progenitors during neurogenic process or disorders.

Drosophila NSCs provide a perfect model to study CNS development, not only on invertebrate level but also for vertebrates, in which some of the outstanding questions underlying the regulations of both physiological neurogenesis or neurological disorders are fundamental and shared. In mammalian brain development after pseudostratified neuroepithelium of ectodermal origin, radial glia cells (RGCs, or RGs) are the primary NSCs in neurogenetic neocortex at embryonic or fetal stages. RGCs are viewed as the analogous of Drosophila NBs (including type I NBs, type II NBs, type 0 NBs, MB NBs or optic lobe NBs) undergo self-renewal and produce a daughter radial glia and a daughter neuron (for direct neurogenesis) or progenitor (for indirect neurogenesis) (Noctor et al., 2004). The self-renewal divisions of radial glia directly producing neurons are reproduced by Drosophila type 0 NBs in late embryonic nerve cords or tOPC (Baumgardt et al., 2014; Bertet et al., 2014; Karcavich & Doe, 2005; Ulvklo et al., 2012). In addition, direct neuron-producing RGCs persist temporally in neurogenesis and spatially in ventricular zone while type 0 NBs are converted from type I NBs at certain point temporally in a lineage-specific dependent manner and spatially in ventral nerve cords or tOPC (Baumgardt et al., 2014; Bertet et al., 2014; Gunnar et al., 2016). RGCs produce either basal intermediate progenitors (bIPs) which terminally divide once to produce differentiated neurons, or outer/basal radial glia cells (oRGs or bRGs) (originally discovered in human and ferret outer SVZ (oSVZ) (Fietz et al., 2010; Hansen et al., 2010; Pinson et al., 2019; Reillo et al., 2011) and then in mouse...
embryonic medial or later neocortex inner SVZ (iSVZ) or oSVZ at lower abundance (Shitamukai et al, 2011; Wang et al, 2011)) which undergo asymmetric divisions to produce self-renewing oRGs and bIPs. Thus, these two types of RGCs are recapitulated by Drosophila type I and type II NBs respectively. The proliferation capability of oRGs is thought to be a crucial advantage for neocortical expansion in mammals, as is shown for INPs in Drosophila type II NB lineages. RGCs and oRGs, like their Drosophila counterpart NBs and INPs, establish hierarchies of the cortex with a diverse type of neurons/glia through spatiotemporal expression of neurogenic transcription factors (Holguera & Desplan, 2018; Penisson et al, 2019; Pollen et al, 2015; Rossi et al, 2017). Therefore, studies on the development and regulation of Drosophila NSCs and INPs will lead to a better understanding of neurogenetic fundamentals, as well as developmental disorders such as microcephaly or CNS tumor genesis. The studies in CHAPTER III and IV demonstrate that INPs, including both immature and mature INPs, present a pivotal node for the regulation of neurogenesis in type II NB lineages through balancing dedifferentiation and differentiation. Type II NB master regulator PntP1 directly binds and recruits mediator complex components Skd and Kto to activate imINP differentiation regulator dfez/Erm in imINPs, which in turn functions together with the homeodomain protein Six4 to antagonize PntP1 function to promote the maturation and differential potential of imINPs and prevent their fate reversion to NBs. We demonstrate that the antagonization effects are mediated by direct protein-protein interactions, although it is still possible that Six4 engages in transcriptional suppression of neural stem cell genes by being recruited with Erm together during chromatin/histone remodeling to repress downstream transcription (Janssens et al., 2017; Koe et al, 2014). The hyper-suppression on NB identity genes including PntP1 in type II NBs or imINPs upon overexpression of Six4, would decrease the INP pool, while insufficient attenuation of PntP1 caused by knockdown of Six4 or Erm, or the failure to activation of Erm due to
reducing/removing Skd/Kto levels, induces imINPs to dedifferentiate to type II NBs and generates brain tumors. Similarly in mammals, defects in oRG generation compromise neurogenesis (Watson et al., 2014) and mis-regulation of proliferation of oRGs contributes to glioblastoma formation by turning on a NSC gene expression profile (Bhaduri et al., 2020; Wang et al., 2020). Six4 is also manifested as a complementary factor to PntP1 to prevent premature expression of Pros and the subsequent precocious loss of INPs, possibly through interacting with Btd (Xie et al., 2014).

