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RESEARCH ARTICLE

Figures and figure supplements

Tailless/TLX reverts intermediate neural progenitors to stem cells driving tumourigenesis via repression of *asense/ASCL1*

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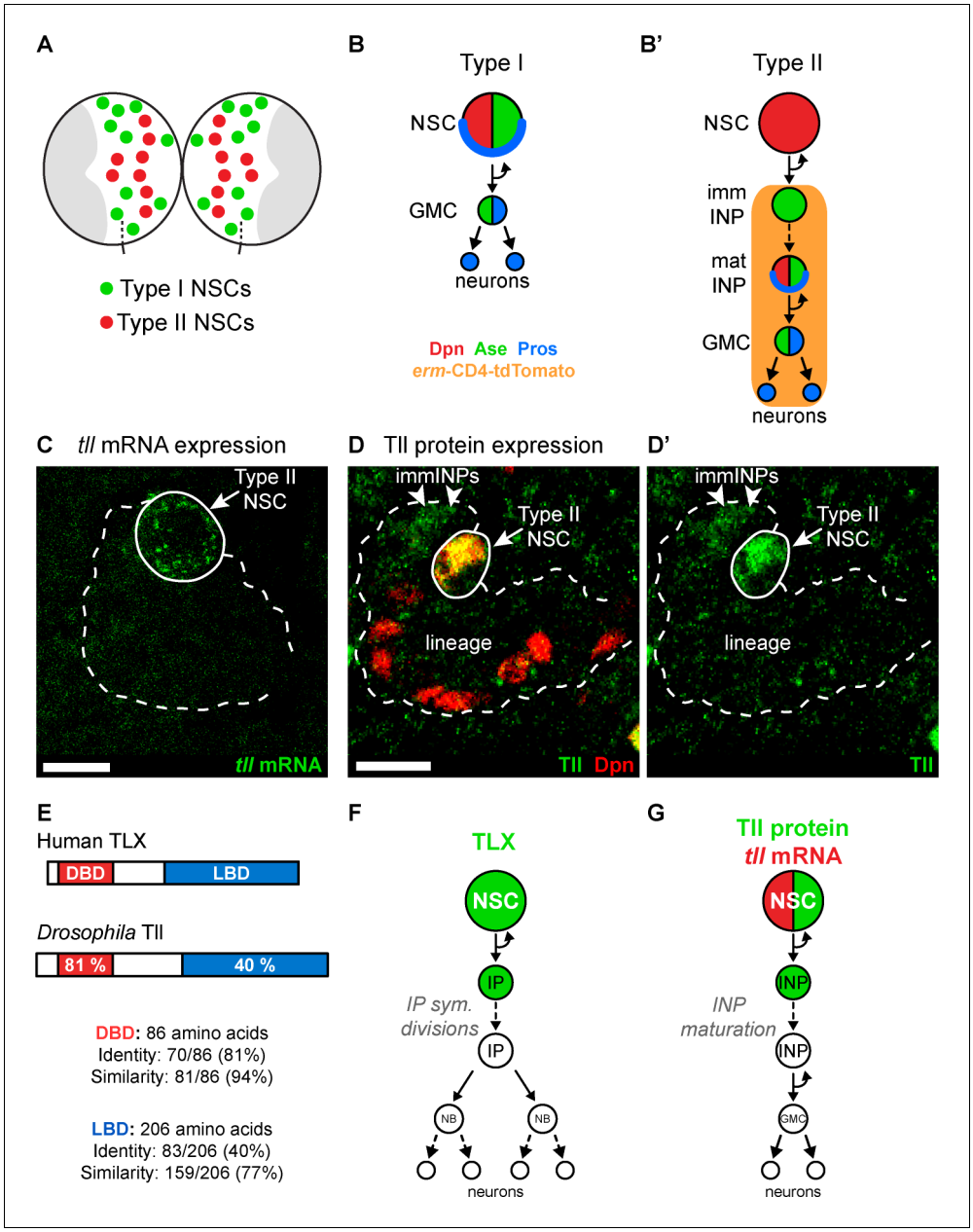

Figure 1. Tll is expressed in *Drosophila* Type II NSCs. (**A**) Schematic showing the position of the eight Type II NSCs (red) in each brain lobe. The majority of stem cells in the *Drosophila* brain are Type I NSCs (green). The optic lobes, which generate the adult visual processing centre, are shown in grey. (**B–B'**) Schematics showing the expression of cell fate markers in (**B**) Type I and (**B'**) Type II lineages. NSC: neural stem cell; imm INP: immature intermediate neural progenitor; mat INP: mature intermediate neural progenitor; GMC: ganglion mother cell. (**C**) RNA FISH shows *tll* mRNA (green) expression in Type II NSCs (solid outline) but not in their lineages (dotted outline). Type II lineages were identified by *pntP1*-GAL4 > *mCD8-GFP* expression in the central brain at wandering third instar larval stage. (**D–D'**) Immunostaining for Tll (green) shows strong expression in Type II NSCs (Dpn^+ (red), solid outline) and weak expression in Dpn^- immature INPs (immINPs, arrow heads). Mature INPs (small Dpn^+ cells in the lineage) do not express Tll. Type II lineages were identified by *pntP1*-GAL4 > *mCD8-GFP* expression in the central brain at wandering third instar larval stage. (**E**) Amino acid conservation between human TLX and *Drosophila* Tll. (**F**) Schematic showing that TLX (green) is expressed in NSCs and intermediate progenitors (IPs) in SVZ of the adult mouse brain (***Li et al., 2012***; ***Obernier et al., 2011***). (**G**) Schematic showing *tll* mRNA (red) and Tll protein (green) expression in *Drosophila* Type II NSC lineages. Single section confocal images. Scale bars represent 10 µm.

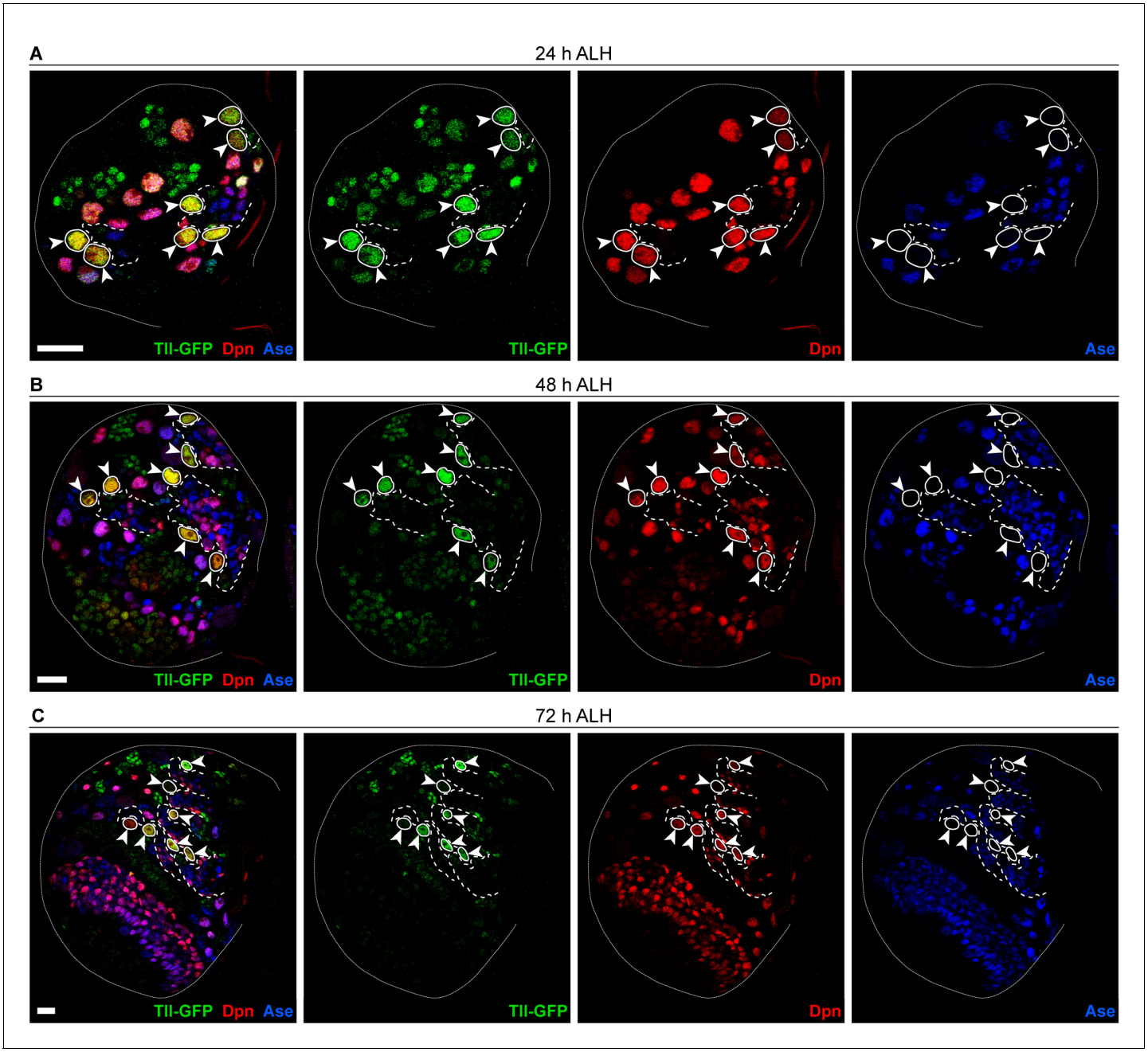

Figure 1—figure supplement 1. Tll is expressed in *Drosophila* Type II NSCs. (**A–C**) Tll-GFP (green) is expressed in Type II NSCs (Dpn^+ (red) and Ase^- (blue)) (arrowheads and outlined with solid white lines) and downregulated in differentiating lineages (dotted white lines) during larval development. (**A**) 24, (**B**) 48, or (**C**) 72 hours (h) after larval hatching (ALH). Seven of eight Type II lineages per brain lobe are shown in each panel. *pntP1*-GAL4 > *myr-RFP* expression was used to identify Type II lineages. Single section confocal images. Scale bars represent 15 μm.

