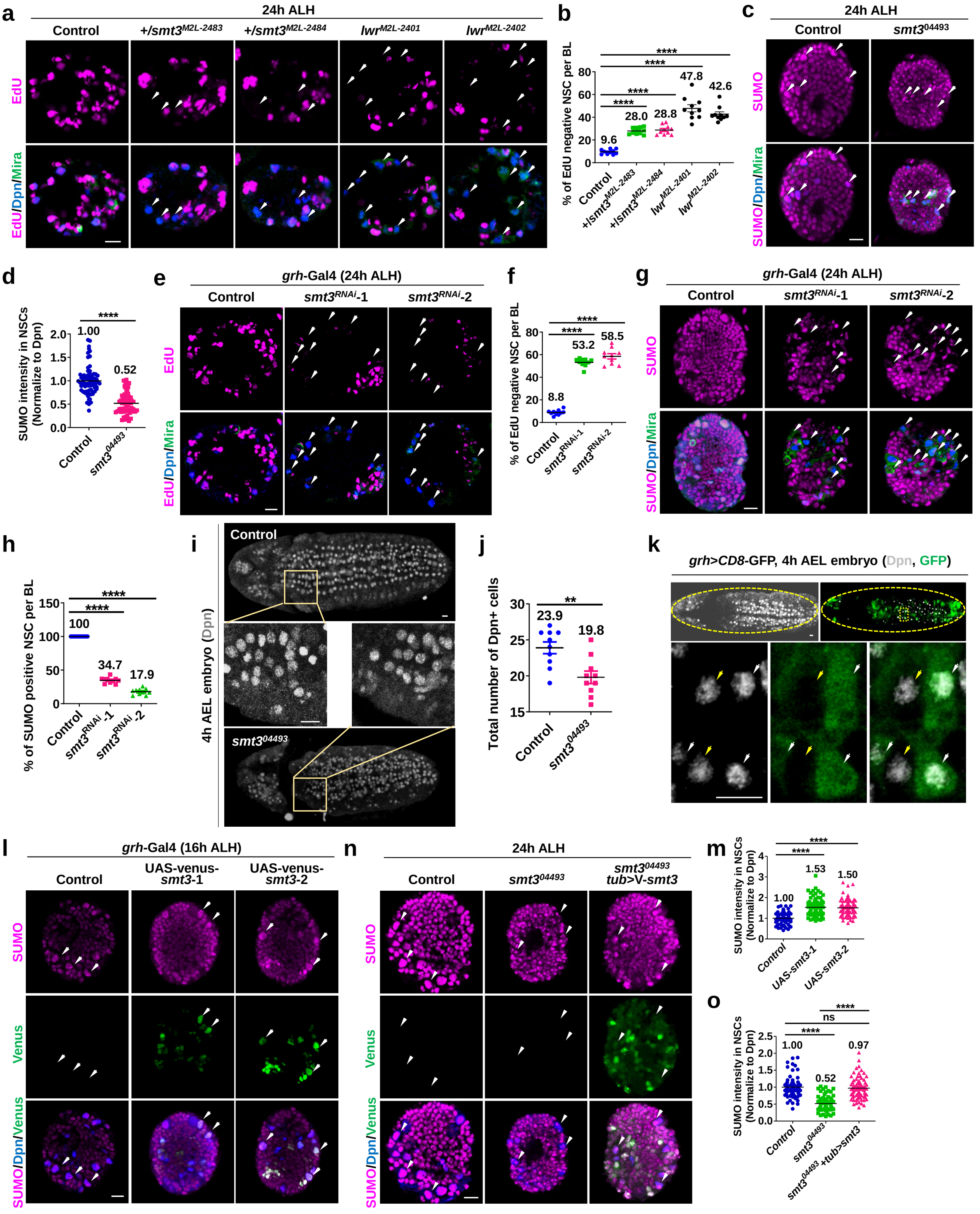
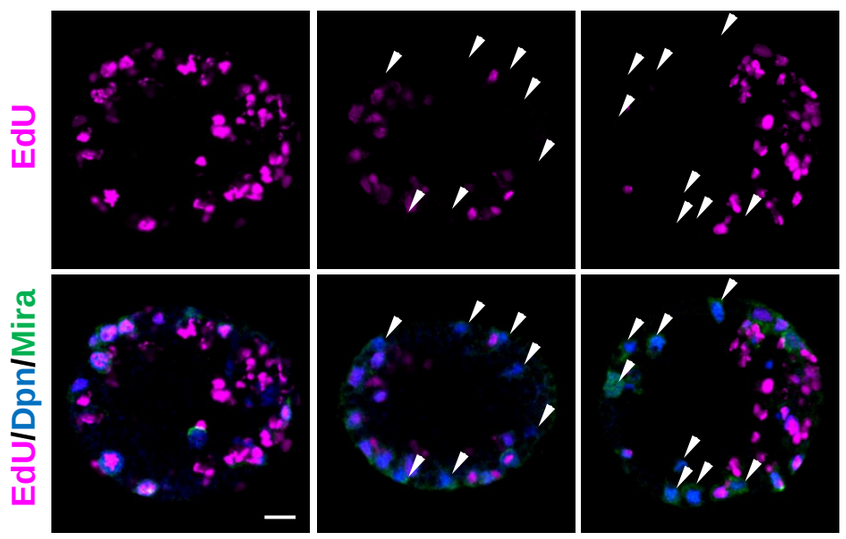

a
24h ALH
Control
+/smt3M2L-2483
+/smt3M2L-2484
lwrM2L-2401
lwrM2L-2402
EdU
EdU/Dpn/Mira
b
% of EdU negative NSC per BL
9.6
28.0
28.8
47.8
42.6
****
Control
+/smt3M2L-2483
+/smt3M2L-2484
lwrM2L-2401
lwrM2L-2402
c
24h ALH
Control
smt304493
SUMO
SUMO/Dpn/Mira
d
SUMO intensity in NSCs (Normalize to Dpn)
1.00
0.52
****
Control
smt304493
e
grh-Gal4 (24h ALH)
Control
smt3RNAi-1
smt3RNAi-2
EdU
EdU/Dpn/Mira
f
% of EdU negative NSC per BL
8.8
53.2
58.5
****
Control
smt3RNAi-1
smt3RNAi-2
g
grh-Gal4 (24h ALH)
Control
smt3RNAi-1
smt3RNAi-2
SUMO
SUMO/Dpn/Mira
h
% of SUMO positive NSC per BL
100
34.7
17.9
****
Control
smt3RNAi-1
smt3RNAi-2
i
4h AEL embryo (Dpn)
Control
smt304493
j
Total number of Dpn+ cells
23.9
19.8
**
Control
smt304493
k
grh>CD8-GFP, 4h AEL embryo (Dpn, GFP)
l
grh-Gal4 (16h ALH)
Control
UAS-venus-smt3-1
UAS-venus-smt3-2
SUMO
Venus
SUMO/Dpn/Venus
n
24h ALH
Control
smt304493
smt304493 tub>V-smt3
SUMO
Venus
SUMO/Dpn/Venus
m
SUMO intensity in NSCs (Normalize to Dpn)
1.00
1.53
1.50
****
Control
UAS-smt3-1
UAS-smt3-2
o
SUMO intensity in NSCs (Normalize to Dpn)
1.00
0.52
0.97
****
ns
Control
smt304493
smt304493 +tub>smt3

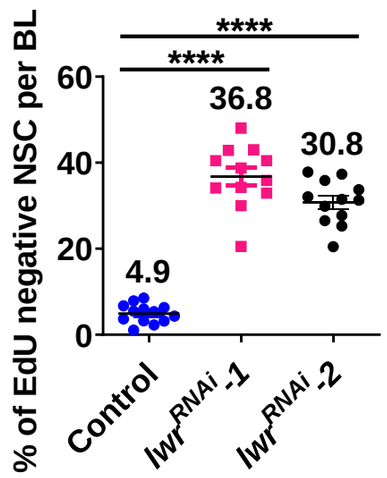
Supplementary Fig 1. Smt3 is required for NSC reactivation and brain development.

(a) At 24h ALH, larval brain lobes from control (*yw*), *smt3*$^{M2L\text{-}2483}$ and *smt3*$^{M2L\text{-}2484}$ heterozygous mutants, *lwr*$^{M2L\text{-}2401}$ and *lwr*$^{M2L\text{-}2402}$ homozygous mutants were analyzed for EdU incorporation. NSCs were marked by Dpn and Mira. White arrows point to EdU negative quiescent NSCs. **(b)** Quantification graph of EdU- NSCs per BL for genotypes in (a). Control, 9.6 ± 2.1, n = 11 BL; +/*smt3*$^{M2L\text{-}2483}$, 28 ± 3, n = 11 BL; +/*smt3*$^{M2L\text{-}2484}$, 28.8 ± 4.1, n = 11 BL; *lwr*$^{M2L\text{-}2401}$, 47.8 ± 9.9, n = 10 BL; *lwr*$^{M2L\text{-}2402}$, 42.6 ± 6.4, n = 10 BL. **(c)** At 24h ALH, larval brain lobes from control (*yw*) and *smt3*^{04493} were labeled with SUMO, Dpn and Mira. White arrows point to Dpn+ and Mira+ NSCs. **(d)** Quantification graph of SUMO intensity (normalized to Dpn) in NSCs in genotypes in (c). Control, 1 ± 0.3, n = 10 BL; *smt3*^{04493}, 0.52 ± 0.23, n = 10 BL. **(e)** At 24h ALH, larval brain lobes from control (*β-gal*^{RNAi}) and two *smt3*^{RNAi} lines driven by *grh*-Gal4 were analyzed for EdU incorporation. White arrows point to EdU negative quiescent NSCs. **(f)** Quantification graph of the EdU- NSCs per BL in various genotypes in (e). Control, 8.8 ± 2.3, n = 10 BL; *smt3*^{RNAi}-1, 53.2 ± 1.5, n = 10 BL; *smt3*^{RNAi}-2, 58.5 ± 7, n = 10 BL. **(g)** At 24h ALH, larval brain lobes from control (*β-gal*^{RNAi}) and two *smt3*^{RNAi} lines driven by *grh*-Gal4 were labeled with SUMO, Dpn and Mira. White arrows point to Dpn+ Mira+ and SUMO negative NSCs. **(h)** Quantification of SUMO negative NSCs per BL in various genotypes in (g). Control, 100 ± 0, n = 10 BL; *smt3*^{RNAi}-1, 34.7 ± 4.4, n = 10 BL; *smt3*^{RNAi}-2, 17.9 ± 4.6, n = 10 BL. **(i)** At 4h after egg laying (AEL), embryos from control (*yw*) and *smt3*^{04493} lines were labeled with Dpn. The middle panels are enlarged views of the embryo in the yellow squares. **(j)** Quantification graph of total number of Dpn+ cells in genotypes in (i). Control, 23.9 ± 2.6, n = 10 BL; *smt3*^{04493}, 19.8 ± 2.7, n = 10 BL. **(k)** At 4h after egg laying (AEL), embryos of *grh>CD8*-GFP were labeled with Dpn and GFP. The oval frame marks the outline of the embryo. The left panel is Dpn staining with higher brightness and background to show the outline of the embryo. The bottom panels are enlarged views of the embryo in the yellow square. White arrows point to GFP+ Dpn+ NSCs, yellow arrows point GFP- Dpn+ NSCs. **(l)** At 16h ALH, larval brain lobes from control (*β-gal*^{RNAi}) and two UAS-venus-*smt3* lines were labeled with SUMO, GFP and Dpn. White arrows point to Dpn+ NSCs. **(m)** Quantification graph of SUMO intensity (normalized to Dpn) in NSCs in genotypes in (l). Control, 1 ± 0.29, n = 10 BL; UAS-venus-*smt3*-1, 1.53 ± 0.42, n = 11 BL; UAS-venus-*smt3*-2, 1.5 ± 0.39, n = 10 BL. **(n)** At 24h ALH, larval brain lobes control (*yw*), *smt3*^{04493} and *smt3*^{04493} + *tub>smt3* were labeled with SUMO, GFP and Dpn. White arrows point to some of the Dpn+ NSCs. **(o)** Quantification graph of the SUMO intensity (normalized with Dpn) in NSCs in various genotypes in (n). Control, 1 ± 0.3, n = 10 BL; *smt3*^{04493}, 0.52 ± 0.23, n = 10 BL; *smt3*^{04493} + *tub>smt3*, 0.97 ± 0.31, n = 10 BL. Data are presented as mean ± SD. **** for P ≤ 0.0001. Scale bars: 10 μm.