Consecutively inactivating neural stem cell genes and sequentially activating differentiation factors in neural progenitors

Aspects of the regulation of the transition from neural stem cells to progenitors are conserved from Drosophila to mammalian neurogenesis, including inactivating neural stem cell genes and activating differentiation factors in neural progenitors. For example, the suppression of Notch signaling is required to bRG formation (Fujita et al., 2020) and the selective LIFR/STAT3 signaling in bRG which is necessary for bRG cell cycle progression (Pollen et al., 2015). ImINPs between type II NBs and mINPs, which are mitotically active and undergo asymmetric divisions to self-renew, provide an optimal location for NB to INP transition by diminishing neural stem cell genes (including those favoring cell fate maintenance/cell growth/proliferation/self-renewal/cell cycle progression), and activating/maintaining differentiation genes (including those promoting imINP maturation or restricting premature differentiation). The studies in CHAPTER III and IV of this dissertation and previous studies consistently demonstrate this paradigm. ImINPs which are formed from the basal part of their parental NBs quickly remove known stemness-supporting factors such as Notch, Zld, Klu, Dpn, Tll, and gradually terminate PntP1, and activate the expression or activity of Ase, Erm, Ham, Brm, Osa, Six4 to facilitate the fate commitment of imINPs to become mitotic mINPs. Based on
studies on the commitment of imINPs to mINPs, sequential phases of regulations can be summarized as follows:

1) The abolishment of self-renewal/proliferation/stemness factor is triggered at the onset of establishing cell polarity, when Brat/Numb sit in the hub for downregulating Notch, Zld, Dpn or Klu.

2) Abolishment of those above factors releases the inhibition on differentiation/maturation-promoting genes, including *erm*, as an example, which is activated by PntP1 and its cofactors Skd/Kto or by components of the integrator complex. Also, it is high likely that the activation of Ham or SWI/SNF complex component Brm/Osa/Snr1 also relies on the removal of the inhibition due to abolishing stemness-supporting factors, given that they function similarly to Erm and directly interact with Erm in the same pathway to regulate the fate commitment of imINPs, though no affirmatory experimental evidence has been reported yet.

3) These differentiation/maturation-promoting factors gradually terminate type II NB identity genes such as *pntP1* (not terminated until mINPs) and *tll* (not terminated until Ase⁺ imINPs) independently or by interacting with other factors including Six4, erm or Ham, although the details of how *tll* is efficiently downregulated in INPs is not clearly understood yet (see also see the discussion section in CHAPTER II). The gradual termination is likely required in preventing precocious activation of the cell cycle exit determinant such as Pros. During this phase, open questions related to the study in CHAPTER IV remain: what exact roles do mediator complex subunits other than Skd/Kto submodule play and how do they function? Based on their genetic interactions with Six4, half of them show variable effects to prevent the generation of supernumerary type II NBs while the other half do not. Thus, it is likely that half of the mediator complex components also prevent the dedifferentiation of imINPs by attenuating the stemness-promoting genes or promoting the expression of differentiation/maturation-promoting factors.
through working with Skd and Kto, while the other half do not. This suggests a possible model which is opposite to the current acknowledgment that Skd or Kto functions generally as an inhibitory subunit to repress all other mediator complex components.

4) To stabilize the self-renewal of mINPs, it is necessary to re-activate some of neural stemness/self-renewal genes (including \textit{dpn/notch}/other cell cycle regulator genes) and maintain some extent of differentiation/maturation genes (including \textit{ase/ham} for stabilizing the self-renewal of mINPs). The mechanisms at work in this process are unknown. However, a few studies have explored how the proliferation/self-renewal, cell growth or cell cycle controlling is regulated in mINPs once they are committed. Considering the similar expression profile of key known regulators between mINPs and type I NBs, including active Notch pathway, Dpn, Ase, cytoplasmic Pros or silenced PntP1 or Tll, and similar asymmetric divisions to produce GMCs, generic regulations of type I NBs may also apply to mINPs. Nevertheless, there must be additional restrictions needed to control INP proliferation or cell growth, considering their limited rounds of mitotic divisions (up to 10) (Bello \textit{et al.}, 2008; Boone & Doe, 2008; Bowman \textit{et al.}, 2008) and smaller cell size of mINPs compared to those of type I NBs. Thus, it is high likely that the de-activation or antagonization of stem cell genes, in a milder manner, persists through the lifespan of mINPs. This is an interesting area of future study.