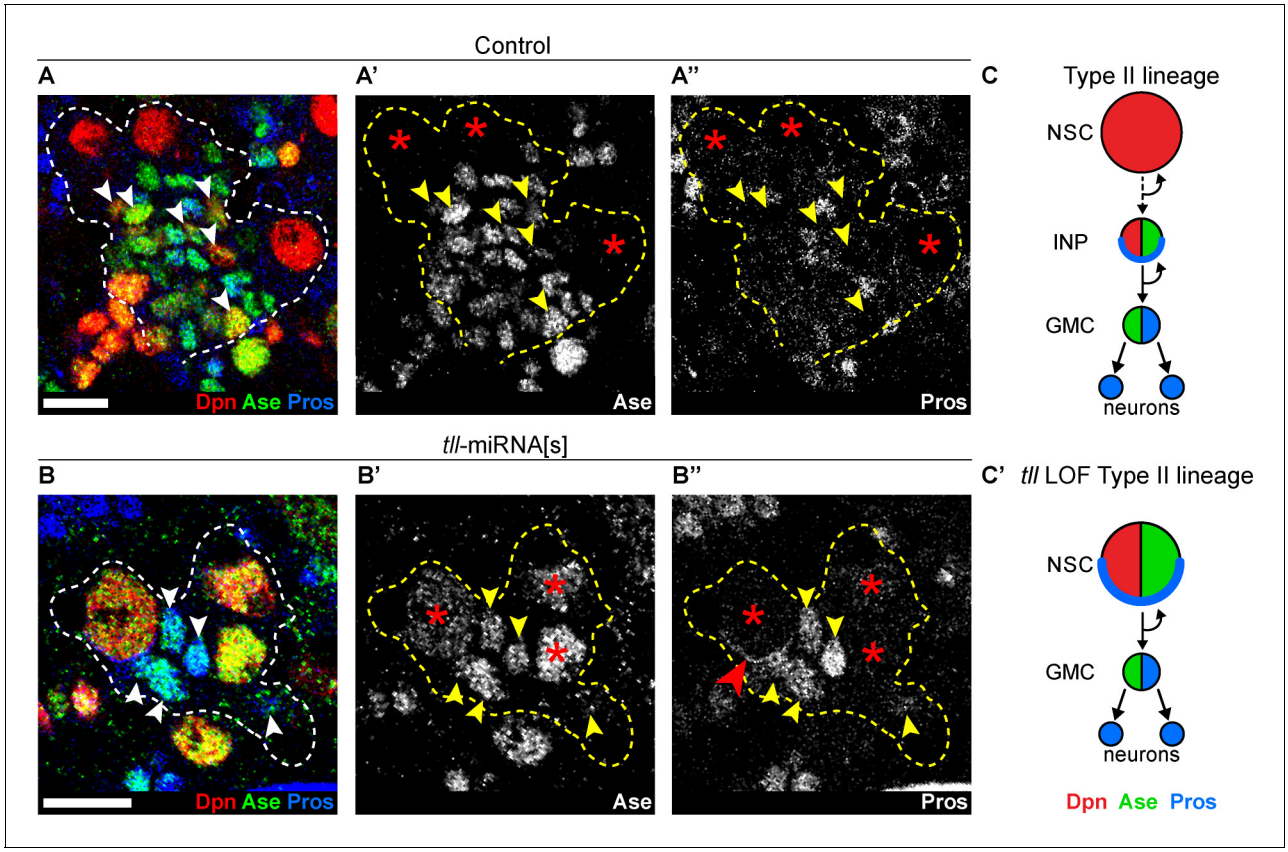

Figure 2. Tll is required for Type II NSC fate and lineage progression. (**A–A''**) Control Type II NSCs (Dpn^{+} (red) and Ase^{-} (green)) generate INPs (arrowheads, Dpn^{+}, Ase^{+} and Pros^{-} (blue)). Dotted lines outline three Type II lineages. Red asterisks (*) indicate Type II NSCs. *n* = 10 brains, dissected at the end of second larval instar stage. (**B–B''**) Upon *tll* knockdown using *pntP1 >act*-GAL4 to drive UAS-*tll*-miRNA[s], Type II NSCs express Ase and generate GMCs directly (arrowheads, Ase^{+} and Pros^{+}) and exhibit Pros crescents (red arrowhead). Dotted lines outline three Type II lineages identified by *pntP1 >act*-GAL4 driving UAS-*GFP*. Red asterisks (*) indicate Type II NSCs. *n* = 10 brains, dissected at the end of second larval instar stage. (**C–C'**) Schematic summarising the *tll* loss of function (LOF) phenotype in Type II NSCs. Single section confocal images. Scale bars represent 10 μm.

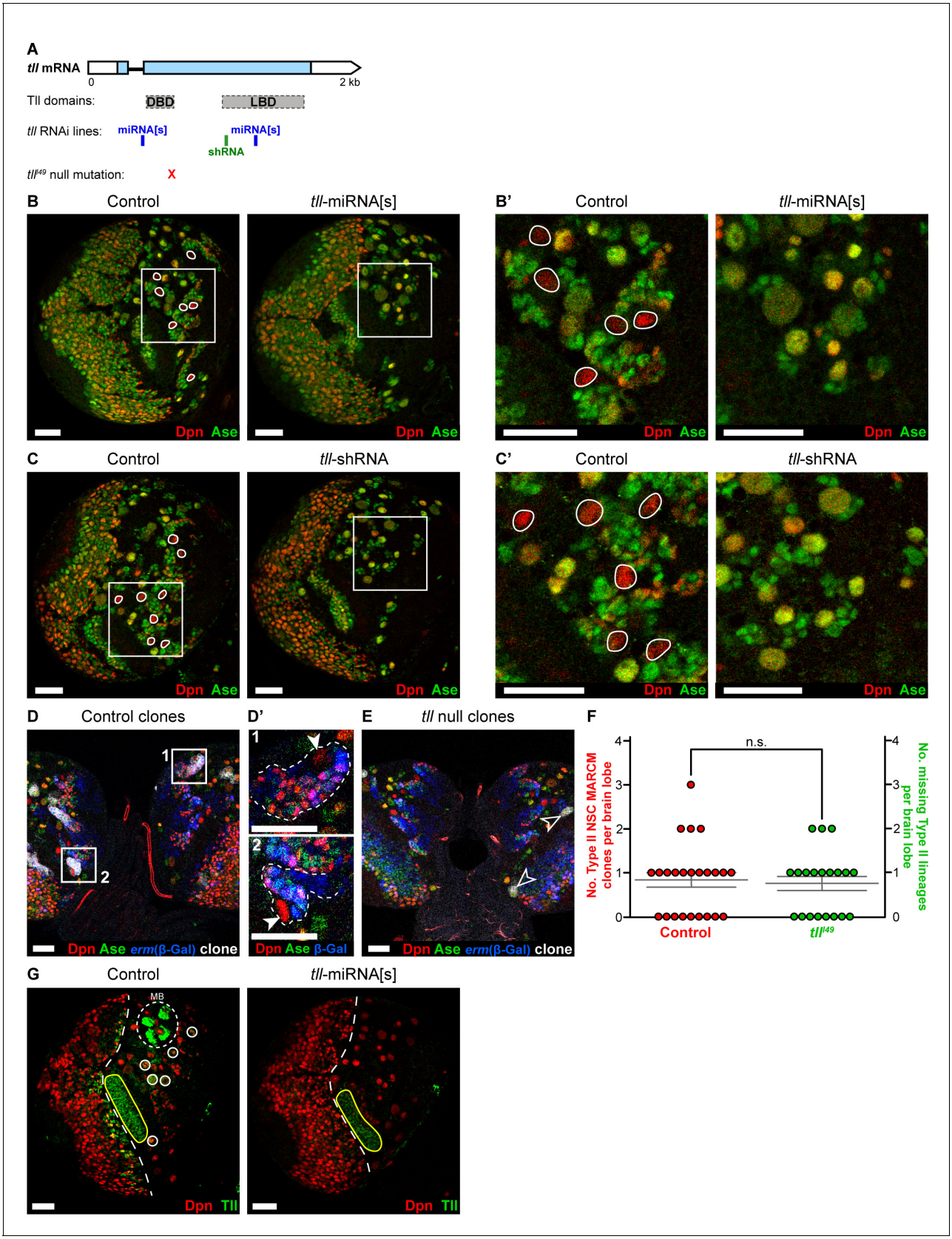

Figure 2—figure supplement 1. TII loss of function in Type II NSCs. (**A**) The *tll* coding sequence (blue regions of *tll* mRNA) codes for two protein domains (grey): DBD and LBD. *tll* RNAi lines miRNA[s] (***Lin et al., 2009***) and shRNA (from VDRC) target different regions of *tll* mRNA. tll^{l49} contains a