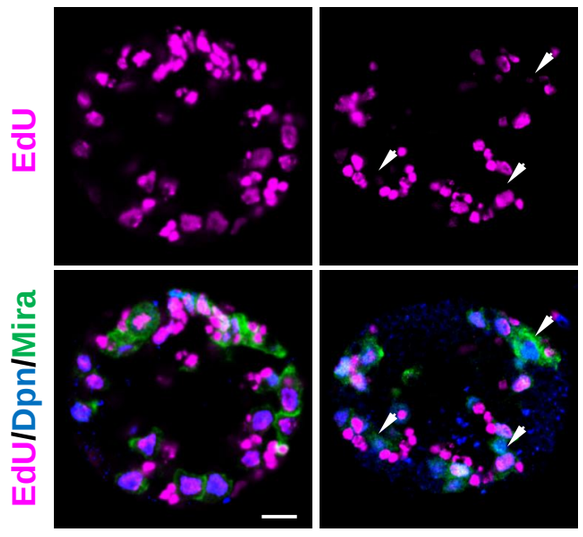
a


grh-Gal4 (24h ALH)
Control
lwrRNAi-1
lwrRNAi-2
EdU
EdU/Dpn/Mira

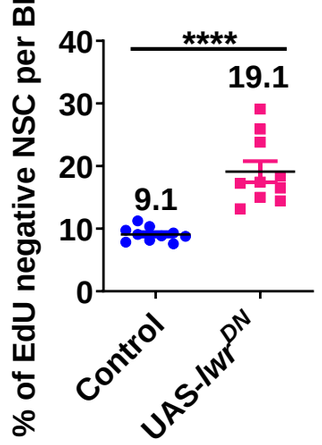
b


% of EdU negative NSC per BL
****
****
4.9
36.8
30.8
Control
lwrRNAi-1
lwrRNAi-2

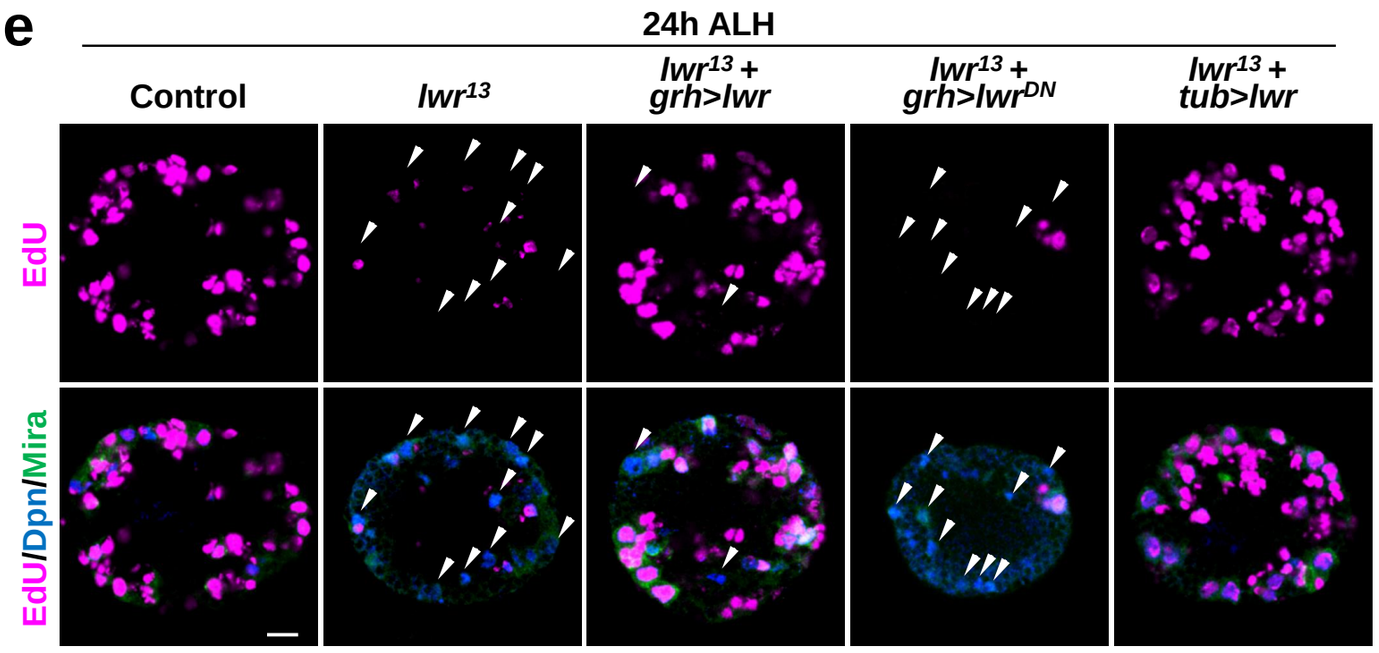
c


grh-Gal4 (24h ALH)
Control
UAS-lwrDN
EdU
EdU/Dpn/Mira

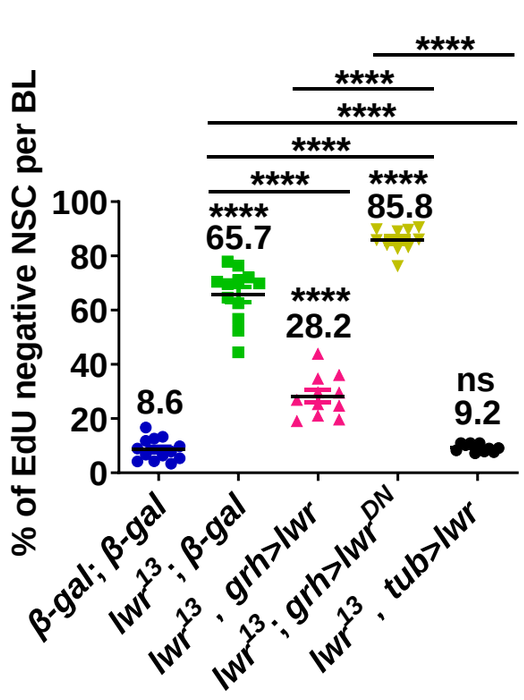
d


% of EdU negative NSC per BL
****
9.1
19.1
Control
UAS-lwrDN

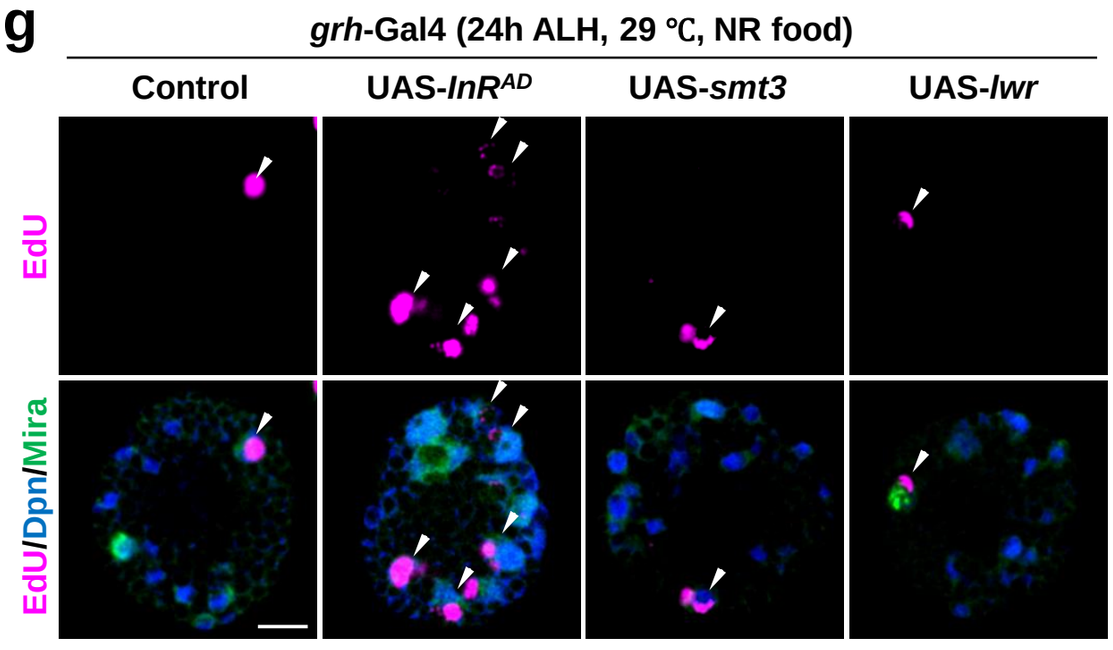
e


24h ALH
Control
lwr13
lwr13 + grh>lwr
lwr13 + grh>lwrDN
lwr13 + tub>lwr
EdU
EdU/Dpn/Mira

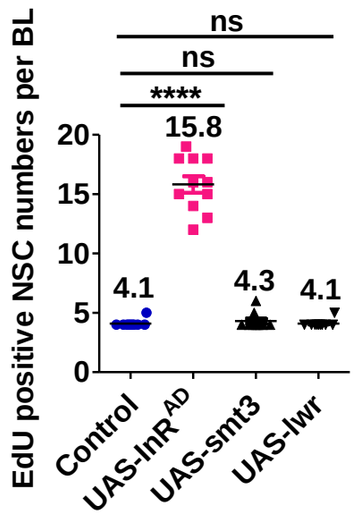
f


% of EdU negative NSC per BL
****
****
****
****
****
8.6
65.7
28.2
85.8
ns
9.2
β-gal; β-gal
lwr13 ; β-gal
lwr13 , grh>lwr
lwr13 ; grh>lwrDN
lwr13 , tub>lwr

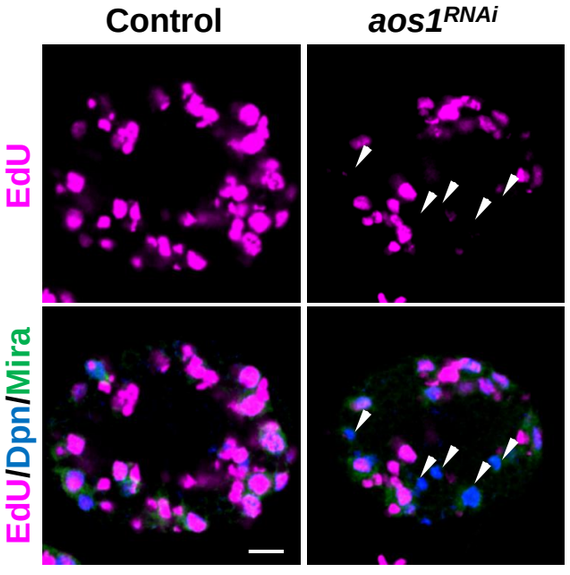
g


grh-Gal4 (24h ALH, 29 ℃, NR food)
Control
UAS-InRAD
UAS-smt3
UAS-lwr
EdU
EdU/Dpn/Mira

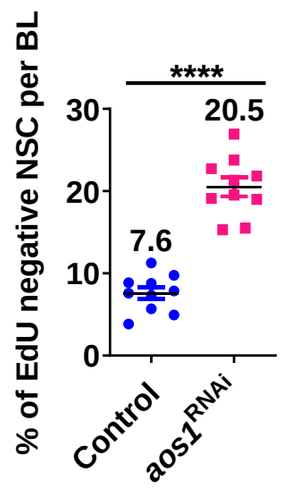
h


EdU positive NSC numbers per BL
ns
ns
****
15.8
4.1
4.3
4.1
Control
UAS-InRAD
UAS-smt3
UAS-lwr