**Context-dependent tumorigenesis in type II NB lineages**

Our experimental results based on specific manipulations of some key regulators, including overexpressing Tll and downregulating of Six4, Skd, Kto or Erm, consistently produce supernumerary type II NBs resulting from dedifferentiation of imINPs, leading to tumor-like overproliferation. This neural progenitor origin of brain tumor formation is consistent with the recent discovery that adult glioblastoma harbors oRG-like cells with tumorigenic and invasive
properties in mammals (Bhaduri et al., 2020; Wang et al., 2020). The imINP-derived tumor generation is clearly distinct from an asymmetric division defect-related tumor genesis in the way that the mitotic machinery and the cell polarity is not affected. This is demonstrated by 1) supernumerary type II NBs resulting from Six4 knockdown in this study; 2) Tll directly triggering the generation of NB fate from specified imINPs (Hakes & Brand, 2020); and knockdown of Skd/Kto in specified imINPs leading to tumorigenic type II NBs. Our study also presents conserved or novel transcriptional defect clues that will lead to brain tumor formation. Rodent or human Tll homologue, Tlx has long been shown to foster neurogenesis by promoting proliferation and cell cycle progression of NSCs/precursors or progenitors in both embryonic and adult mice brains (Li et al., 2012; Li et al., 2008; Niu et al., 2011). Tll upregulation leads to spontaneous development of glioma-like lesions and gliomas arising from SVZ NSCs with high migratory ability for further invasive progression (Liu et al., 2010) and cells in primary glioblastoma with Tll expression behave cancer stem cells to self-renew and trigger transplantable tumor formation (Zhu et al., 2014). The mutual exclusion of TLX and ASCL1 (human homolog of Ase) in human glioblastoma and Ase restoration preventing Tll tumor generation provide the possibility that Ase is involved in promoting imINP differentiation and make TLX-ASCL1 likely therapeutic targets for progenitor-derived brain tumor (Hakes & Brand, 2020). Emerging incidence of mutation in MED12 (Kto in Drosophila) shows its role in diverse cancer types, including thyroid cancer, leukemia, uterine leiomyomas, fibroadenomas or phyllodes tumors of the breast (Ibrahimpasic et al., 2017; Piscuoglio et al., 2015; Zhang et al., 2020). Although no convincing evidence yet demonstrates mis-regulation of MED12 in brain tumor except that our results for the first time to show that MED12 may also function in restricting uncontrolled proliferation of tumor generated from neural progenitors in mammalian CNS. Similar situation fits to MED13. Emerging studies have shown Six4 functions in regulating various types of tumor formation, including colorectal cancer (Sun et al., 2019), lung cancer (Tang et al., 2019; Zhang et
al, 2019), breast cancer (Sun et al, 2020), and hepatocellular carcinoma (He et al, 2020).

However, the finding in this current study of Six4 functioning to prevent brain tumor generation in this current study is completely novel, possibly due to the lack of evidence showing Six4 expression in neural progenitor pools in mammals.

In addition to Skd and Kto, knocking down some, but not all, of other components of mediator complex, particularly when together with knocking down of Six4, leading to the generation of supernumerary type II NBs, which resembles tumor formation. Although it needs to be further validated by additional genetic tests, it is highly likely that these supernumerary type II NBs arise from the dedifferentiation of (im)INPs given that simultaneous knockdown of Six4 synergistically boosts the phenotypes. These observations suggest that mediator complex subunits prevent the imINP-derived tumor generation. However, our results show that only a subset of mediator complex subunits involved this process, suggesting that individual subunits of mediator complex may not function uniformly in imINPs to maintain their differentiation. Thus, it is essential to further determine the precise composition of mediator complex subunits in the subgroup that specifically regulates the differentiation of imINPs, which can be potentially determined by examining interactions of other components of the mediator complex with Skd, Kto, or Six4 in imINP context or isolating the possible subcomplex.

A brief conclusion

Studies in this dissertation first discovered the detailed molecular mechanisms in maintaining the identity of type II NBs in drosophila central brains, that is a novel pathway of “PntP1→Tll→Ase” identified in type II NBs. The studies also further dissected the mechanism underlying the promotion of development of imINPs, that is the synergistic interaction of Six4 and Erm (which is activated by PntP1 and its novel cofactors, Skd and Kto) to antagonize PntP1 to ensure imINPs
become fate-committed mINPs. Thus, detailed mechanisms are demonstrated in this dissertation to provide novel insights at the transcriptional level to better understand the development of both type II NBs and INPs in *Drosophila* CNS (Fig. V-2).

**Figure V-2. A working model of indicated factors in regulating the development of type II NBs and INPs.**

In type II NBs, PntP1 directly activate the repressor Tll to suppress Ase expression and thus maintains the identity of type II NBs. In newly born imINPs, Six4 contributes to the inhibition of nuclear Pros expression and thus prevents premature differentiation of imINPs to become GMCs. In early and late imINPs, except the newly born imINPs, Skd and Kto function as cofactors of PntP1 to activate Erm, which in turn directly binds Six4 to antagonize PntP1’s function to promotes the maturation of imINPs and prevent their dedifferentiation, thus ensuring that imINPs become fate committed mINPs instead of dedifferentiating to type II NBs.
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APPENDIX: PUBLICATIONS


- Zhu, S., **Chen, R.** Soba, P. and Jan, Y.N., 2019. JNK signaling coordinates with ecdysone signaling to promote pruning of *Drosophila* sensory neuron dendrites. Development, 146(8).