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point mutation (x) at the 3' end of the DBD that creates a stop codon, resulting in a null mutant (***Diaz et al., 1996***; ***Pignoni et al., 1990***). (**B–B'**) Knockdown of *tll* during larval stages (*wor*-GAL4,UAS-*mCD8-GFP;tub*-GAL80^{ts}>*tll*-miRNA[s]) resulted in the absence of all Type II NSCs. Control brains contained eight Type II NSCs (Dpn^{+} (red) and Ase^{-} (green), white outlines; seven visible in the section shown). In *tll*-miRNA[s] brains, all NSCs expressed Dpn and Ase. Images in (B') are magnifications of the boxed regions in (B). *n* = 10 brain lobes for Control; *n* = 11 brain lobes for *tll*-miRNA[s]. (**C–C'**) Using *wor*-GAL4,UAS-*mCD8-GFP;tub*-GAL80^{ts} to drive *tll*-shRNA also resulted in the loss of all Type II NSCs (Dpn^{+} and Ase^{-}, white outlines in Control). Images in (C') are magnifications of the boxed regions in (C). *n* = 11 brain lobes for Control and *tll*-shRNA. (**D–D'**) Type II NSCs (Dpn^{+} and Ase^{-}) could be encompassed within control MARCM clones at 72 hr after clone induction. *wor*-GAL4 >*mCD8-mCherry* (white) identified NSC clones and *erm-lacZ* (β-Gal (blue)) labelled Type II lineages. (D') shows magnifications of the boxed regions in (D). Type II NSCs (arrowheads) and their lineages marked by clones are highlighted by dotted white lines. 20 Type II NSC MARCM clones were observed in 24 brain lobes analysed (from 12 brains). (**E**) Central brain Type I tll^{l49} NSCs could be visualised (arrowheads), but no tll^{l49} Type II lineage clones could be recovered at 72 hours after clone induction. *n* = 20 brain lobes (from 10 brains). (**F**) Quantifications of the number of Type II lineages labelled by control MARCM clones per brain lobe (20 clones in 24 brain lobes) compared to the number of Type II lineages absent in tll^{l49} brain lobes (15 absent lineages in 20 brains). Mann-Whitney U test, P = n.s. (P = 0.849). (**G**) Driving expression of *tll*-miRNA[s] with *insc*-GAL4;*tub*-GAL80^{ts} during larval development effectively knocks down Tll (green) in all NSCs (Dpn^{+} (red)). In the control panel, Type II NSCs are highlighted by solid circles (6 of 8 are visible in the section shown) and mushroom body (MB) lineages express high levels of Tll (dotted circle) as reported previously (***Kurusu et al., 2009***). The dotted line in both panels indicates the boundary between the optic lobe (left) and the central brain (right). Note that *insc*-GAL4 is not expressed in neuroepithelial cells of the optic lobe and so Tll expression is unaffected in these cells (yellow outline highlights the neuroepithelium of the optic lobe inner proliferation centre). Brains dissected at wandering third instar stage.

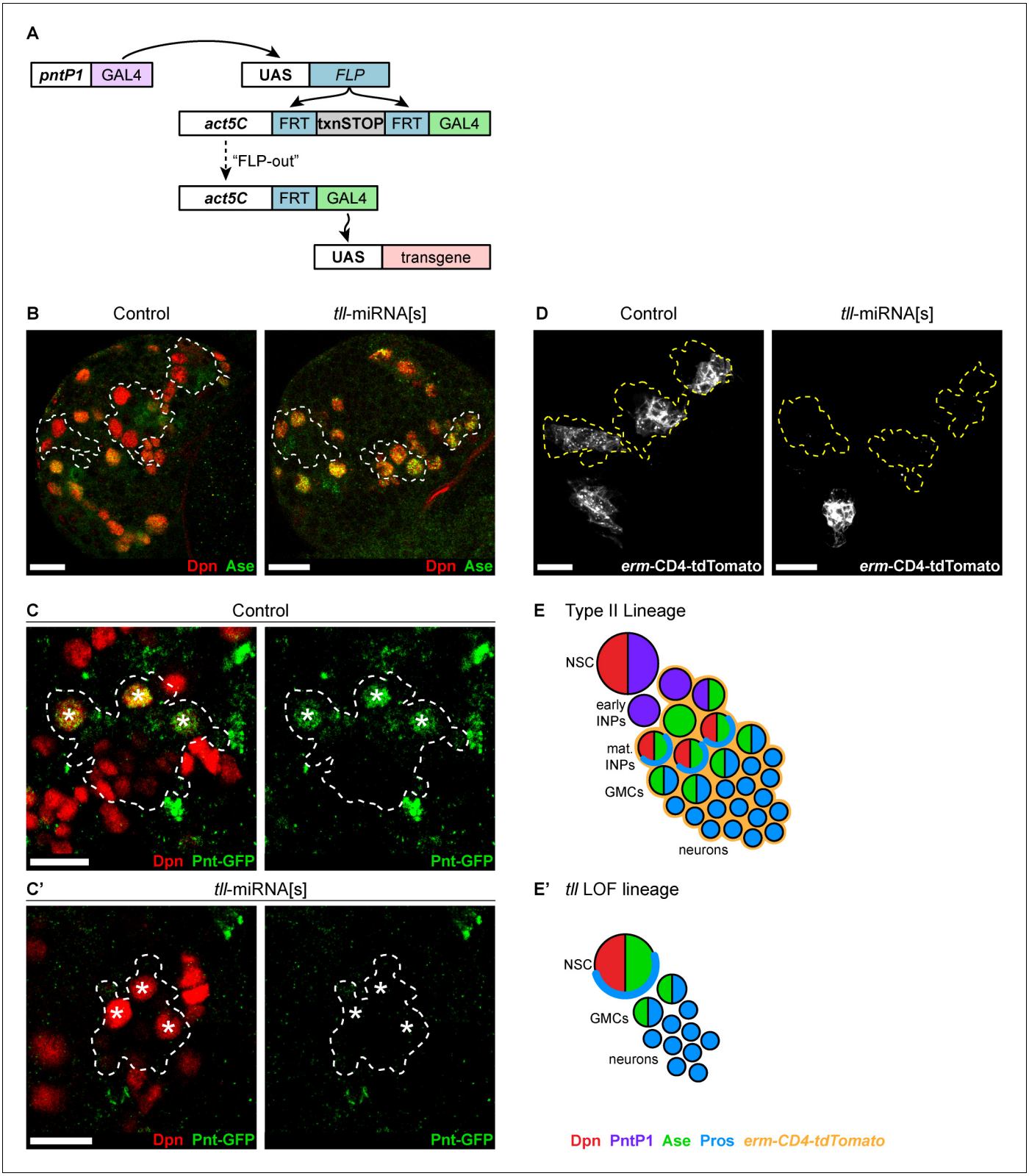

Figure 2—figure supplement 2. Generating an immortalised Type II NSC driver to assess cell fate changes. (**A**) Schematic showing *pntP1 >act*-GAL4, an immortalised Type II NSC driver. (**B**) Driving UAS-*tll*-miRNA[s] with *pntP1 >act*-GAL4 resulted in the derepression of Ase (green) in all Type II NSCs (Dpn^{+} (red)) at the end of the first larval instar. *n* = 14 brains for Control; *n* = 11 brains for *tll*-miRNA[s]). Type II lineages identified by *pntP1 >act*-GAL4 driving UAS-*GFP* are outlined with dotted white lines. (**C-C'**) Driving UAS-*tll*-miRNA[s] with *pntP1 >act*-GAL4 resulted in the loss of Pnt-GFP (green)

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expression from Type II NSCs (Dpn$^+$ (red)). Pnt-GFP was lost from all Type II NSCs in 11 brains assessed with the exception of 4 dorsal-lateral lineages, which maintained weak Pnt-GFP expression (*i.e.* 172 Type II NSCs out of 176 total lost Pnt-GFP expression upon *tll* knockdown). *n* = 12 brains for Control; *n* = 11 brains for *tll*-miRNA[s]. Brains were dissected at the end of second larval instar stage. Dotted lines outline three Type II lineages identified by *pntP1 >act*-GAL4 driving UAS-*lacZ(nls)*. White asterisks (*) indicate Type II NSCs. (**D**) *erm-CD4-tdTomato* (white) is absent in all *tll*-miRNA[s] Type II lineages at the end of the first larval instar. In control brains, *erm-CD4-tdTomato* is expressed in INPs and is a Type II lineage marker. *n* = 14 brains for Control; *n* = 11 brains for *tll*-miRNA[s]. Type II lineages identified by *pntP1>act*-GAL4 driving UAS-*GFP* are outlined with dotted yellow lines. Images are max projections over 10 μm. (**E–E'**) Schematic summarising the *tll* loss of function (LOF) phenotype in Type II NSCs, showing that *tll* LOF lineages switch to Type I fate. Single section confocal images unless stated otherwise. Scale bars represent 15 μm.