i

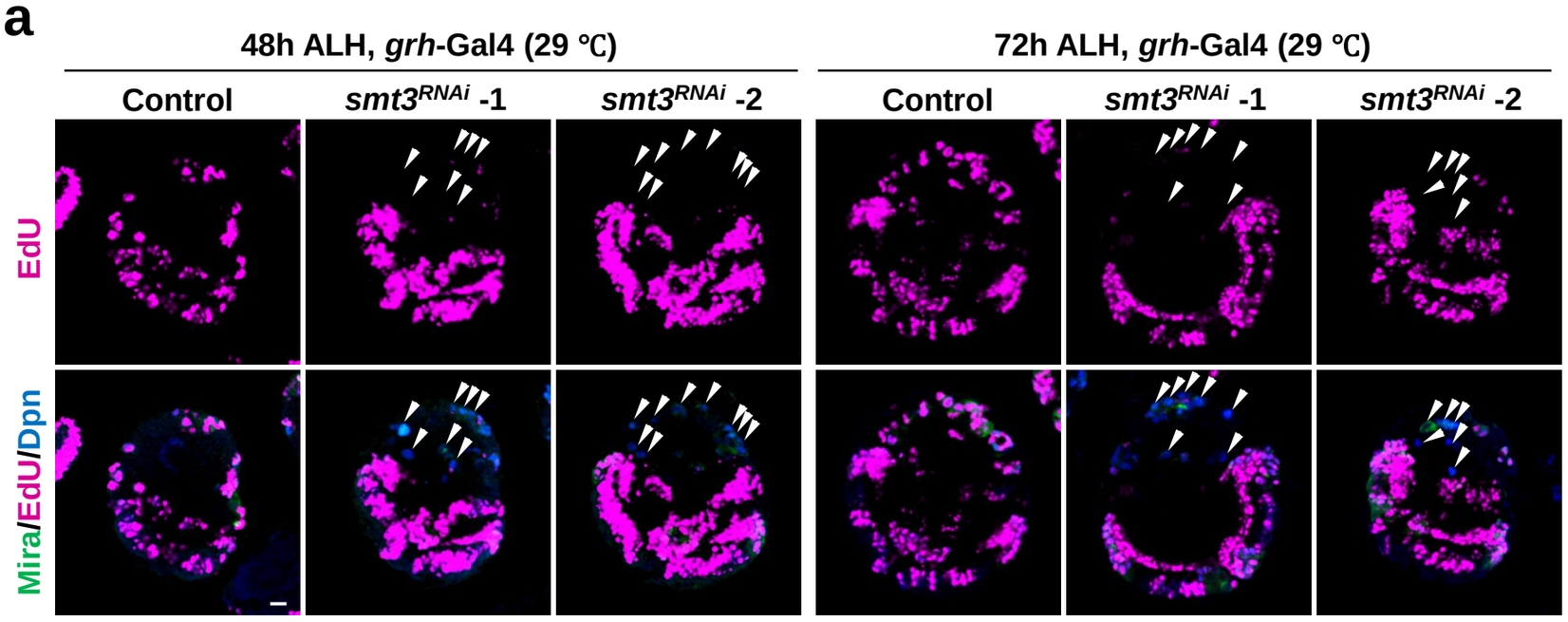

grh-Gal4 (24h ALH)
Control
aos1RNAi
EdU
EdU/Dpn/Mira

j

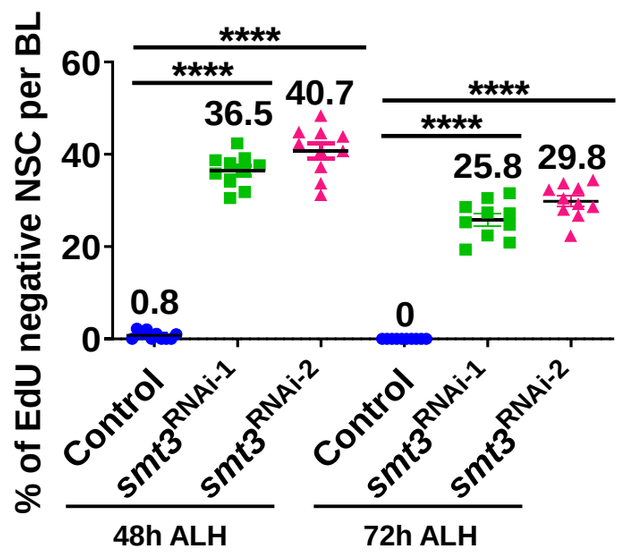

% of EdU negative NSC per BL
****
7.6
20.5
Control
aos1RNAi

Supplementary Fig 2. SUMO E1 and E2 enzymes are required for NSC reactivation.
(a) At 24h ALH, larval brain lobes from control (*β-gal*^{RNAi}) and two *lwr*^{RNAi} lines driven by *grh*-Gal4 were analyzed for EdU incorporation. NSCs were marked by Dpn and Mira. White arrows point to EdU negative quiescent NSCs. **(b)** Quantification graph of EdU- NSCs per BL for genotypes in (a). Control, 4.9 ± 2.2, n = 13 BL; *lwr*^{RNAi}-1, 36.8 ± 6.9, n = 12 BL; *lwr*^{RNAi}-2, 30.8 ± 5.2, n = 12 BL. **(c)** At 24h ALH, larval brain lobes from control (*β-gal*^{RNAi}) and a *lwr*^{DN} line driven by *grh*-Gal4 analyzed for EdU incorporation. NSCs were marked by Dpn and Mira. White arrows point to EdU negative quiescent NSCs. (d) Quantification graph of EdU- NSCs per BL for genotypes in (c). Control, 9.1 ± 1.1, n = 10 BL; UAS-*lwr*^{DN}, 19.1 ± 5.3, n = 10 BL. **(e)** At 24h ALH, larval brain lobes from control (*yw*), *lwr*^{13}, *lwr*^{13} + *grh>lwr*, *lwr*^{13} + *grh>lwr*^{DN} and *lwr*^{13} + *tub>lwr* were analyzed for EdU incorporation. NSCs were marked by Dpn and Mira. White arrows point to EdU negative quiescent NSCs. (f) Quantification graph of EdU- NSCs per BL for genotypes in (e). Control, 8.6 ± 4.1, n = 13 BL; *lwr*^{13}, 65.7 ± 10, n = 12 BL; *lwr*^{13} + *grh>lwr*, 28.2 ± 7.6, n = 11 BL; *lwr*^{13} + *grh>lwr*^{DN}, 85.8 ± 8.5, n = 10 BL; *lwr*^{13} + *tub>lwr*, 9.2 ± 5.8, n = 10 BL. **(g)** At 24h ALH, larval brain lobes under nutrition deprivation condition from control (*β-gal*^{RNAi}), UAS-*InR*^{AD} (positive control), UAS-*smt3* and UAS-*lwr* driven by *grh*-Gal4 analyzed for EdU incorporation. NSCs were marked by Dpn and Mira. White arrows point to EdU positive active NSCs. **(h)** Quantification graph of EdU+ NSC number per BL for genotypes in (g). Control, 4.1 ± 0.32, n = 10 BL; UAS-*InR*^{AD}, 15.8 ± 2.27, n = 11 BL; UAS-*smt3*, 4.3 ± 0.67, n = 10 BL; UAS-*lwr*, 4.1 ± 0.32, n = 10 BL. **(i)** At 24h ALH, larval brain lobes from control (*β-gal*^{RNAi}) and an *aos1*^{RNAi} line driven by *grh*-Gal4 were analyzed for EdU incorporation. NSCs were marked by Dpn and Mira. White arrows point to EdU negative quiescent NSCs. **(j)** Quantification graph of EdU- NSCs per BL for genotypes in (i). Control, 7.6 ± 2.3, n = 10 BL; *aos1*^{RNAi}, 20.5 ± 3.6, n = 10 BL. Data are presented as mean ± SD. **** for P ≤ 0.0001, ns indicates p > 0.05. Scale bars: 10 μm.

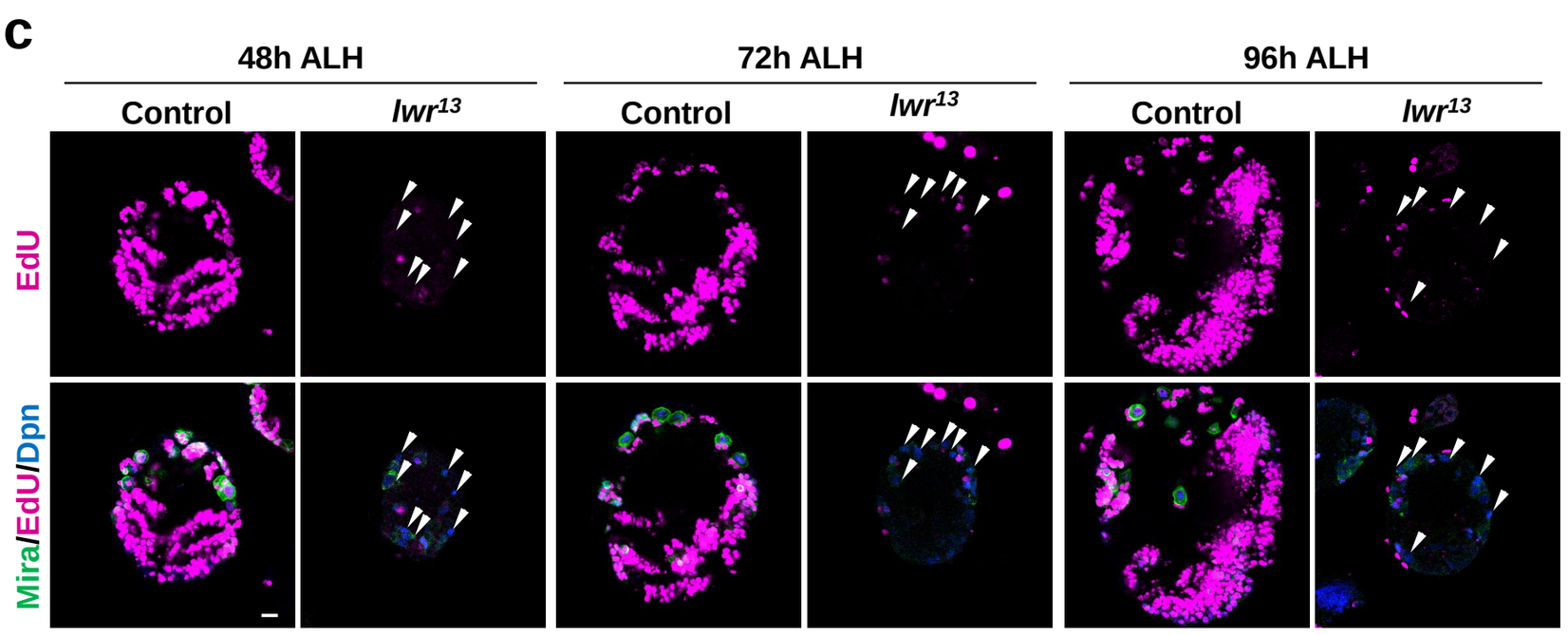
a


48h ALH, grh-Gal4 (29 ℃)
72h ALH, grh-Gal4 (29 ℃)
Control
smt3RNAi -1
smt3RNAi -2
Control
smt3RNAi -1
smt3RNAi -2
EdU
Mira/EdU/Dpn

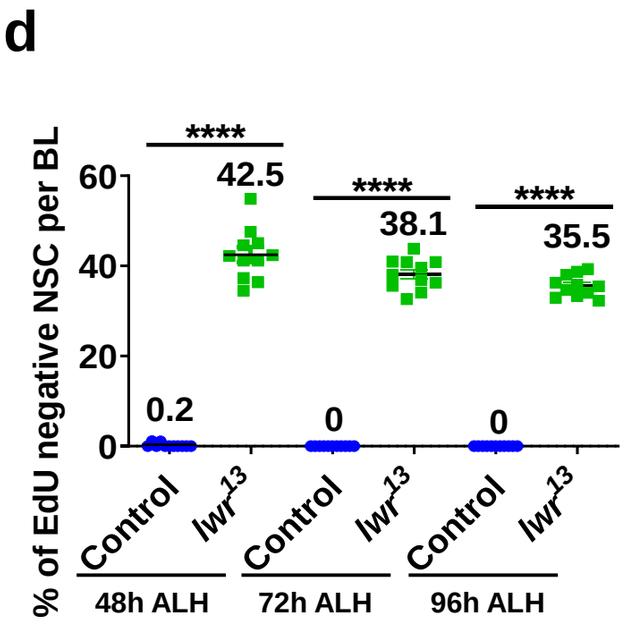
b


% of EdU negative NSC per BL
60
40
20
0
****
****
****
****
36.5
40.7
25.8
29.8
0.8
0
Control
smt3RNAi-1
smt3RNAi-2
Control
smt3RNAi-1
smt3RNAi-2
48h ALH
72h ALH

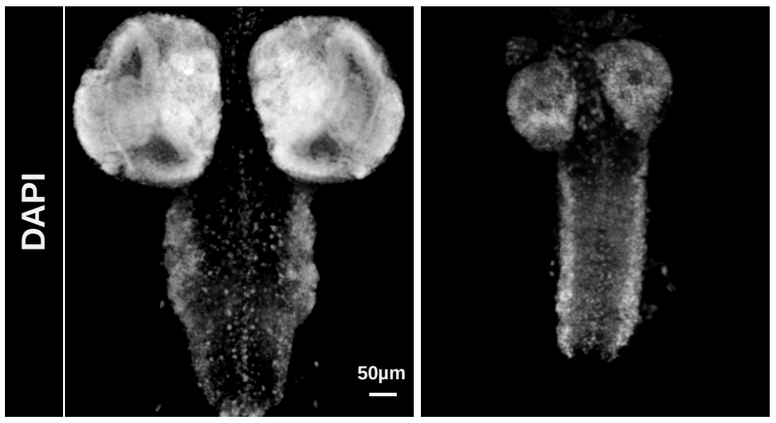
c


48h ALH
72h ALH
96h ALH
Control
lwr13
Control
lwr13
Control
lwr13
EdU
Mira/EdU/Dpn

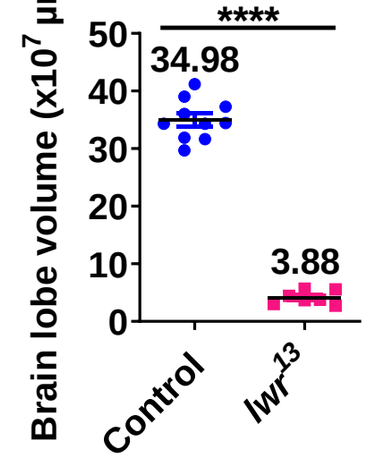
d


% of EdU negative NSC per BL
60
40
20
0
****
****
****
42.5
38.1
35.5
0.2
0
0
Control
lwr13
Control
lwr13
Control
lwr13
48h ALH
72h ALH
96h ALH

