Figures and figure supplements

Multiple Wnts act synergistically to induce Chk1/Grapes expression and mediate G2 arrest in Drosophila tracheoblasts

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Figure 1. Wnt signaling is required for G2 arrest in Tr2 tracheoblasts. (A) Cartoon representing the cell cycle phasing of cells in wild type Tr2 DT at different larval stages based on FUCCI. (B-C) Impact of knockdown of Chk1 and components of the Wnt signaling pathway on cell numbers in Tr2 DT. Figure 1 continued on next page.
(B) Graph shows cells numbers in wild type (btl-GAL4), Btl-Chk1RNAi (btl-GAL4/UAS-Chk1RNAi), and Btl-Wnt pathway components RNAi (btl-GAL4/+; UAS-TCF RNAi/+; btl-GAL4/UAS-Dsh RNAi; btl-GAL4/UAS-Fz2 RNAi) at L2 and 16–24 h L3. (C) Graph shows cell numbers in wild type (tub-GAL80ts/+; UAS-ArmRNAi/Tb) and Armadillo mutants (tub-GAL80ts/+; btl-GAL4/UAS-ArmRNAi) at 24–26 h L3. Larvae were grown at 18°C and transferred to 29°C at 0–2 hr into L3. (D-E) Impact of reduction in levels of Chk1 or in levels of different components of the Wnt signaling pathway on mitotic indices in Tr2 DT. (D) Graph shows frequency of pH3+ nuclei at 16–24 h L3 in wild type (btl-GAL4), Chk1 mutant (btl-GAL4/UAS-Chk1RNAi), and Wnt pathway mutants (btl-GAL4/+; UAS-TCF RNAi/+; btl-GAL4/UAS-Dsh RNAi; btl-GAL4/UAS-Fz2 RNAi). (E) Graph shows the frequency of pH3+ nuclei in wild type (tub-GAL80ts/+; UAS-ArmRNAi/Tb) and Armadillo mutants (tub-GAL80ts/+; btl-GAL4/UAS-ArmRNAi) at 24–26 h L3. Larvae were grown as stated above. (F) Expression pattern of Wnt reporters Fz3-GFP (fz3-GFP) and Nkd-LacZ (nkd-lacZ) in Tr2 DT at different larval stages. Panels show immunostaining for GFP in Fz3-GFP (fz3-GFP) (top panel) and β-Gal in Nkd-LacZ (nkd-lacZ) (Bottom panel) and their respective secondary controls. (G) Quantitative PCR analysis of Fz3 mRNA levels in micro-dissected Tr2 DT fragments at different stages. Graph shows fold change in mRNA levels with respect to L2 (n = 3 experiments, n ≥ 15 Tr2 DT fragments/condition/experiment, mean ± standard deviation). DT = Dorsal Trunk, DB = Dorsal Branch, TC = Transverse Connective. Scale bar = 20 μm (mean ± standard deviation, n ≥ 7 tracheae) Student’s paired t-test: *p<0.05.
**Figure 1—figure supplement 1.** List of developmental signaling pathway knockdowns not showing proliferation at 16–24 h L3. (A) Impact of reduction of components of various developmental signaling pathways on cell numbers in Tr2 DT. Table shows cells numbers in wild type (btl-GAL4), Btl-hopRNAi (btl-GAL4/UAS-hopRNAi), Btl-CiRNAi (btl-GAL4/UAS-CiRNAi), Btl-pi3KRNAi (btl-GAL4/UAS-pi3KRNAi), Btl-BtldN (btl-GAL4/+, UAS-BtlDN/+), Btl-TkRNAi (btl-GAL4/UAS-TkRNAi), Btl-YkRNAi (btl-GAL4/+; UAS-YkRNAi/+), Btl-BskRNAi (btl-GAL4/+; UAS-BskRNAi/+), Btl-EcRNAi (btl-GAL4/+; UAS-EcRNAi/+), Btl-EGFRRNAi (btl-GAL4/+; UAS-EGFRRNAi/+), at 16–24 h L3 (mean values ± standard deviation, n ≥ 7 tracheae).