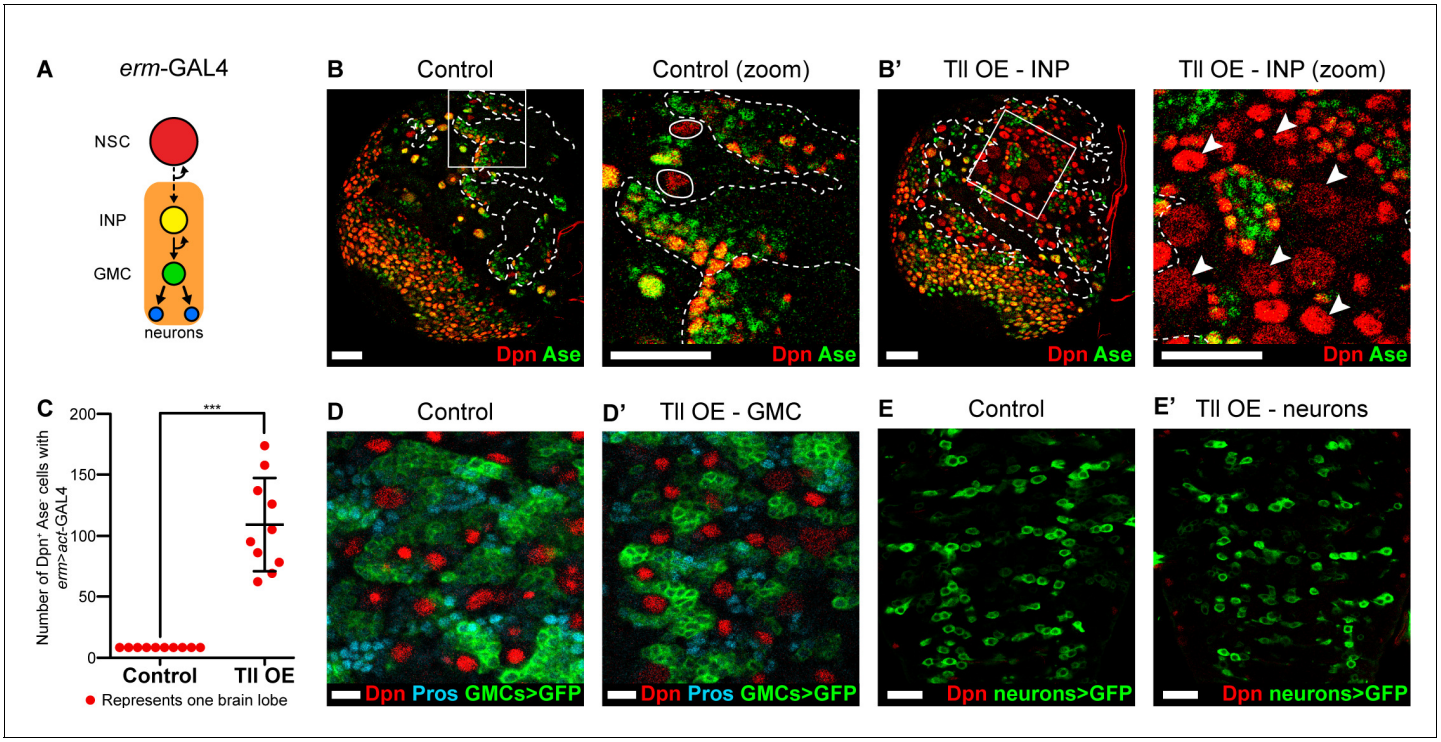

Figure 3. Tll overexpression in INPs generates ectopic NSCs. (**A**) Schematic showing the expression of *erm*-GAL4, which begins to be expressed in Type II lineages during the final stages of INP maturation. (**B–B'**) In Control (zoom), solid white outlines indicate Type II NSCs and *erm >act*-GAL4 is expressed in their lineages (dotted white lines). Tll OE in INPs with *erm >act*-GAL4 resulted in a large expansion of Type II NSCs (Dpn^+ (red) and Ase^- (green)) in Type II lineages. Arrowheads in Tll OE – INP (zoom) highlight ectopic Type II NSCs. Zoom panels are magnifications of boxed regions in Control and Tll overexpression (OE) – INP. *n* = 10 brain lobes for Control and Tll. UAS-*tll* expression was restricted to larval stages with *tub*-$GAL80^{ts}$ and brains were dissected at wandering third instar larval stage. (**C**) Quantification of the total number of Type II NSCs (Dpn^+ Ase^-) in Control or Tll OE *erm >act*-GAL4 brains. Kolmogorov-Smirnov test ***, p<0.001 (p=0.000091). (**D–D'**) Expressing Tll in GMCs (using GMR71C09-GAL4 > *mCD8-GFP* (green)) does not result in ectopic NSCs (*i.e.* no Dpn^+ GFP^+ cells) nor defects in differentiation, as assessed by Pros (blue) staining. *n* = 10 brains for Control, *n* = 12 brains for Tll OE. Brains were dissected at wandering third instar larval stage. (**E–E'**) Expressing Tll in neurons using OK371-GAL4 > *mCD8-GFP* (green) does not result in ectopic NSCs (*i.e.* no Dpn^+ GFP^+ cells). *n* = 4 brains for Control and Tll. Brains were dissected at wandering third instar larval stage. Single section confocal images. Scale bars represent 30 µm in (**B, B', E, E'**) and 10 µm in (**D, D'**).

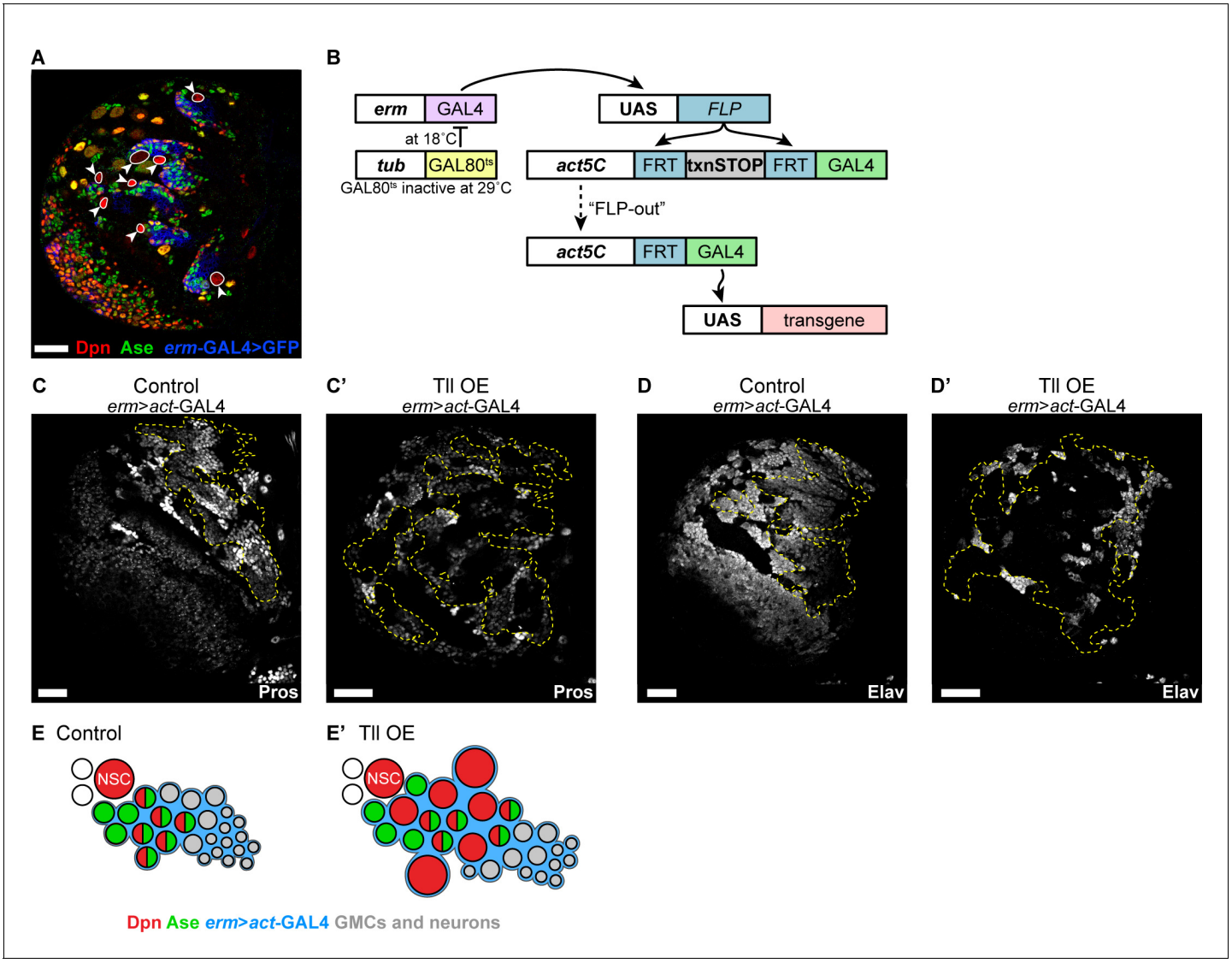

Figure 3—figure supplement 1. TII overexpression in INPs results in ectopic Type II NSCs. (**A**) erm-GAL4 >*mCD8-GFP* (blue) is not expressed in Type II NSCs (Dpn^{+} (red), Ase^{-} (green) arrowheads and white outlines) but is expressed in their lineages. Brains were dissected at wandering third instar larval stage. (**B**) Schematic showing *erm* >*act*-GAL4, an immortalised Type II INP driver, which includes *tub*-GAL80^{ts} to allow for temporal regulation of GAL4 activity. (**C–C'**) Expressing high levels of TII with *erm* >*act*-GAL4 during larval development resulted in a decrease in differentiating progeny compared to Control, as assessed by Pros staining (white). *n* = 10 brain lobes for Control and TII OE. Brains dissected at wandering third instar larval stage. (**D–D'**) Expressing high levels of TII with *erm* >*act*-GAL4 during larval development reduced the generation of neurons from INPs, as assessed by staining for Elav (white). *n* = 13 brain lobes for Control; *n* = 12 brain lobes for TII overexpression (TII OE). Brains dissected at wandering third instar larval stage. (**E–E'**) Schematic depicting the effect of TII OE on Type II lineages. Single section confocal images. Scale bars represent 30 μm.