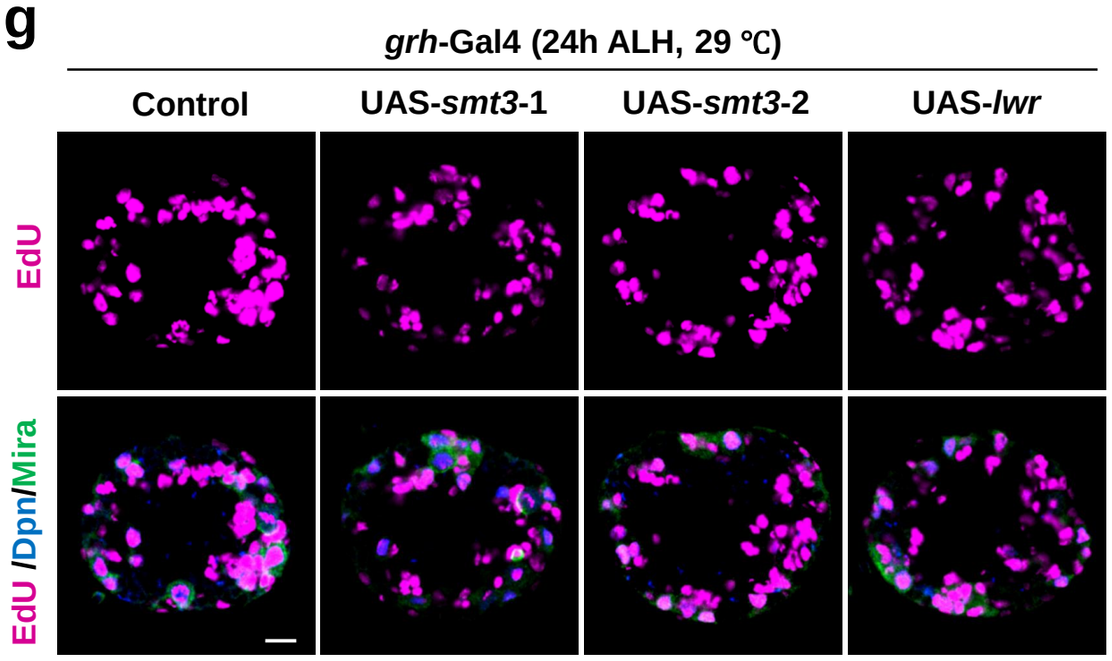
e


96h ALH
Control
lwr13
DAPI
50μm

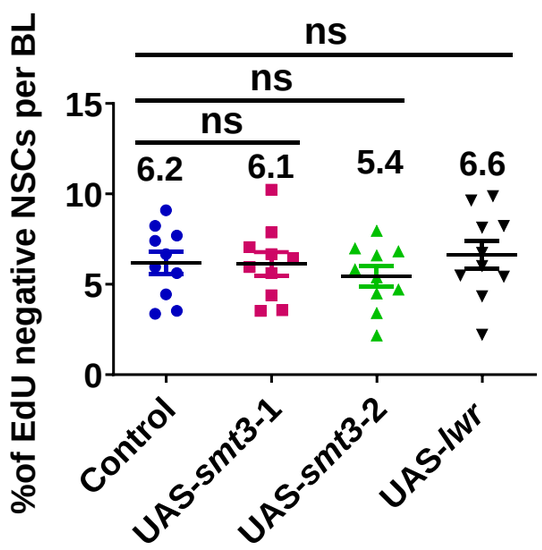
f


Brain lobe volume (x10^7 μm^3)
50
40
30
20
10
0
****
34.98
3.88
Control
lwr13

g


grh-Gal4 (24h ALH, 29 ℃)
Control
UAS-smt3-1
UAS-smt3-2
UAS-lwr
EdU
EdU /Dpn/Mira

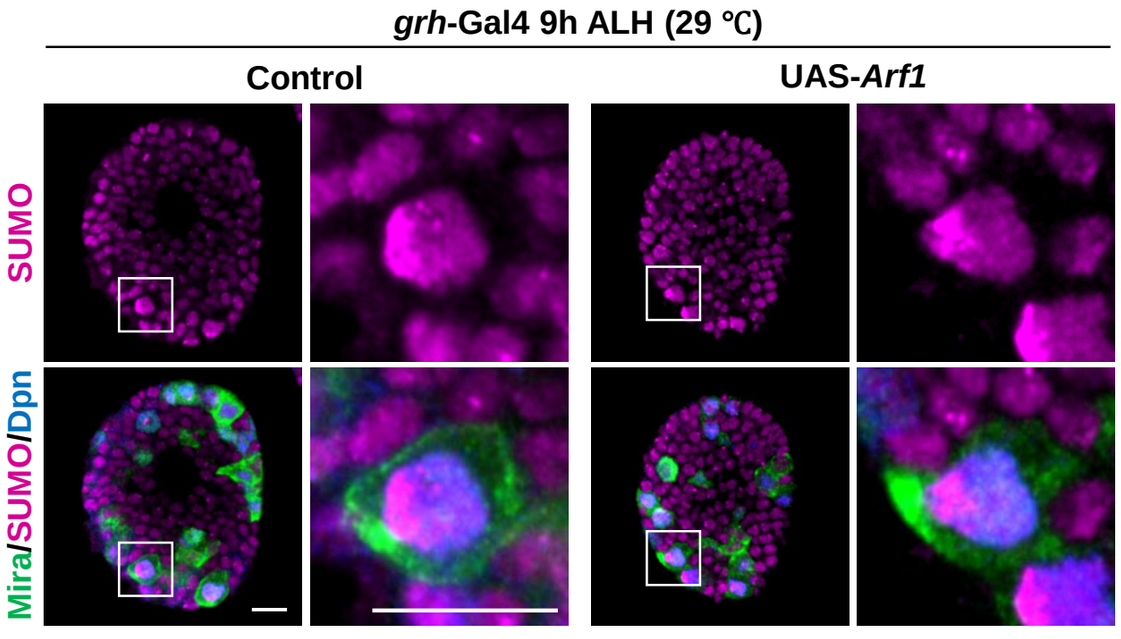
h


%of EdU negative NSCs per BL
15
10
5
0
ns
ns
ns
6.2
6.1
5.4
6.6
Control
UAS-smt3-1
UAS-smt3-2
UAS-lwr

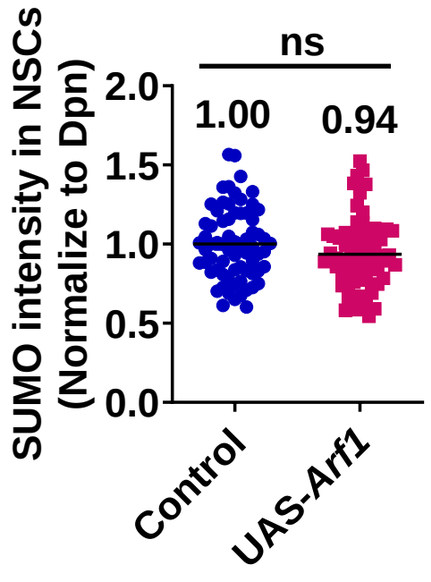
Supplementary Fig 3. Depletion of *SUMO* or *lwr* blocks NSC reactivation.

(a) At 48h and 72h ALH, larval brain lobes from control (*β-gal*^{RNAi}) and *smt3*^{RNAi} lines driven by *grh*-Gal4 were labeled with EdU, Dpn and Mira. NSCs were marked by Dpn and Mira. White arrows point to EdU negative NSCs. **(b)** Quantification graph of EdU negative NSC per brain lobe in (a). At 48h ALH, control, 0.8 ± 0.9, n = 10 BL; *smt3*^{RNAi} -1, 36.5 ± 3.6, n = 10 BL; *smt3*^{RNAi} -2, 40.7 ± 5.3, n = 10 BL; At 72h ALH, control, 0, n = 10 BL; *smt3*^{RNAi} -1, 25.8 ± 4, n = 10 BL; *smt3*^{RNAi} -2, 29.8 ± 3.7, n = 10 BL. **(c)** At 48h, 72h and 96h ALH, larval brain lobes from control (*yw*) and *lwr*^{13} were analyzed for EdU incorporation. NSCs were marked by Dpn and Mira. White arrows point to EdU negative quiescent NSCs. **(d)** Quantification graph of EdU- NSCs per BL for genotypes in (c). 48h ALH, control, 0.2 ± 0.4, n = 11 BL; *lwr*^{13}, 42.5 ± 5.7, n = 11 BL; 72h ALH, control, 0, n = 11 BL; *lwr*^{13}, 38.1 ± 3.4, n = 11 BL; 96h ALH, control, 0, n = 11 BL; *lwr*^{13}, 35.5 ± 2.4, n = 11 BL. **(e)** Maximum intensity z-projection of larval brains from control (*yw*) and *lwr*^{13} at 96h ALH, which were stained with DAPI. **(f)** Quantification graph of brain volume in various genotypes in (e). (×10^7 μm^3), Control, 34.98 ± 03.51, n = 10 BL; *lwr*^{13}, 3.88 ± 1.16, n = 7 BL. **(g)** At 24h ALH, larval brain lobes from control (*β-gal*^{RNAi}), UAS- *smt3*-1, UAS- *smt3*-2 and UAS- *lwr* lines driven by *grh*-Gal4 were analyzed for EdU incorporation. NSCs were marked by Dpn and Mira. **(h)** Quantification graph of EdU negative NSC per brain lobe in (g). Control, 6.2 ± 2, n = 10 BL; UAS- *smt3*-1, 6.1 ± 2, n = 10 BL; UAS- *smt3*-2, 5.4 ± 1.8, n = 10 BL; UAS- *lwr*, 6.6 ± 5.3, n = 10 BL. Data are presented as mean ± SD. ∗∗∗∗ indicates $p \leqslant 0.0001$, ns indicates $p > 0.05$. Scale bar in E is 50 μm, the rest are 10 μm.

a

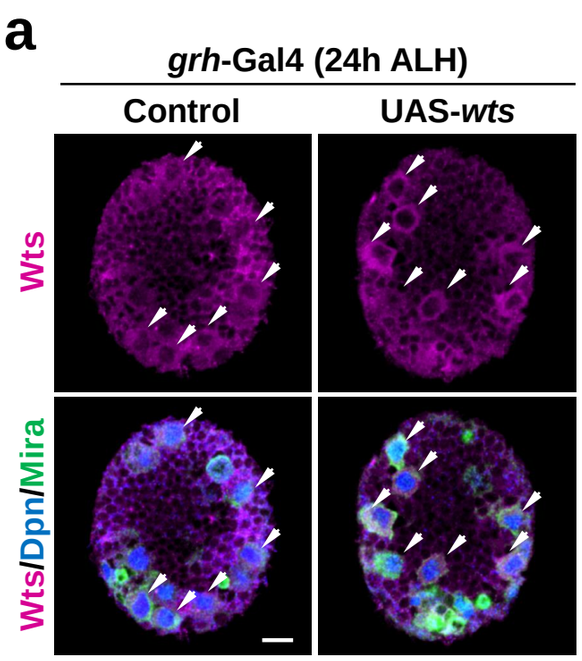

grh-Gal4 9h ALH (29 ℃)
Control
UAS-Arf1
SUMO
Mira/SUMO/Dpn

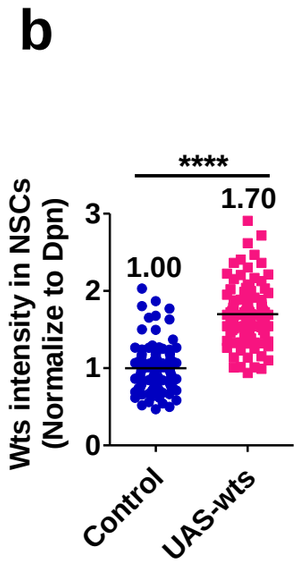
b

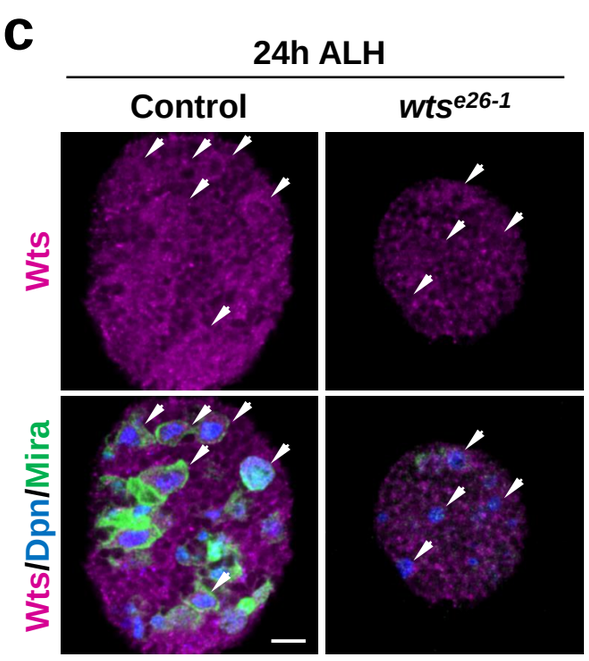

SUMO intensity in NSCs
(Normalize to Dpn)
ns
1.00
0.94
2.0
1.5
1.0
0.5
0.0
Control
UAS-Arf1

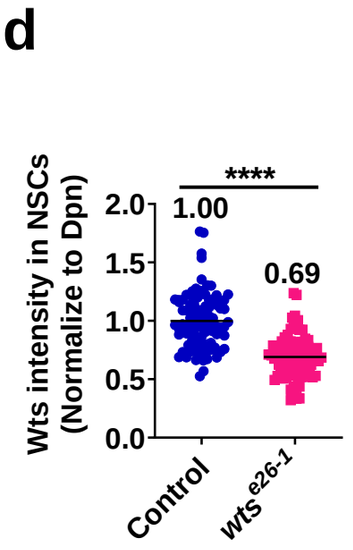
Supplementary Fig 4. *Arf1* overexpression in NSCs does not affect SUMO intensity in NSCs.