Figure 1—figure supplement 2. Loss of Chk1 or Wnt signaling leads to continuous proliferation of tracheoblasts through L3. (A) Impact of knockdown of Chk1 and TCF on cell numbers in Tr2 DT. Graph shows cell numbers in wild type (btl-GAL4), Btl-Chk1RNAi (btl-GAL4/UAS-Chk1RNAi), and Btl-TCF RNAi (btl-GAL4/+; UAS-TCF RNAi/+). L2 and 16–24 h L3 data same as Figure 1B, mean values ± standard deviation, n ≥ 7 tracheae. Student’s paired t-test: *p<0.05.
Figure 2. Phosphorylated Chk1 levels are diminished in the absence of Wnt signaling. (A) Activated Chk1 (phospho-Chk1$^{Ser345}$, pChk1) immunostaining (white) in Tr2 DT in Chk1 mutants and Wnt pathway mutants. pChk1 immunostaining in Tr2 DT in (i) wild type (btl-GAL4), (ii) Btl-Chk1$^{RNAi}$ (btl-GAL4/UAS-Chk1$^{RNAi}$) (iii) Btl-TCF$^{RNAi}$ (btl-GAL4/+; UAS-TCF$^{RNAi}$/+) at L2 and (iv) wild type treated with secondary antibody alone. Scale bar = 20 μm.
Figure 3. Wnt signaling regulates Chk1 transcription. (A) Quantitative PCR analysis of Chk1 mRNA levels in micro-dissected Tr2 DT fragments at different stages. Graph shows fold change in Chk1 mRNA in Tr2 DT fragments from wild type (btl-GAL4), Wnt pathway loss-of-function (btl-GAL4/+; UAS-TCF\textsuperscript{RNAi}/+) and Wnt pathway gain-of-function (UAS-ArmS10/+; btl-GAL4/) larvae. Fold change has been represented with respect to L2 (n = 3 experiments, n ≥ 15 Tr2 DT fragments/condition/experiment, mean ± standard deviation). (B) Analysis of Chk1-lacZ expression in Tr2 DT in wild type and Wnt pathway mutants. β-Gal immunostaining in larvae expressing Chk1-lacZ at L2, 0–8 h L3 and 32–40 h L3 and in Btl-TCF\textsuperscript{RNAi} (btl-GAL4/Chk1 lacZ; UAS-TCF\textsuperscript{RNAi}/+) at L2. (C) Impact of overexpression of Chk1 and ATR in TCF mutants. Graph shows cell numbers in wild type (btl-GAL4, UAS-FUCCI/ Cyo GFP; +/Tb), Btl-TCF\textsuperscript{RNAi}, Chk1 (btl-GAL4, UAS-FUCCI/+; UAS-TCF\textsuperscript{RNAi}/+), Btl-TCF\textsuperscript{RNAi}, ATR (btl-GAL4, UAS-FUCCI/+; UAS-ATR) at 16–24 h L3. (D) Impact of reduction of TCF and overexpression of Chk1 on levels of pChk1 in Tr2 DT. pChk1 immunostaining (white) in Btl-TCF\textsuperscript{RNAi}, UAS-Chk1 (btl-GAL4, UAS-FUCCI/+; UAS-TCF\textsuperscript{RNAi}/UAS-Chk1) animals at L2 (This image has been acquired at a lower laser power and gain setting compared to Figure 2A to prevent saturation of white pixels). (E) Impact of reduction of TCF and overexpression of ATR on levels of pChk1 in Tr2 DT. pChk1 immunostaining (white) in Btl-TCF\textsuperscript{RNAi}, UAS-ATR (btl-GAL4, UAS-FUCCI/+; UAS-TCF\textsuperscript{RNAi}/UAS-ATR) animals at L2 (mean values ± standard deviation, n ≥ 7 tracheae) Scale bar = 20 μm. Student’s paired t-test: *p<0.05.