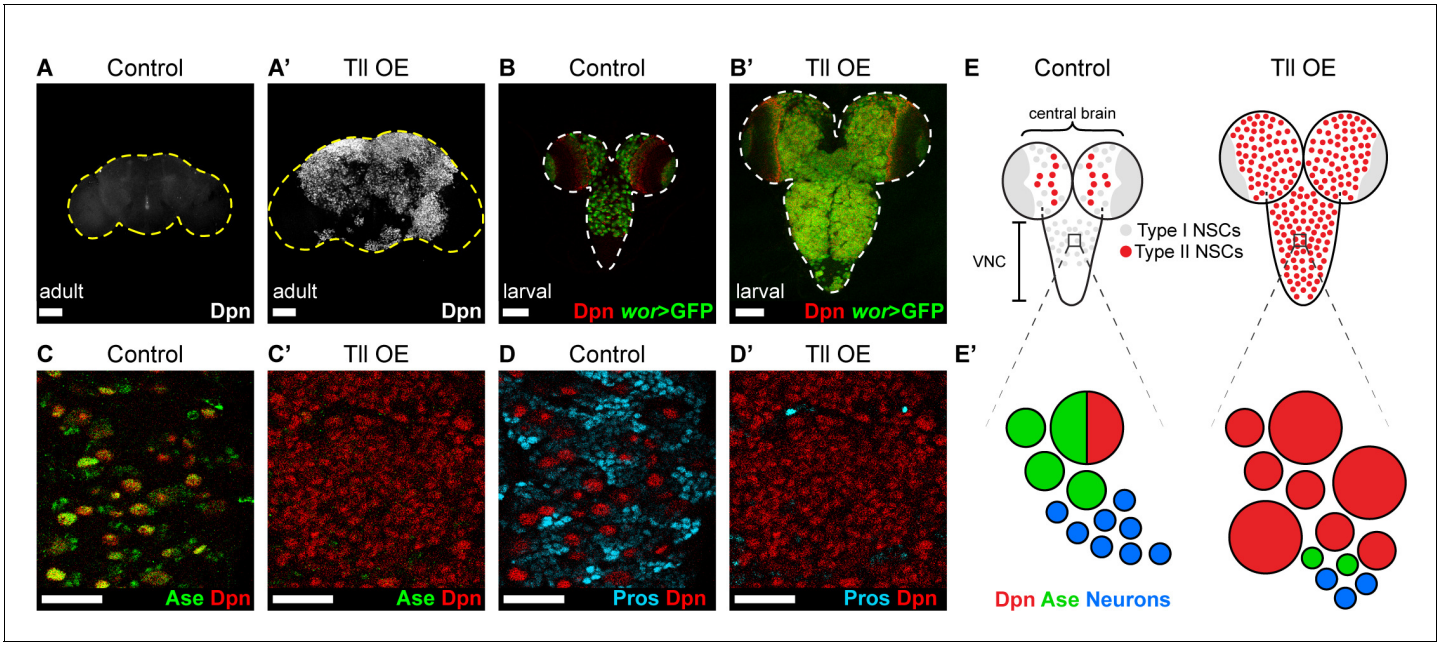

Figure 4. Tll can initiate Type II NSC tumours from Type I NSCs. (**A–A'**) Overexpression of *Drosophila* Tll in neural lineages using *wor*-GAL4 resulted in NSC tumours (Dpn^{+} (white)) in all adult brains assessed. Control adult brains did not contain any NSCs. *n* = 7 brains for Control and Tll OE. UAS-*tll* expression was restricted to late larval stages with *tub*-GAL80^{ts} and brains were dissected from newly-eclosed adult flies. Images are projections over 15 µm (Control) or 17 µm (Tll). (**B–B'**) Overexpression of Tll during larval development with *wor*-GAL4 resulted in large tumours consisting of ectopic NSCs (Dpn^{+} (red) and *wor*-GAL4 >*mCD8-GFP* (green)) in the central brain and VNC of all brains assessed. UAS-*tll* expression was restricted to larval stages with *tub*-GAL80^{ts} and brains were dissected at wandering third instar larval stage. (**C–C'**) NSCs in the VNC are Type I (Dpn^{+} (red) and Ase^{+} (green)) in Control brains. Tll-induced tumours (ectopic Dpn^{+} cells) derived from Type I NSCs in the VNC are negative for Ase. UAS-*tll* expression was restricted to larval stages with *tub*-GAL80^{ts} and brains were dissected at wandering third instar larval stage. (**D–D'**) Tll tumours in the VNC occur at the expense of differentiating progeny (Pros (blue)). UAS-*tll* expression was restricted to larval stages with *tub*-GAL80^{ts} and brains were dissected at wandering third instar larval stage. (**E**) Schematic showing the organisation of Type I NSCs (Ase^{+} (grey)) and Type II NSCs (Ase^{-} (red)) in Control brains and Tll OE brains. Note that in Control brains the VNC contains only Type I NSCs, whereas Tll OE VNCs contain many ectopic Type II NSCs. (**E'**) Schematic showing transformation of Type I NSC lineages in the VNC to ectopic Type II NSCs when Tll is expressed at high levels. Single section confocal images unless stated otherwise. Scale bars represent 100 µm in (A-B') and 30 µm in (C-D'). *n* = 10 brains for all conditions unless stated otherwise.

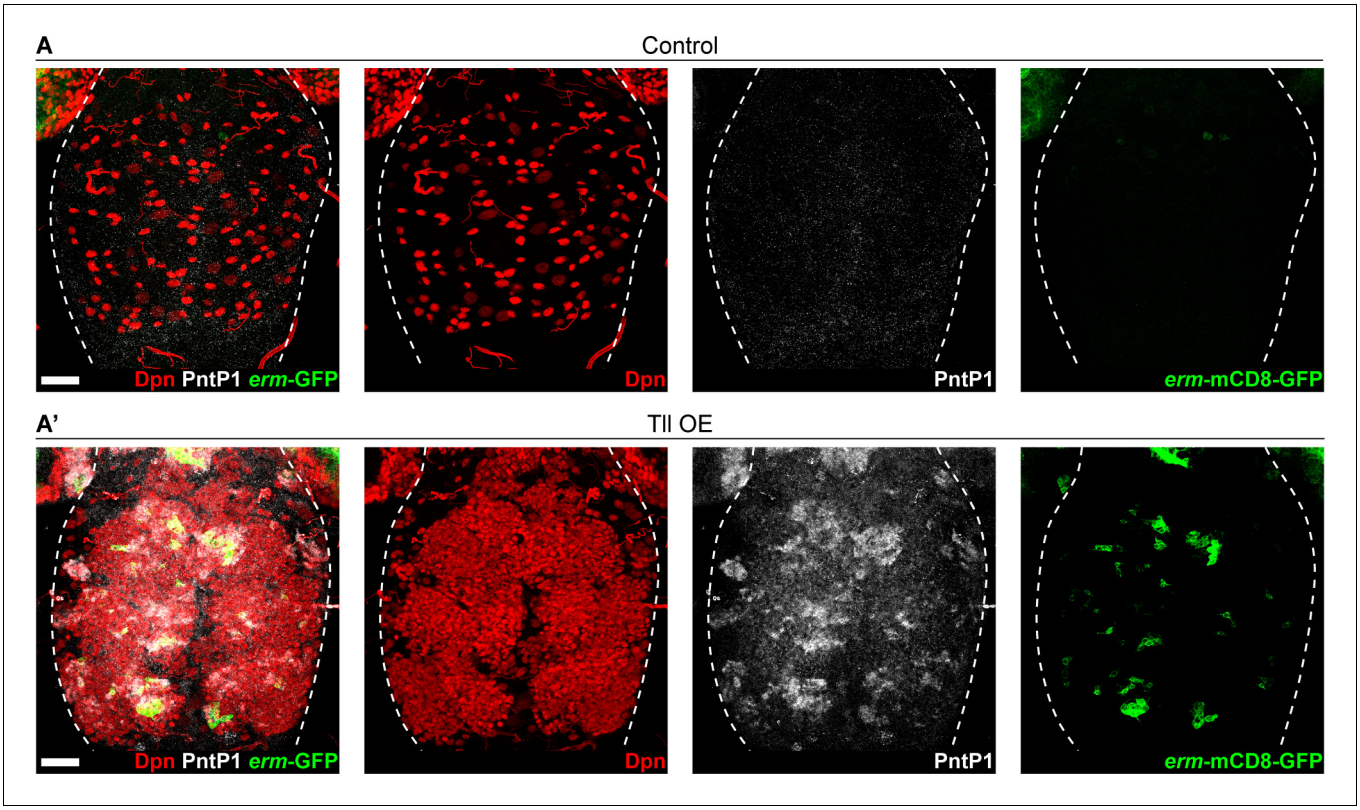

Figure 4—figure supplement 1. Tll is sufficient to induce the generation of INPs from a subset of Type I NSCs. (**A**) In Control VNCs, all lineages are generated from Type I NSCs (Dpn^{+} (red)). Type I NSCs do not express PntP1 (white) and do not give rise to INPs (as assessed by *erm*-mCD8-GFP expression (green)). *n* = 10 VNCs for Control. Images are a projection over 25 µm. (**A'**) When Tll is expressed at high levels in VNC NSCs, a subset of the ectopic NSCs (Dpn^{+}) express PntP1 and generate INPs (*erm*-mCD8-GFP^{+}). UAS-*tll* expression was restricted to larval stages with *tub*-GAL80^{ts}. *n* = 10 VNCs for Tll overexpression (Tll OE). Brains dissected at wandering third instar larval stage. Images are a projection over 58 µm. Scale bars represent 30 µm.