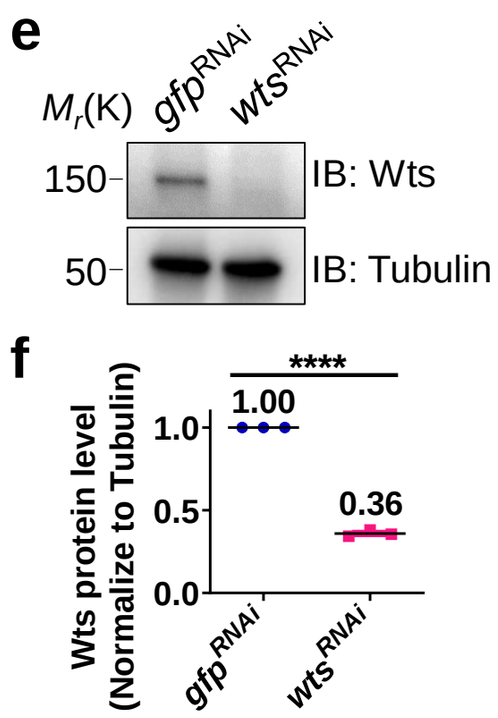
(a) At 9h ALH, larval brain lobes from control (*β-gal*^{RNAi}) and UAS-*Arf1* lines driven by *grh*-Gal4 were labeled with SUMO, Dpn and Mira. NSCs were marked by Dpn and Mira. White square point to EdU negative NSCs. The right panels are enlarged views of the cells in the white squares in the left panels. **(b)** Quantification graph of SUMO intensity in NSCs in (a). At 9h ALH, control, 1 ± 0.23, n = 10 BL; UAS-*Arf1*, 0.94 ± 0.23, n = 10 BL. Data are presented as mean ± SD. ns indicates $p > 0.05$. Scale bars are 10 μm.

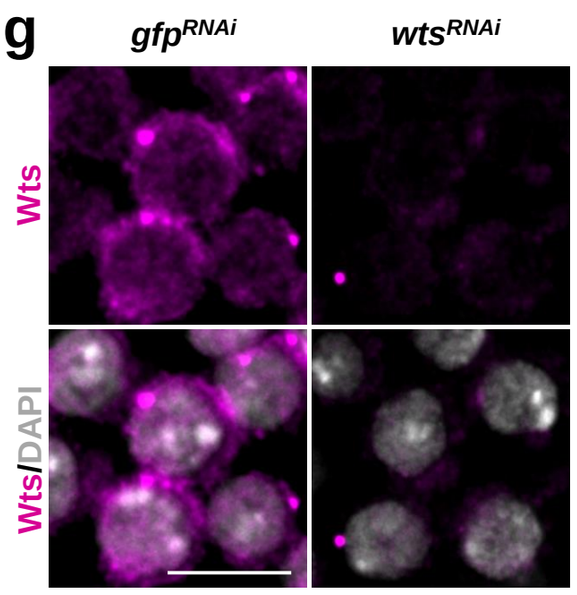

a
grh-Gal4 (24h ALH)
Control
UAS-wts
Wts
Wts/Dpn/Mira

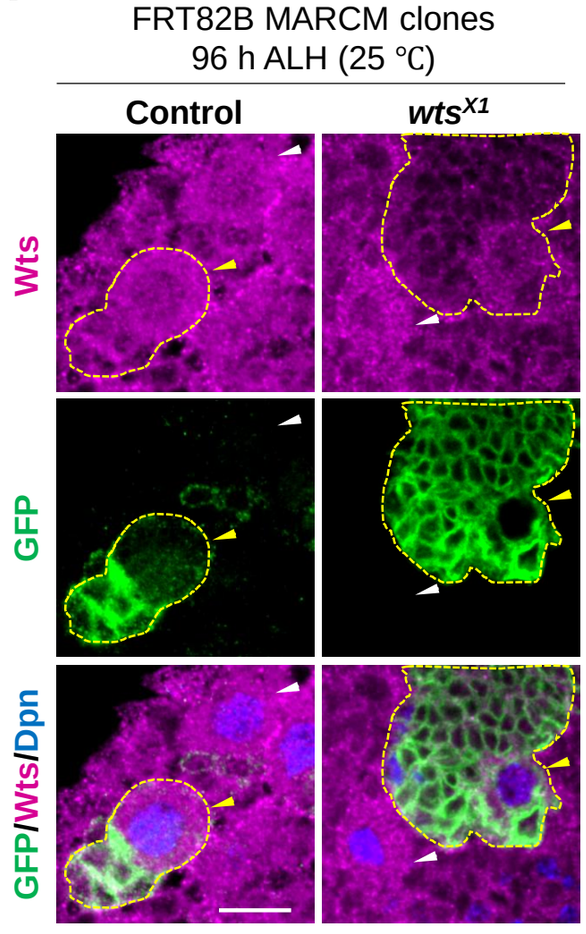

b
Wts intensity in NSCs (Normalize to Dpn)
****
1.00
1.70
Control
UAS-wts

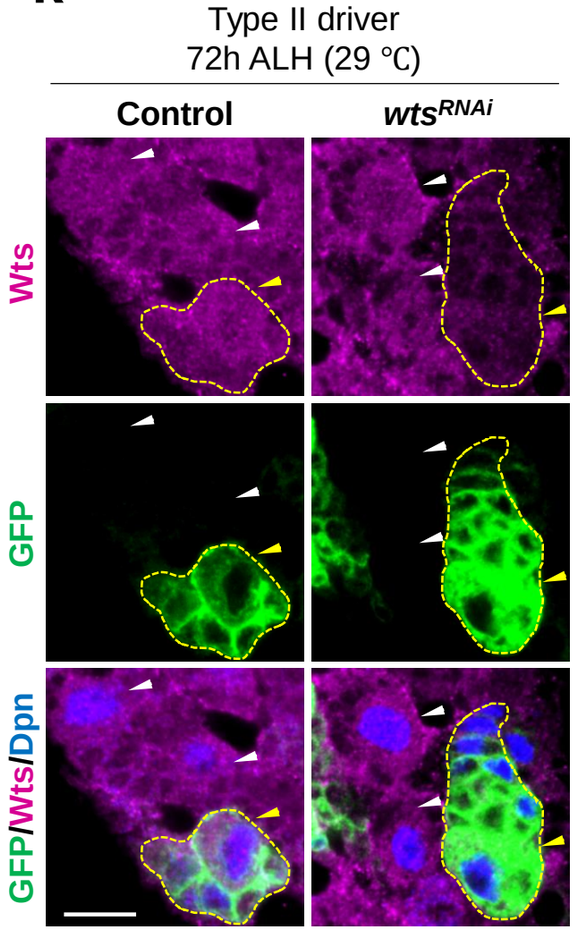

c
24h ALH
Control
wts e26-1
Wts
Wts/Dpn/Mira

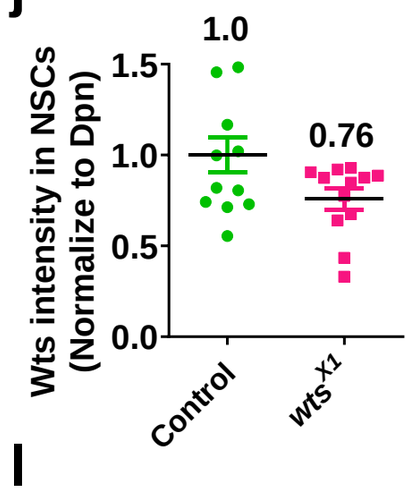

d
Wts intensity in NSCs (Normalize to Dpn)
****
1.00
0.69
Control
wts e26-1

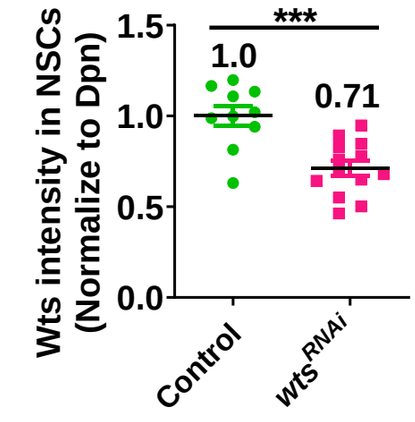

e
Mr(K)
gfp RNAi
wts RNAi
150
50
IB: Wts
IB: Tubulin
f
Wts protein level (Normalize to Tubulin)
****
1.00
0.36
gfp RNAi
wts RNAi