Figure 4. Wg, Wnt5, Wnt6 and Wnt10 are required to maintain Chk1 expression. (A) Quantitative PCR analysis for levels of Wg, Wnt5, Wnt6 and Wnt10 mRNA in micro-dissected Tr2 DT fragments at different stages. Graph shows fold change in Wg, Wnt5, Wnt6 and Wnt10 mRNA in Tr2 DT fragments from wild type (btl-GAL4) larvae at L2, 0–8 h L3 and 32–40 h L3. Fold change has been represented with respect to L2 (n = 3 experiments, n ≥ 15 Tr2 DT fragments/condition/experiment, mean ± standard deviation). (B) smFISH for Wg, Wnt5, Wnt6 and Wnt10 mRNA in Tr2 DT at L2 and 32–40 h L3. Arrowheads point to the sites of mRNA accumulation. (Scale bar = 5 μm) (C) Impact of knockdown of components of the Wnt signaling pathway on cell numbers in Tr2 DT. Graph shows cells numbers in wild type (btl-GAL4, Same as Figure 1B) and Btl-Wnt pathway components RNAi (btl-GAL4/+; UAS-WgRNAi/+, btl-GAL4/+; UAS-Wnt5RNAi/+, btl-GAL4/+; UAS-Wnt6RNAi/+, btl-GAL4/+; UAS-Wnt10RNAi/+) at L2 and 16–24 h L3. (D) Impact of reduction in levels of different components of the Wnt signaling pathway on mitotic indices in Tr2 DT. Graph shows frequency of pH3+ nuclei at 16–24 h L3 in wild type (btl-GAL4, Same as Figure 1D) and Wnt pathway mutants (btl-GAL4/+, UAS-WgRNAi/+, btl-GAL4/+, UAS-Wnt5RNAi/+, btl-GAL4/+, UAS-Wnt6RNAi/+, btl-GAL4/+, UAS-Wnt10RNAi/+) (E) Impact of expression of mutant Wg, Wnt5 and Wnt6 on cell numbers in Tr2 DT. Graphs show cell Figure 4 continued on next page
numbers in wild type and Wg mutants (Wg<sup>ts</sup>) at 24–26 h L3. (Larvae were grown at 18°C and transferred to 29°C at 0–2 hr into L3) Wnt5 mutant (Wnt5<sup>400</sup> FRT19A) and Wnt6 (Wnt6 KO) on cell numbers in Tr2 DT. (F) Activated Chk1 (phospho-Chk1<sup>Ser345</sup>, pChk1) immunostaining (white) in Tr2 DT in Wnt pathway mutants. pChk1 immunostaining in Tr2 DT in (i) Btl-Wg<sup>RNAi</sup>(btl-GAL4/+; UAS-Wg<sup>RNAi</sup>/+), (ii) Btl-Wnt5<sup>RNAi</sup>(btl-GAL4/+; UAS-Wnt5<sup>RNAi</sup>/+), (iii) Btl-Wnt6<sup>RNAi</sup>(btl-GAL4/+; UAS-Wnt6<sup>RNAi</sup>/+) and Btl-Wnt10<sup>RNAi</sup>(btl-GAL4/+; UAS-Wnt10<sup>RNAi</sup>/+) at L2. (G) Quantitative PCR analysis of Chk1 and Fz3 mRNA levels in micro-dissected Tr2 DT fragments at L2. Graphs show fold change in Chk1 and Fz3 mRNA in Wild type, Btl-TCF<sup>RNAi</sup>(btl-GAL4/+; UAS-TCF<sup>RNAi</sup>/+), Btl-Wg<sup>RNAi</sup> (btl-GAL4/+; UAS-Wg<sup>RNAi</sup>/+), Btl-Wnt5<sup>RNAi</sup>(btl-GAL4/+; UAS-Wnt5<sup>RNAi</sup>/+), Btl-Wnt6<sup>RNAi</sup>(btl-GAL4/+; UAS-Wnt6<sup>RNAi</sup>/+) and Btl-Wnt10<sup>RNAi</sup>(btl-GAL4/+; UAS-Wnt10<sup>RNAi</sup>/+) in Tr2 DT fragments. Fold change has been represented with respect to Wild type (n = 3 experiments, n ≥ 15 Tr2 DT fragments/condition/experiment, mean ± standard deviation) (mean values ± standard deviation, n ≥ 7 tracheae) Scale bar = 20 μm. Student’s paired t-test: *p<0.05.