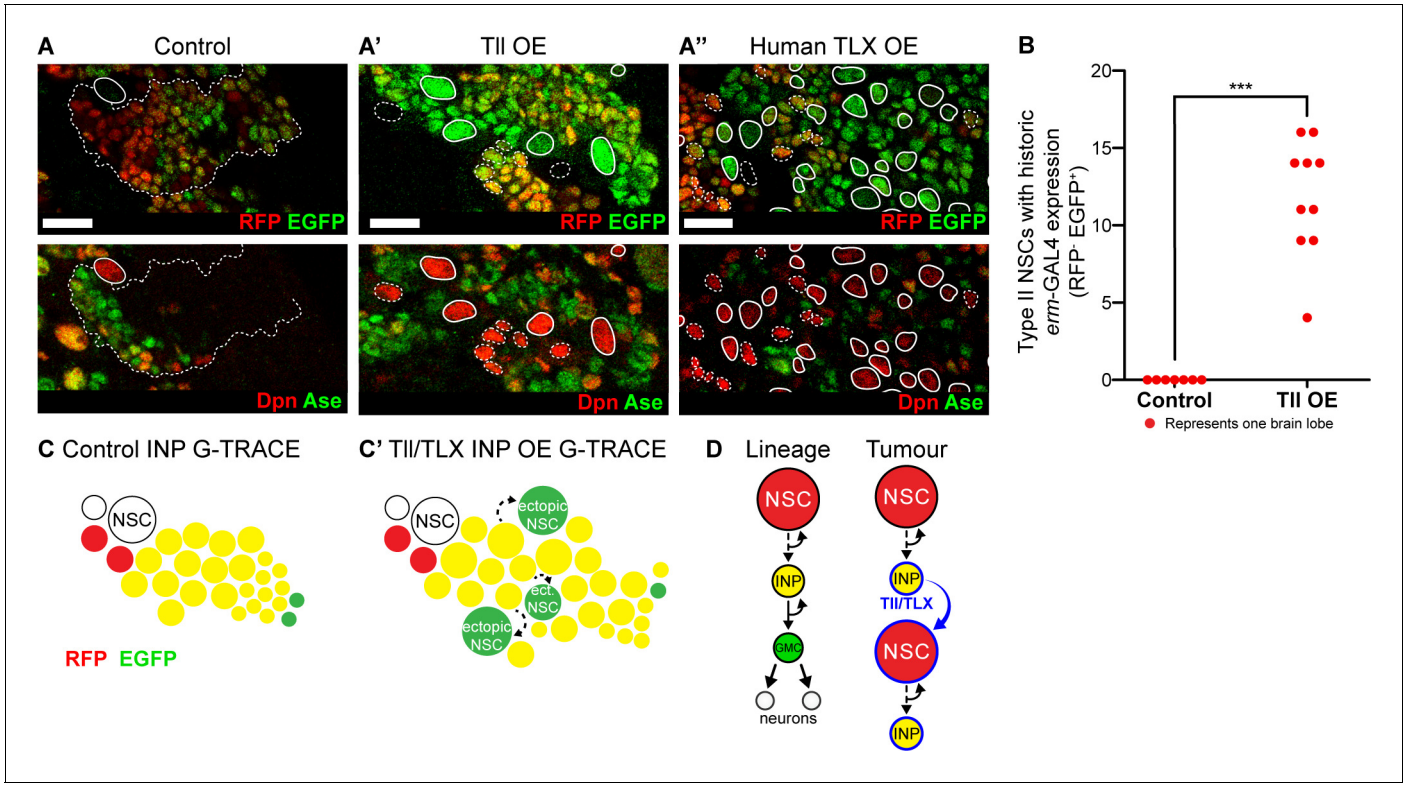

Figure 5. Tll/TLX overexpression results in reversion of INPs to NSC fate. (**A–A''**) G-TRACE reveals current (RFP (red)) and historic (EGFP (green)) *erm*-GAL4 expression (top panels). Dpn (red) and Ase (green) were used to assess the reversion of INPs to Type II NSCs (bottom panels). (**A**) In Control Type II lineages, NSCs (Dpn^+ Ase^-, solid outline) are negative for both components of G-TRACE, whereas lineages show transition from RFP to EGFP (dotted outline). Overexpression (OE) of (**A'**) Tll or (**A''**) human TLX in INPs resulted in ectopic Type II NSCs (Dpn^+ Ase^-, white outlines) that express the EGFP component of the G-TRACE only (solid outline). Dpn^+ Ase^- NSCs with dotted white outline either express neither G-TRACE component (as in Control) or express both RFP and GFP (indicating current expression of *erm*-GAL4). *n* = 8 brain lobes for Control and *n* = 10 for Tll and human TLX. Brains were dissected at wandering third instar stage. (**B**) Quantification of Type II NSCs expressing G-TRACE memory only (*i.e.* Dpn^+ Ase^- and RFP^- $EGFP^+$). Kolmogorov-Smirnov test ***, p<0.001 (p=0.000103). *n* = 7 brain lobes for Control; *n* = 10 brain lobes for Tll overexpression (OE). Brains were dissected at wandering third instar larval stage. (**C–C'**) Schematic showing the expression of G-TRACE with the INP-specific *erm*-GAL4 in Control brains or with Tll/TLX OE. (**D**) A model for how Tll/TLX generates ectopic NSCs and, consequently, tumours from INPs. Single section confocal images. Scale bars represent 15 µm.

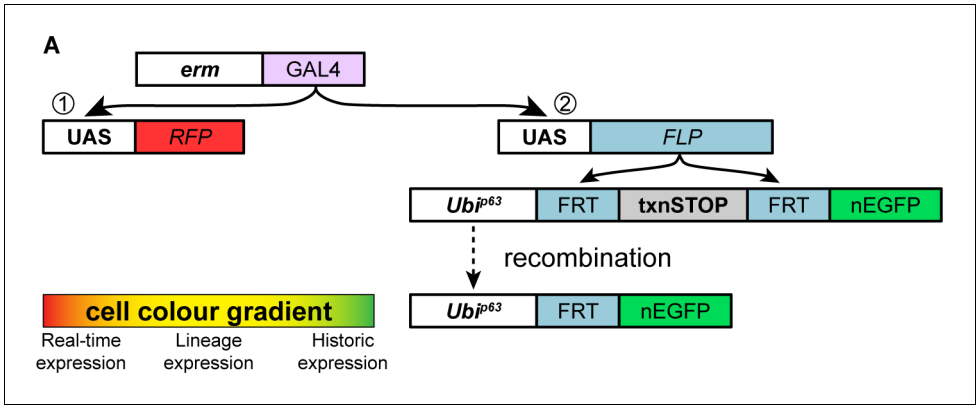

Figure 5—figure supplement 1. Tll/TLX overexpression induces reversion of INPs to NSC fate. (**A**) Schematic showing the G-TRACE system. When *erm*-GAL4 is used to drive the G-TRACE cassette, GAL4 drives the expression of (1) UAS-*RFP* (real-time expression) and (2) UAS-*FLP*, which excises a transcriptional stop sequence to allow a *Ubi* promoter to drive EGFP expression (historic expression). The cell colour gradient shows the transition from current (red) to historic (green) GAL4 expression: RFP-only (real-time), to RFP and EGFP co-expression (lineage), to EGFP-only (historic).

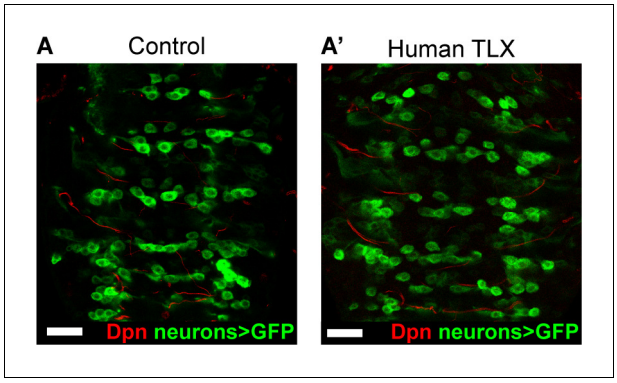

Figure 5—figure supplement 2. Expressing TLX in post-mitotic neurons does not result in ectopic NSCs. (**A–A'**) Expressing Human TLX in neurons with OK371-GAL4 > *mCD8-GFP* (green) does not result in ectopic NSCs (Dpn^{+} (red)). *n* = 8 brains for Control and Human TLX overexpression. Brains were dissected at wandering third instar larval stage. Single section confocal images. Scale bars represent 30 μm.