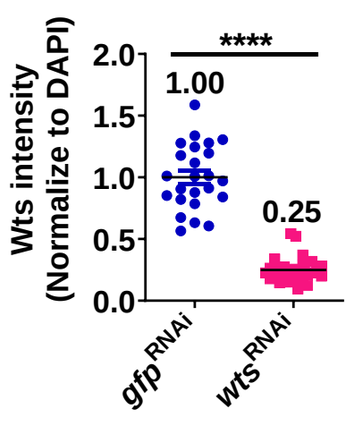

g
gfp RNAi
wts RNAi
Wts
Wts/DAPI

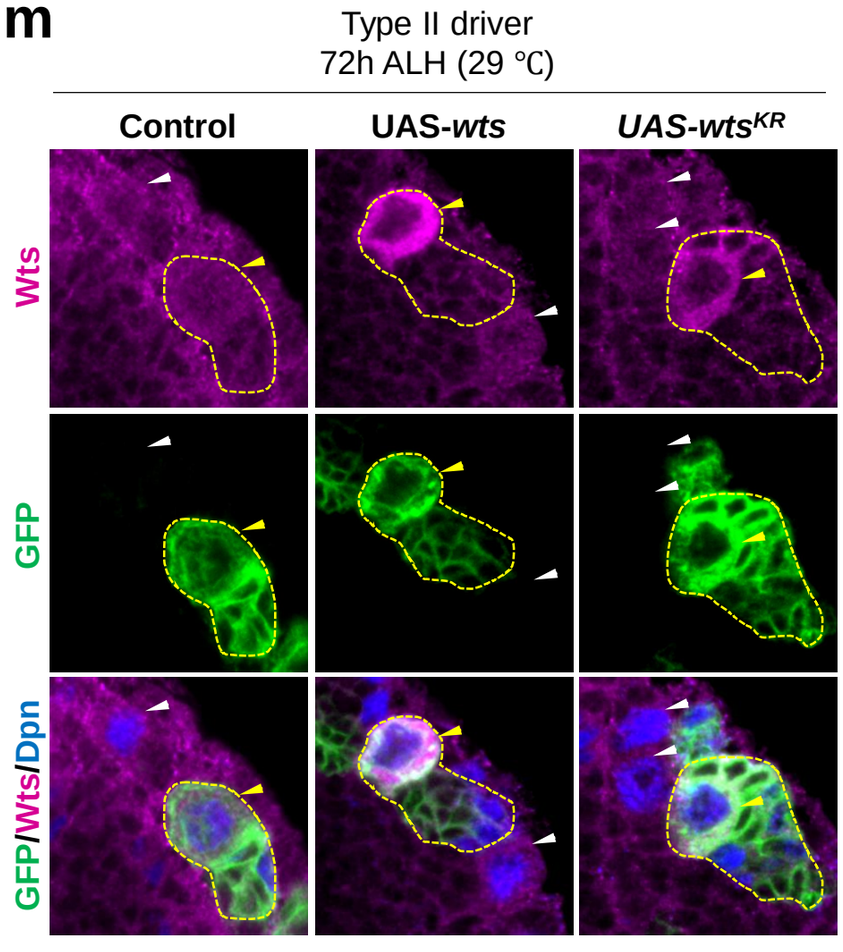

i
FRT82B MARCM clones
96 h ALH (25 ℃)
Control
wts X1
Wts
GFP
GFP/Wts/Dpn

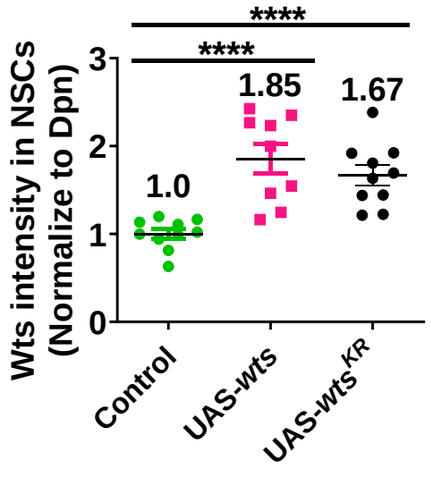

k
Type II driver
72h ALH (29 ℃)
Control
wts RNAi
Wts
GFP
GFP/Wts/Dpn


j
*
1.0
0.76
Wts intensity in NSCs (Normalize to Dpn)
Control
wts X1


l
***
1.0
0.71
Wts intensity in NSCs (Normalize to Dpn)
Control
wts RNAi

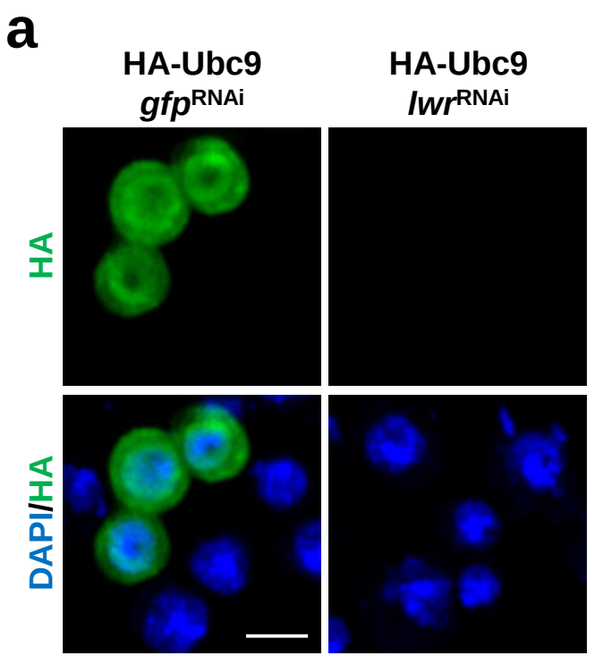

h
Wts intensity (Normalize to DAPI)
****
1.00
0.25
gfp RNAi
wts RNAi

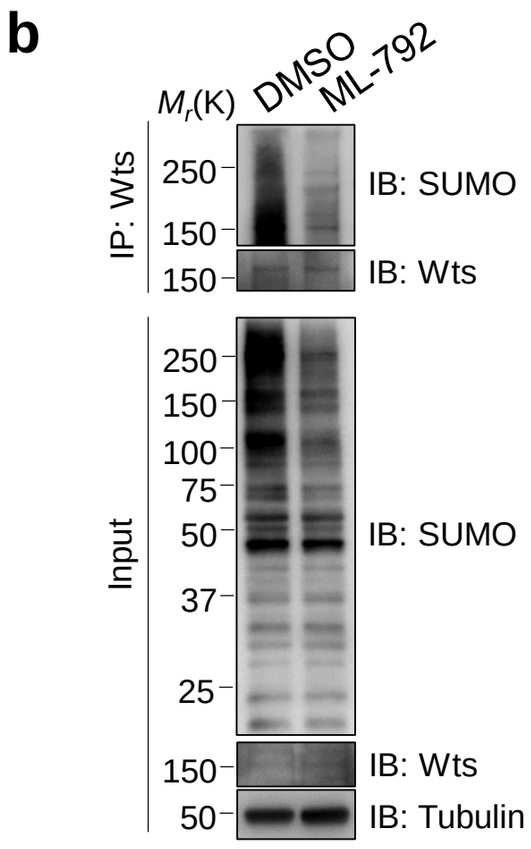

m
Type II driver
72h ALH (29 ℃)
Control
UAS-wts
UAS-wts KR
Wts
GFP
GFP/Wts/Dpn

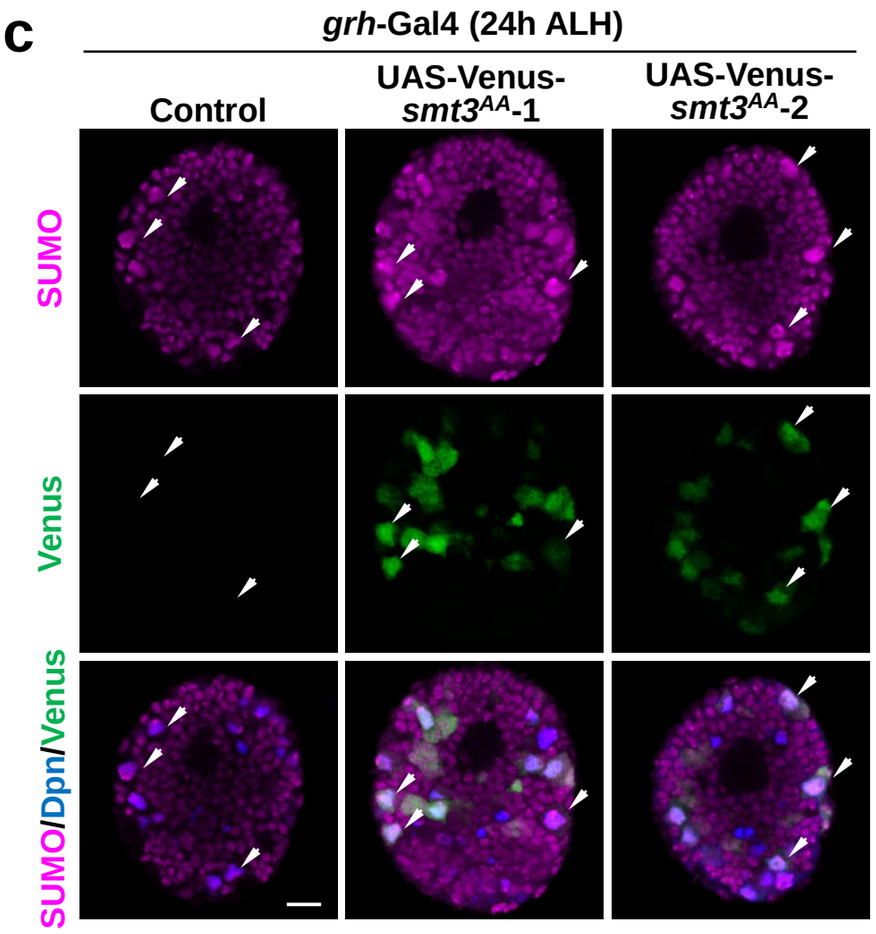

n
****
****
Wts intensity in NSCs (Normalize to Dpn)
1.0
1.85
1.67
Control
UAS-wts
UAS-wts KR

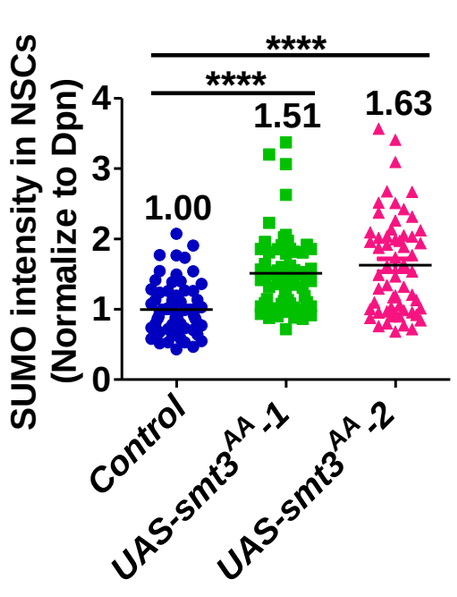
Supplementary Fig 5. Anti-Wts antibodies specifically recognize Wts in larval brains and S2 cells.

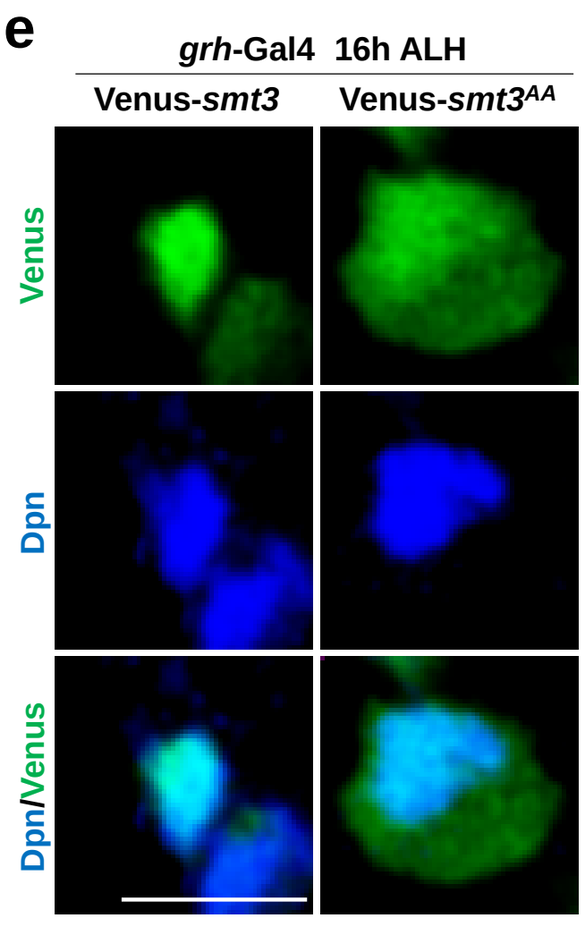
(a, c) At 24h ALH, larval brain lobes from control (*β-gal*^{RNAi}) and UAS-*wts* lines driven by *grh*-Gal4 (a) or control (*yw*) and *wts*$^{e26-1}$ (c) were labeled with Wts, Dpn and Mira. NSCs were marked by Dpn and Mira. White arrows point to NSCs. **(b, d)** Quantification graph of Wts intensity (normalized to Dpn) in NSCs in (a, c) respectively. (B), Control, 1 ± 0.3, n = 10 BL; UAS-*wts*, 1.7 ± 0.36, n = 10 BL; (d), Control, 1 ± 0.23, n = 10 BL; *wts*$^{e26-1}$, 0.69 ± 0.18, n = 10 BL. **(e)** S2 cells were treated with dsRNA targeting *gfp* (control) or *wts*. Western blot tested the Wts knocking down efficiency by using anti-Wts antibody. Tubulin works as loading control. **(f)** Quantification graph of Wts protein level (normalized to Tubulin) in three independent repeats in (e). *gfp*^{RNAi}, 1; *wts*^{RNAi}, 0.36 ± 0.02. **(g)** S2 cells were treated with *gfp*- or *wts*-dsRNA. Immunostaining tested the Wts knocking down efficiency by using anti-Wts antibody. DAPI labels cell nucleus. **(h)** Quantification graph of Wts intensity (normalized to DAPI) in (g). *gfp*^{RNAi}, 1 ± 0.26; *wts*^{RNAi}, 0.25 ± 0.11. **(i)** At 96h ALH, larval brain lobes from control (*yw*) and *wts*^{X1} lines were labeled with Wts, Dpn and GFP. NSCs were marked by Dpn and Mira. MARCM clones were marked by GFP. The yellow dotted circles labels MARCM clones, yellow arrows point to the NSCs in MARCM clones, the white arrows point to the neighboring NSCs. **(j)** Quantification graph of Wts intensity (normalized to Dpn) in NSCs in (i). Control, 1 ± 0.33, n = 12; *wts*^{X1}, 0.76 ± 0.2, n = 12. **(k, m)** At 72h ALH, larval brain lobes from (k) control (*β-gal*^{RNAi}), *wts*^{RNAi} lines and (m) control (*β-gal*^{RNAi}), UAS-*wts* and UAS-*wts*^{KR} driven by Type II NSC driver were labeled with Wts, Dpn and GFP. NSCs were marked by Dpn and Mira. Type II NSC clones were marked by GFP and yellow dotted circles, yellow arrows point to the NSCs in Type II NSC clones, the white arrows point to the neighboring NSCs. **(l, n)** Quantification graph of Wts intensity (normalized to Dpn) in NSCs in (k, m) respectively. (k), Control, 1 ± 0.17, n = 10; *wtsRNAi*, 0.71 ± 0.15, n = 13; (m), Control, 1 ± 0.17, n = 10; UAS-*wts*, 1.85 ± 0.5, n = 9; UAS-*wts*^{KR}, 1.67 ± 0.36, n = 10. Data are presented as mean ± SD. ∗ indicates $0.05 \leqslant p \leqslant 0.01$, ∗∗∗ indicates $p \leqslant 0.001$, ∗∗∗∗ indicates $p \leqslant 0.0001$. Scale bars: 10 μm.


a
HA-Ubc9
gfpRNAi
HA-Ubc9
lwrRNAi
HA
DAPI/HA


b
Mr(K)
DMSO
ML-792
IP: Wts
250
150
IB: SUMO
150
IB: Wts
Input
250
150
100
75
50
37
25
IB: SUMO
150
IB: Wts
50
IB: Tubulin