Figure 4—figure supplement 1. Wnt ligands do not affect each other's expression. (A) Impact of knockdown of Wnt10 on cell numbers in Tr2 DT. Graph shows cell numbers in wild type (btl-GAL4, Same as Figure 1B) and Btl-Wnt10RNAi (btl-GAL4/UAS-Wnt10RNAi) at L2 and 16–24 h L3 (mean values ± standard deviation, n > 7 tracheae). (B) smFISH for Wnt5 mRNA in Wnt5 mutant (Wnt5[400] FRT19A) Tr2 DT at L2. (Scale bar = 5 μm). (C) smFISH for GAPDH mRNA in Wild type (btl-GAL4) Tr2 DT at 32–40 h L3. Arrowheads point to the sites of mRNA accumulation. (Scale bar = 5 μm). (D) Quantitative PCR analysis of Wg, Wnt5, Wnt6 and Wnt10 mRNA levels in micro-dissected Tr2 DT fragments at L2. Graph shows fold change in Wg, Wnt5, Wnt6 and Wnt10 mRNA in Wild type (btl-GAL4), Btl-WgRNAi (btl-GAL4/++; UAS-WgRNAi/+), Btl-Wnt5RNAi (btl-GAL4/++; UAS-Wnt5RNAi/+), Btl-Wnt6RNAi (btl-GAL4/++; UAS-Wnt6RNAi/+), and Btl-Wnt10RNAi (btl-GAL4/++; UAS-Wnt10RNAi/+). Fold change has been represented with respect to Wild type (n = 3 experiments, n > 15 Tr2 DT fragments/condition/experiment, mean ± standard deviation).
Figure 4—figure supplement 2. Loss of Wnt2, Wnt4 or WntD does not lead to precocious proliferation in Tr2 DT. (A) Impact of reduction of Wnt2, Wnt4 and WntD on cell numbers in Tr2 DT. Table shows cells numbers in wild type (btl-GAL4), Btl-Wnt2RNAi (btl-GAL4/+; UAS-Wnt2RNAi/+), Btl-Wnt4RNAi (btl-GAL4/+; UAS-Wnt4RNAi/+), and Btl-WntDRNAi (btl-GAL4/+; UAS-WntDRNAi/) at 16–24 h L3 (mean values ± standard deviation, n ≥ 7 tracheae).
Figure 5. Exit from G2 arrest is required for activation of Dpp signaling. (A) phospho-Mad (pMad) immunostaining in wild type Tr2 DT at L2, 0–8 h L3, 16–24 h L3 and 32–40 h L3. (B) Impact of reduction of Chk1 or Wnt pathway components on pMad expression in Tr2 DT. pMad immunostaining in Tr2 DT from Btl-Chk1RNAi (btl-GAL4/UAS-Chk1RNAi) and Btl-Wnt6RNAi (btl-GAL4/+; UAS-Wnt6RNAi/+ ) animals at 16–24 h L3. (C) Impact of overexpression of activated form of Tkv (Btl-TkvQD) on pMad expression in Tr2 DT. pMad immunostaining in Tr2 DT from wild type and Btl-TkvQD (btl-GAL4/+; UAS-TkvQD/+ ) animals at L2. Scale bar = 20 μm.
Figure 6. Loss of Stg, Cdc2 or Cyclin B perturbs hypertrophic growth of Tr2 DT. (A) Impact of reduction of Chk1 or different drivers of G2-M on the size of Tr2 DT at 32–40 h L3. Scatter plot shows length and width of Tr2 DT from Wild type, Btl-Chk1RNAi (btl-GAL4/UAS-Chk1RNAi), Btl-StgRNAi (btl-GAL4/+; UAS-StgRNAi/+), Btl-Cdc2RNAi (btl-GAL4/UAS-Cdc2RNAi) and Btl-CyclinBRNAi (btl-GAL4/+; UAS-CyclinBRNAi/+)) at 32–40 h L3. Values shown in red are from data previously published in Kizhedathu et al., 2018. (n ≥ 6 tracheae). (B) Model for the regulation of G2 arrest by Wnt signaling. Wnt signaling negatively regulates G2-M by transcriptionally upregulating Chk1. Arrest in G2 negatively regulates Dpp signaling, preventing precocious proliferation and allowing for hypertrophic growth of Tr2 DT.