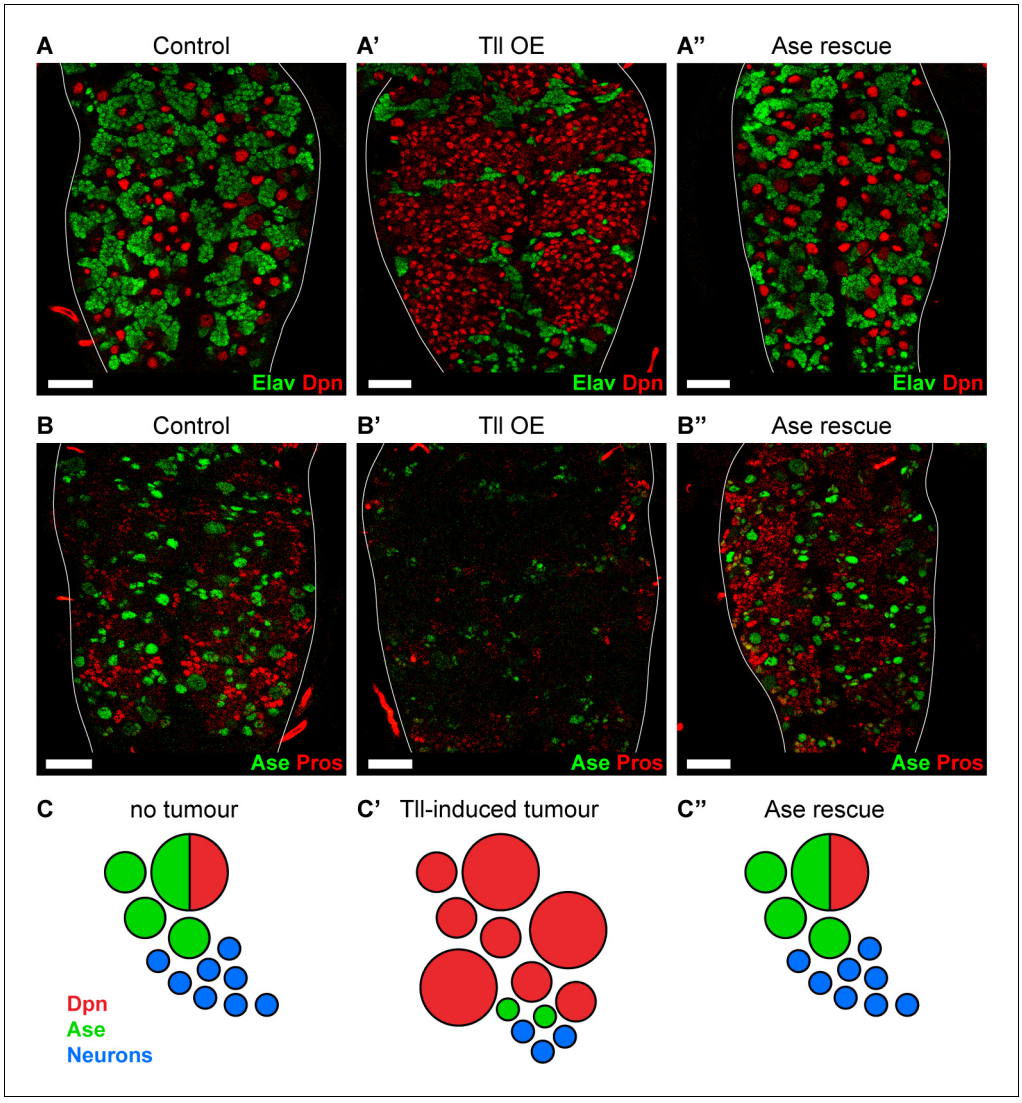

Figure 6. Reinstating progenitor identity prevents the formation of Tll tumours. (**A–A''**) Expressing Ase in combination with Tll during larval development using *wor*-GAL4 prevents tumour formation (ectopic Dpn^{+} cells (red)) and restores neuronal differentiation (Elav (green)) in all brains assessed. *n* = 10 brains for all conditions. Brains were dissected at wandering third instar stage. (**B–B''**) Ase (green) rescues Tll tumours by promoting differentiation (Pros (red)). *n* = 9 brains for Control; *n* = 10 brains for Tll overexpression (Tll OE) and Ase rescue. Brains were dissected at wandering third instar larval stage. (**C–C''**) Schematic depicting Type NSC I lineages (**C**) during development, (**C'**) with Tll-induced tumours and (**C''**) with Ase expression in Tll tumours. Single section confocal images. Scale bars represent 30 μm.

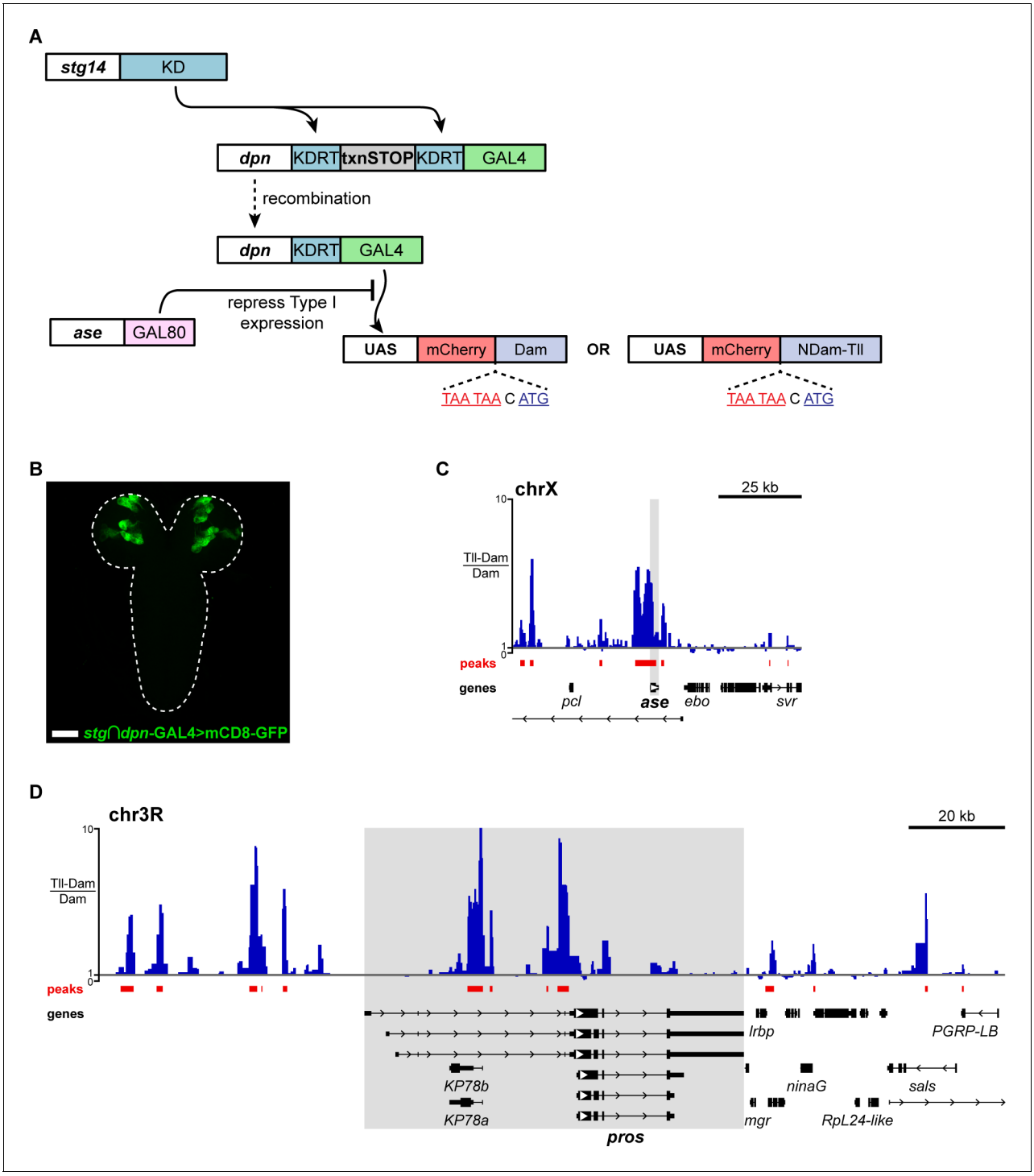

Figure 6—figure supplement 1. Determining Tll target genes in Type II NSCs using targeted DamID (TaDa). (**A**) Schematic showing the recombinase-dependent GAL4 tool used to perform Targeted DamID in Type II NSCs. *stg14* drives the expression of KD recombinase in Type II NSCs (and a small number of Type I NSCs), which excises a transcriptional stop (txnSTOP) resulting in the *dpnEE* promoter driving GAL4. *ase*-GAL80 was included to prevent GAL4 activity in Type I lineages. This system resulted in the expression of TaDa constructs (under the control of UAS) in Type II NSCs specifically. (**B**) *stg14*-patterned *dpn*-GAL4 (*stg* ⋂ dpn-GAL4) driving UAS-*mCD8-GFP* (green) with *ase*-GAL80 is expressed only in Type II NSCs and their lineages. The image is a projection over 75 µm in z. Brains dissected 50 hours ALH at 25 °C. Scale bar represents 50 µm. (**C**) Tll binding (Tll-Dam/Dam) across the *ase* locus in Type II NSCs. (**D**) Tll binding (Tll-Dam/Dam) across the *pros* locus in Type II NSCs. Tll binding in (C) and (D) is represented as scores in GATC fragments on an untransformed scale (y-axis).