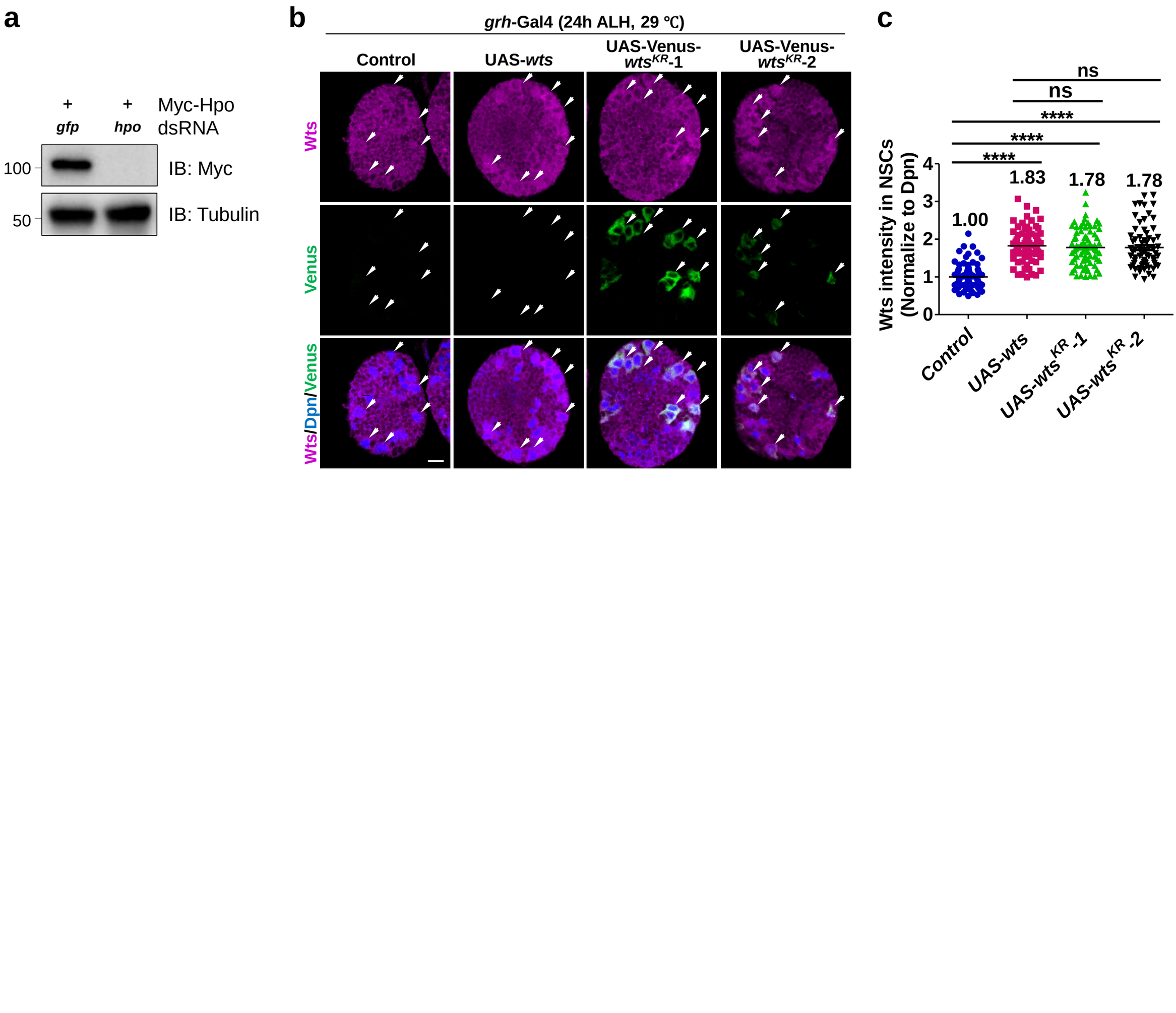

c
grh-Gal4 (24h ALH)
Control
UAS-Venus-smt3AA-1
UAS-Venus-smt3AA-2
SUMO
Venus
SUMO/Dpn/Venus


d
****
1.00
1.51
1.63
SUMO intensity in NSC
(Normalize to Dpn)
0
1
2
3
4
Control
UAS-smt3AA-1
UAS-smt3AA-2


e
grh-Gal4 16h ALH
Venus-smt3
Venus-smt3AA
Venus
Dpn
Dpn/Venus

Supplementary Fig 6. Wts SUMOylation is dependent on Smt3 and Lwr.

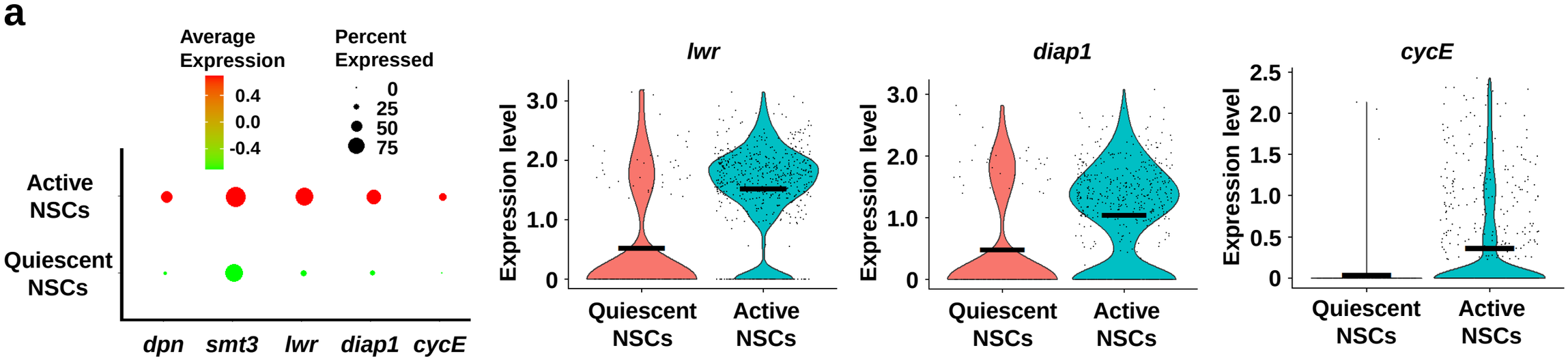
(a) S2 cells were treated with double stranded RNA against. *gfp* or *lwr* and transfected with HA-Ubc9. Cells were labeled with anti-HA antibody and DAPI. **(b)** S2 cells were treated with DMSO or ML-792. IP was conducted with anti-Wts antibody and western blots were performed by using anti-SUMO and anti-Wts antibodies. **(c)** At 24h ALH, larval brain lobes from control (*β-gal*^{RNAi}) and two Venus tagged *smt3*^{AA} overexpression lines driven by *grh*-Gal4 were labeled with SUMO, Venus and Dpn. NSCs were marked by Dpn. White arrows point to NSCs. **(d)** Quantification graph of SUMO intensity (normalized to Dpn) in NSCs in (c). Control, 1 ± 0.38, n = 10 BL; UAS-*smt3*^{AA}-1, 1.51 ± 0.55, n = 10 BL; UAS-*smt3*^{AA}-2, 1.63 ± 0.7, n = 10 BL.**(e)** At 16h ALH, NSCs from Venus tagged *smt3* overexpression line and Venus tagged *smt3*^{AA} overexpression line driven by *grh*-Gal4 were labeled with Venus (GFP) and Dpn. Dpn labels the nucleus of the NSCs. Data are presented as mean ± SD. $****$ indicates $p \leqslant 0.0001$, Scale bars: 10 μm.


a
+
gfp
+
hpo
Myc-Hpo dsRNA
100
IB: Myc
50
IB: Tubulin
b
grh-Gal4 (24h ALH, 29 ℃)
Control
UAS-wts
UAS-Venus-wtsKR-1
UAS-Venus-wtsKR-2
Wts
Venus
Wts/Dpn/Venus
c
Wts intensity in NSCs (Normalize to Dpn)
ns
ns
****
****
****
1.00
1.83
1.78
1.78
Control
UAS-wts
UAS-wtsKR-1
UAS-wtsKR-2

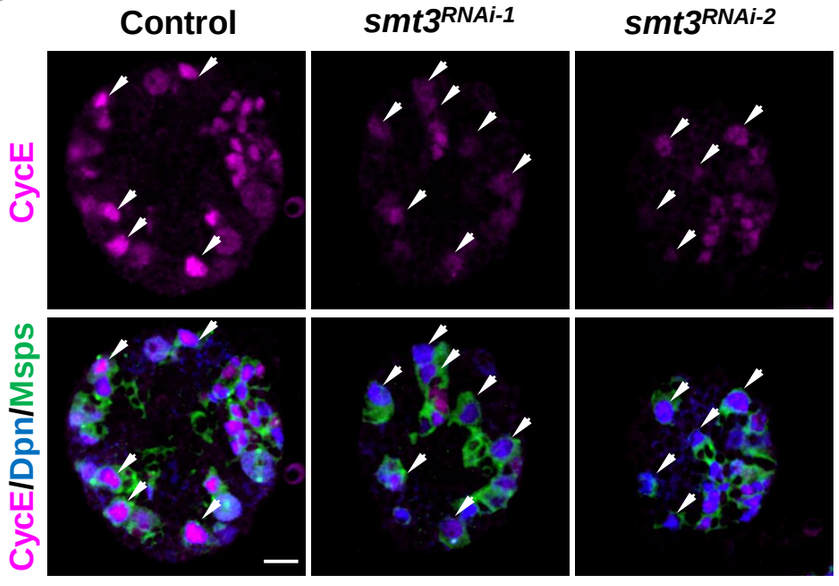
Supplementary Fig 7. SUMOylation of Wts at Lys766 suppresses its phosphorylation and attenuates its kinase activity.

(a) S2 cells were treated with gfp- or hpo-dsRNA, and overexpressed Myc-Hpo. Western blot tested the Hpo knocking down efficiency by using anti-Myc antibody. Tubulin works as loading control. **(b)** At 24h ALH, larval brain lobes from control (*β-gal*^{RNAi}), UAS-*wts* and Venus tagged *wts*^{KR} lines driven by *grh*-Gal4 were labeled with Wts, Dpn and Venus. NSCs were marked by Dpn. White arrows point to NSCs. **(c)** Quantification of Wts intensity (normalized to Dpn) in NSCs in (b). Control, 1 ± 0.21, n = 10; UAS-*wts*, 1.92 ± 0.28, n = 10; UAS-*wts*^{KR}-1, 2.1 ± 0.64, n = 10; UAS-*wts*^{KR}-2, 2.13 ± 0.46, n = 10. Data are presented as mean ± SD. ∗∗∗∗ indicates p ⩽ 0.0001, Scale bars: 10 μm.

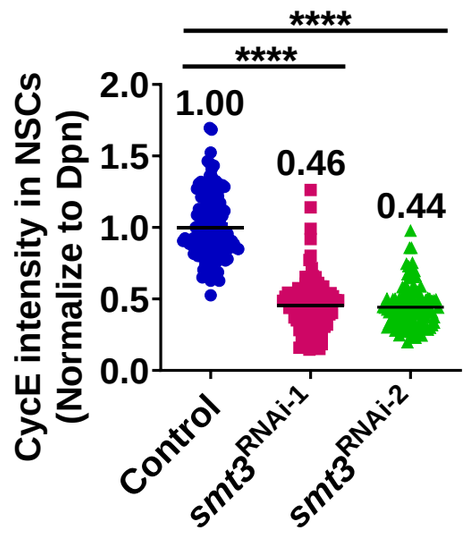
a


Average Expression
0.4
0.0
-0.4
Percent Expressed
0
25
50
75
Active NSCs
Quiescent NSCs
dpn
smt3
lwr
diap1
cycE
lwr
diap1
cycE
Expression level
Quiescent NSCs
Active NSCs

b


grh-Gal4 (24h ALH)
Control
smt3RNAi-1
smt3RNAi-2
CycE
CycE/Dpn/Msps

c


****
****
1.00
0.46
0.44
CycE intensity in NSCs (Normalize to Dpn)
Control
smt3RNAi-1
smt3RNAi-2

Supplementary Fig 8. The expression of the Hippo pathway target genes during NSC reactivation.