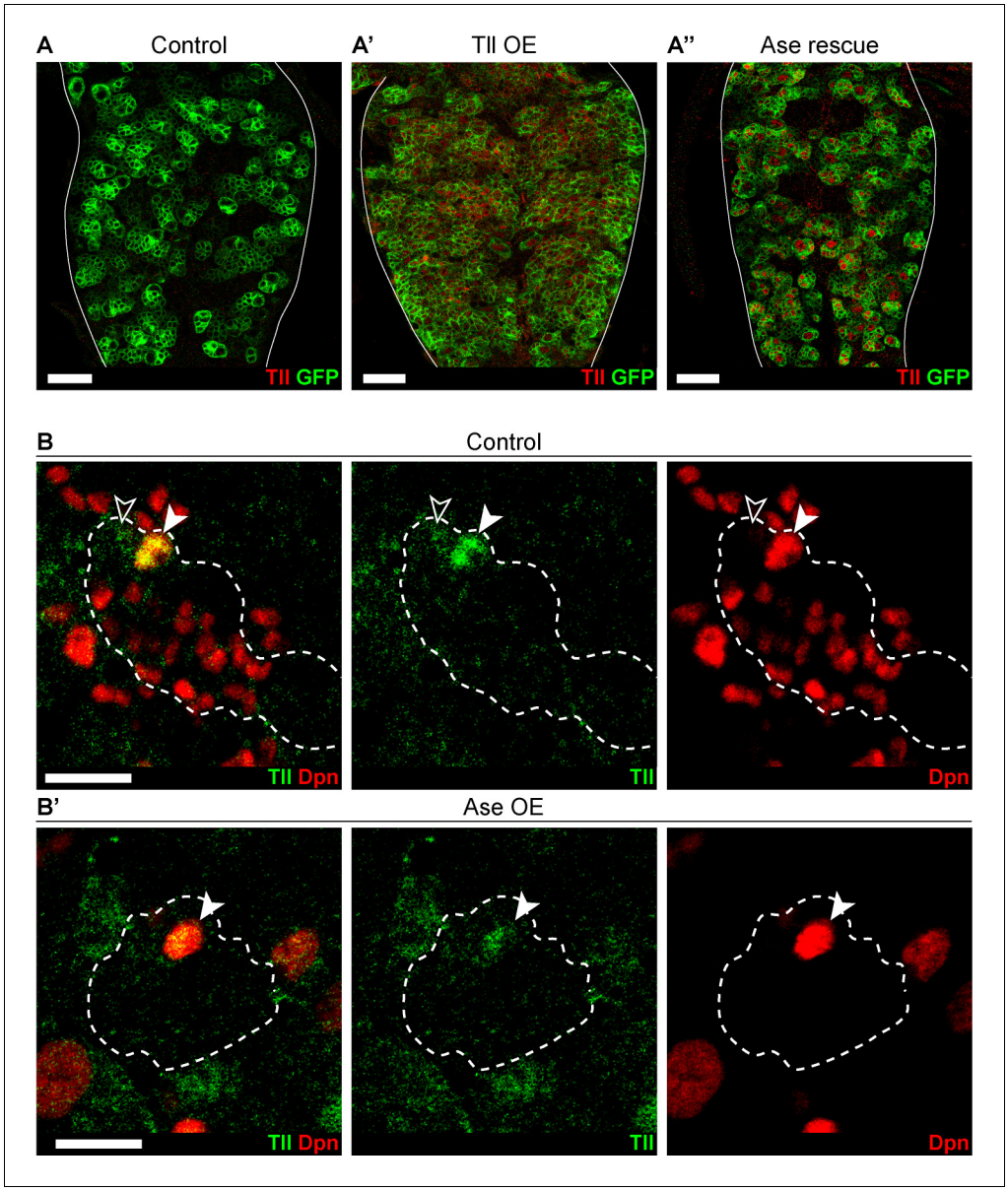

Figure 6—figure supplement 2. Ectopic Ase expression does not repress Tll. (**A–A''**) Tll (red) is not expressed in (**A**) Control VNCs. (**A'**) Tll is expressed at high levels in Tll OE tumours. (**A''**) Despite rescuing the formation of Tll-induced tumours, expressing Ase does not repress the expression of Tll. *wor*-GAL4,UAS-*mCD8-GFP;tub*-GAL80^{ts} (green) was used to express transgenes under the control of UAS during larval stages. *n* = 10 brains for all conditions. Brains were dissected at wandering third instar stage. Single section confocal images. Scale bars represent 30 µm. (**B–B'**) Tll (green) is expressed in (**B**) Control Type II NSCs (Dpn^{+} (red) filled arrowhead) and at weaker levels in immature INPs (Dpn^{-}, arrowhead outline) but not in other cells in the Type II lineages. (**B'**) Tll is still expressed in Ase Overexpression (Ase OE) Type II NSCs (arrowhead). Dotted outlines highlight three Type II lineages identified by *pntP1 >act*-GAL4 driving UAS-*GFP*. *n* = 11 brain lobes for Control and Ase OE. Brains were dissected at wandering third instar larval stage. Single section confocal images. Scale bars represent 15 µm.

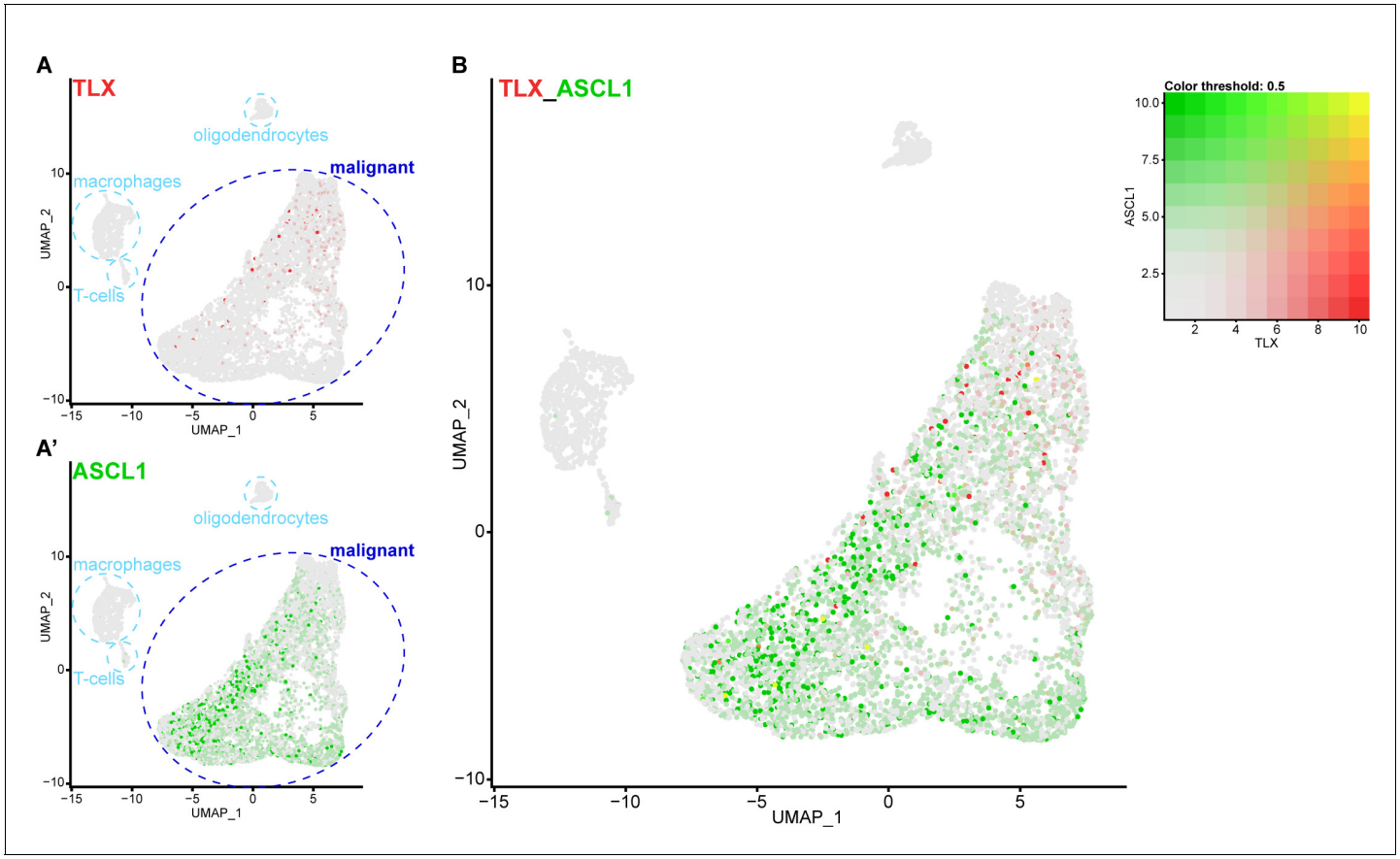

Figure 7. Single cell RNA sequencing reveals that TLX and ASCL1 appear to be mutually exclusive in human glioblastoma. (**A**) Uniform Manifold Approximation and Projection (UMAP) plot of 7,835 single cells coloured by TLX expression (red). Clusters were annotated based on previously known markers. TLX expression is only detected in the malignant cells. (**A'**) UMAP plot coloured by ASCL1 expression (green). ASCL1 expression is only detected in the malignant cells. (**B**) UMAP plot coloured by expression of both TLX (red) and ASCL1 (green), which appear mutually exclusive. Yellow indicates cells that express high levels of both TLX and ASCL1. Single cell RNA sequencing data and cluster markers obtained from ***Neftel et al. (2019)***.

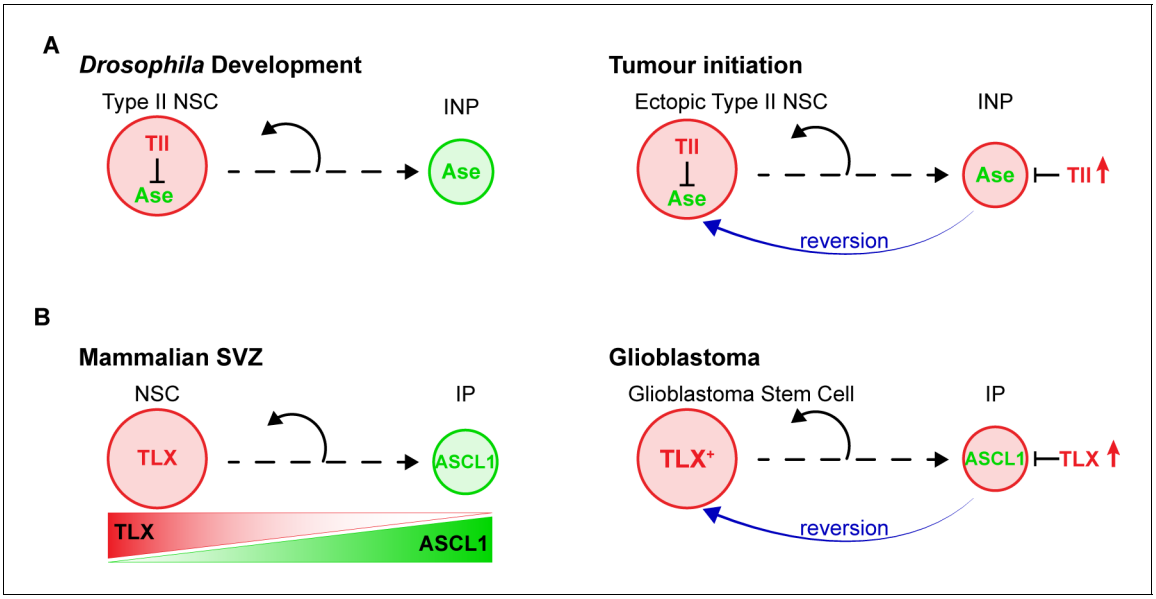

Figure 8. Model – Tll reverts INPs to NSC fate to initiate tumourigenesis. (**A**) Schematics depicting the promotion of Type II NSC fate by Tll (red) in development and tumourigenesis. Tll must be down regulated in Type II lineages to allow differentiation. Ase (green) expression is activated during differentiation. If Tll is high in INPs, or in Type I lineages, Type II NSC fate is maintained, or induced, and tumours form. (**B**) In the adult mouse SVZ, TLX expression is high in NSCs and lower in intermediate progenitors (IPs) (***Li et al., 2012***; ***Obernier et al., 2011***), whereas ASCL1 is high in IPs and low in NSCs (***Kim et al., 2011***; ***Parras et al., 2004***). Based on our results, we predict that high levels of TLX associated with aggressive glioblastoma revert IPs through the repression of ASCL1 to promote the generation of glioblastoma stem cells.