(a) Analysis of *smt3*, *lwr, diap1, cycE* and NSC marker *dpn* mRNA expression in quiescent like NSCs and active like NSCs from the dataset of single-cell RNA seq (C. B. Avalos, et al., 2019). **(b)** At 24h ALH, larval brain lobes from control (*β-gal*^{RNAi}), *smt3*^{RNAi}-1 and *smt3*^{RNAi}-2 lines driven by *grh*-Gal4 were labeled with Dpn, Msps, and CycE. NSCs were marked by Dpn. **(c)** Quantification of CycE intensity (normalized to Dpn) of Dpn+ NSCs in various genotypes in (b). Control, 1 ± 0.21, n = 10 BL; *smt3*^{RNAi}-1, 0.45 ± 0.22, n = 10 BL; *smt3*^{RNAi}-2, 0.51± 0.15, n = 10 BL. White arrows point to NSCs. Data are presented as mean ± SD. **** for P ≤ 0.0001. Scale bars: 10 μm.

a

24h ALH (25 ℃)

wts^{Venus}

Venus

Wts

Venus/Wts/Dpn

b

24h ALH (25 ℃)

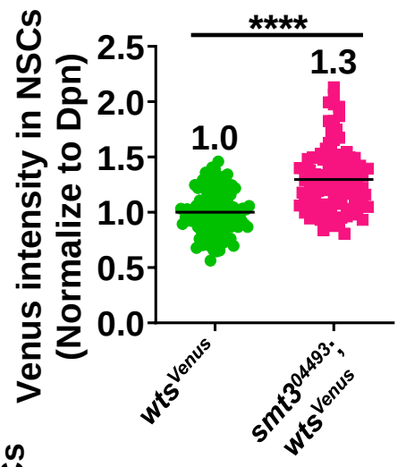
wts^{Venus} | *smt3*^{04493}; *wts*^{Venus}

Venus

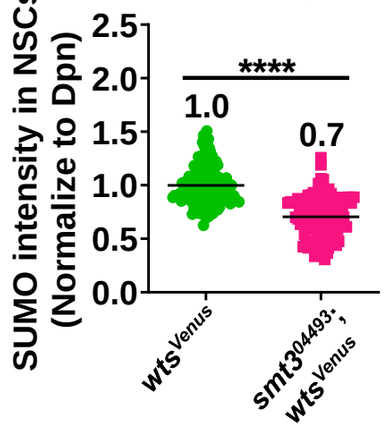
SUMO

Venus/SUMO/Dpn

c



d



e

grh > *wts*^{Venus} 24h ALH (29 ℃)

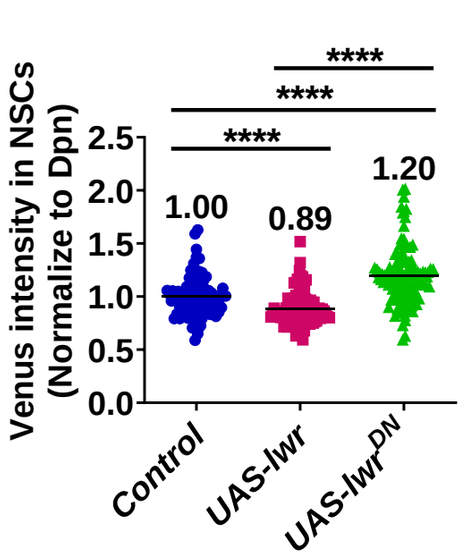
Control | UAS-*lwr* | UAS-*lwr*^{DN}

Venus

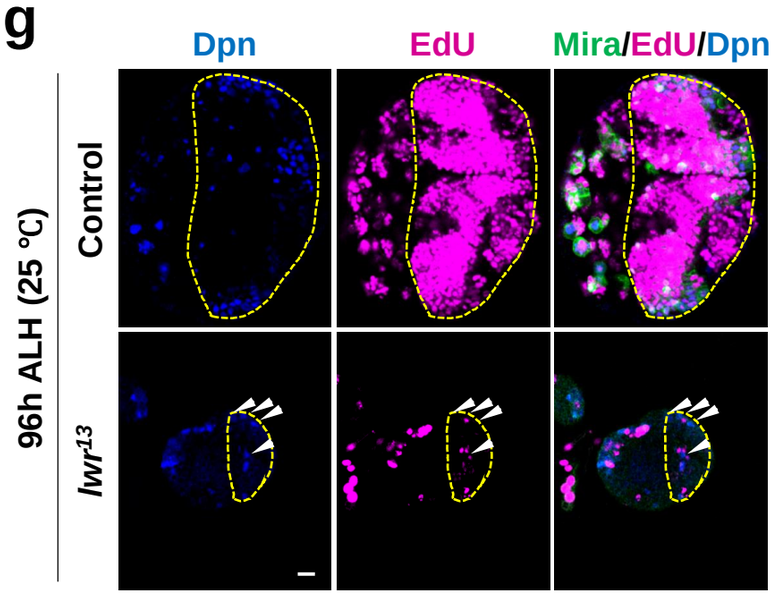
Venus/Dpn

Venus

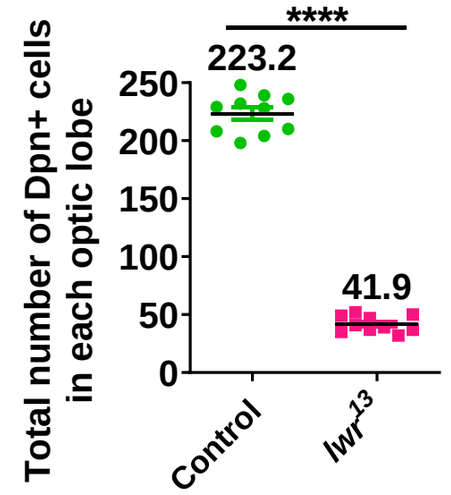
f



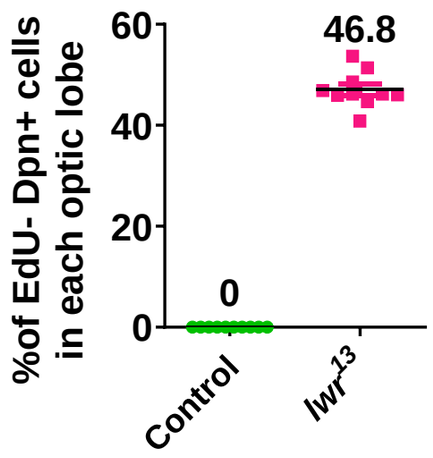
g



h



i



j

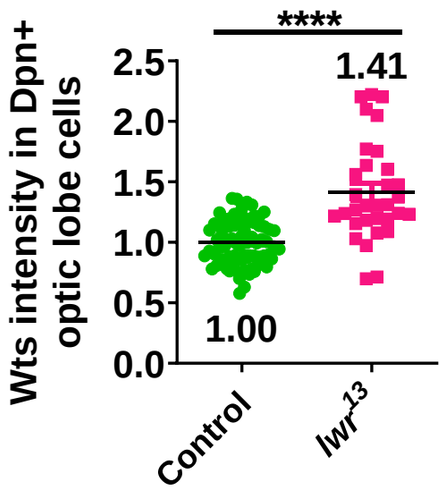
96h ALH (25 ℃)

Control | *lwr*^{13}

Wts

Mira/Wts/Dpn

k



Supplementary Fig 9. The SUMO pathway downregulates Wts protein level in NSCs.

(a, b, e) At 24h ALH, the brains of endogenous Venus tagged Wts line were stained with Venus (GFP), Wts and Dpn. The yellow circles label the Dpn+ NSCs, the yellow dotted circles label the nucleus of the NSCs. The right panels are enlarged views of the cells in the white square on the left panels. **(b)** At 24h ALH, the brains of endogenous Venus tagged Wts line and the stable line combines endogenous Venus tagged Wts with $smt3^{04493}$ were stained with Venus (GFP), SUMO and Dpn. The yellow circles label the Dpn+ NSCs, the yellow dotted circles label the nucleus of the NSCs. The right panels are enlarged views of the cells in the white square on the left panels. **(c, d)** Quantification analysis of Wts protein levels (c) and SUMO protein levels (d) normalized to Dpn for (b). (c), wts^{Venus}, 1 ± 0.2, n = 10 BL; $smt3^{04493}$; wts^{Venus}, 1.3 ± 0.29, n = 10 BL; (d), wts^{Venus}, 1 ± 0.18, n = 10 BL; $smt3^{04493}$; wts^{Venus}, 0.7 ± 0.19, n = 10 BL. **(e)** At 24h ALH, the larval brain lobes from indicated genotypes were labeled with Venus (GFP) and Dpn. The yellow dotted circles label the Dpn+ NSCs. The lower panels are enlarged views of the NSCs in the white squares in the upper panels. **(f)** Quantification analysis of Wts protein levels normalized to Dpn for (e). Control, 1 ± 0.19, n = 10 BL; UAS-*lwr*, 0.89 ± 0.16, n = 10 BL; UAS-lwr^{DN}, 1.2 ± 0.28, n = 10 BL. **(g)** At 96h ALH, larval brain lobes from control (*yw*) and lwr^{13} were analyzed for EdU incorporation. Cells were stained by Dpn and Mira. The yellow dotted circles label the optic lobes. White arrows point to Dpn+ EdU negative quiescent optic lobe cells. **(h, i)** Quantification graph of total number of Dpn+ cells in each optic lobe (h) and EdU- Dpn+ cells per optic lobe (I) for genotypes in (g). (h), Control, 223.2 ± 16.9, n = 10 BL; lwr^{13}, 41.9 ± 7, n = 10 BL. (i), Control, 0, n = 10 BL; lwr^{13}, 46.8 ± 3.5, n = 10 BL. **(j)** At 96h ALH, larval brain lobes from control (*yw*) and lwr^{13} were stained by Wts, Dpn and Mira. The yellow circles label the optic lobes. The right panels are enlarged views of the cells in the white square on the left panels. The white dotted circles label the outline of the Dpn+ optic lobe cells. **(k)** Quantification graph of the Wts intensity in Dpn+ optic lobe cells in (j). Control, 1 ± 0.18, n = 10 BL; lwr^{13}, 1.41 ± 0.4, n = 8 BL. Data are presented as mean ± SD. **** for $P \leqslant 0.0001$. Scale bars: 10 μm.

Supplementary Table 1. Mutation details of *smt3*^{M2L} and *lwr*^{M2L}

a

smt3^{WT}: …C GAG GAC AAC GCC GTC GTC CAG TTC AAG ATC AAG AAG CAC ACA CCC TTG AGG AAG CTG ATG AAC GCC TAC TGC GAC CGT…
smt3$^{M2L-2483}$: …C GAG GAC AAC GCC GTC GTC CAA GAT CAA GAT CAA GAA GCA CAC ACC CTT GAG GAA GCT GAT GAA CGC CTA CTG CGA CCG…
smt3$^{M2L-2484}$: …C GAG GAC AAC GCC GTC GTC CAG TTG TCA AGA TCA AGA AGC ACA CAC CCT TGA GGA AGC TGA TGA ACG CCT ACT GCG ACC…
Smt3^{WT}: MSDEKKGGETEHINLKVLGQDNAVVQQFKIKKHTPLRKLMNAYCDRAGLSMQVVRFRFDGQPINENDTPTTSLEMEEGDTIEVYQQQTGGAP-
Smt3$^{M2L-2483}$: MSDEKKGGETEHINLKVLGQDNAVVQDQEAHTLEEADERLLRPCRTLHAGGALPFRRTAHQRERHSDLAGDGGGRHHRGLPAADWWRSIRLLS-
Smt3$^{M2L-2484}$: MSDEKKGGETEHINLKVLGQDNAVVQLSRSTHP-

b

lwr^{WT}: …A CCC GCC AAG AAC CCT GAC GGC ACC CTC AAC CTG ATG ATC TGG GAG TGC GCC ATT CCC GGC AAG AAG TCC ACC CCC…
lwr$^{M2L-2401}$: …A CCC GCC AAG AAC CCT GAC GGC ACC CTC AAC CTG ATG ATCTGGG AGT GCG CCA TTC CCG GCA AGA AGT CCA CCC CCT…
lwr$^{M2L-2402}$: …A CCC GCC AAG AAC CCT GAC GGC ACC CTC AAC CTG ATGA TCT GGG AGT GCG CCA TTC CCG GCA AGA AGT CCA CCC CCT
Ubc9^{WT}: MSGIAITRLGEERKAWRKDHPFGFVARPAKNPDGTLNLMIWECAIPGKKSTPWEGGLYKLRMIFKDDYPTSPPKCKFEPPLFHPNVYPSGTVCLS…
Ubc9$^{M2L-2401}$: MSGIAITRLGEERKAWRKDHPFGFVARPAKNPDGTLNLMSAPFPARSPPPGRAGSTSCA-
Ubc9$^{M2L-2402}$: MSGIAITRLGEERKAWRKDHPFGFVARPAKNPDGTLNLISGSAPFPARSPPPGRAGSTSCA-

(a) *smt3*$^{M2L-2483}$ has a silent mutation on Q26 and 2 extra nucleotide base pairs (bps), which results in a missense mutation from the 27th amino acid onwards. *smt3*$^{M2L-2484}$ has two extra nucleotide bps, leading to a missense mutation from 27th amino acid and early termination of translation at 34th amino acid. **(b)** Mutations in *lwr*$^{M2L-2401}$ and *lwr*$^{M2L-2402}$ are caused by a 7- and 1-bp deletions, resulting in missense mutations from the 40th or 39th amino acids, respectively, and early termination of translation at 59th and 61th amino acids, respectively.

Supplementary Table 2. Total number of Dpn+ neural stem cells in various genotypes

	yw	*smt3*^{04493}	*lwr*^{13}	*aos1*^{c06048}	UAS-β gal	*smt3*^{RNAi}-1	*smt3*^{RNAi}-2	UAS-β gal	UAS-*smt3*-1	UAS-*smt3*-2	UAS-β gal	UAS-*lwr*
Total number of Dpn+ NSCs	87.1	68	79.2	80.2	83.3	82.3	79.3	60.1	68.1	68.9	65.5	73.2
Number of Dpn+ EdU- NSCs	9	43.6	43.8	26	7.4	43.7	46.5	17.1	40.7	41.7	20	36
% of Dpn+ EdU- NSCs per BL	10.4	64.2	55.2	32.8	8.8	53.2	58.5	28.4	59.4	59.6	30.3	49.2

Supplementary Table 3. Wts K766 was predicted to be SUMOylated

a

Results for Wts putative SUMOylation sites						
Position K	Sequence	Best PS	Consensus direct		Consensus Inverted	
K25	KYTAEALESIKQDLTRFEVQN	Low	Strong Consensus	Low	None	None
K126	PKMMTALMPNKLIRKPSIERD	None	None	None	None	None
K224	PVPPTSQAYVKRRSPALNNRP	None	None	None	None	None
K266	NPQQQLTQQLKSLNLYPGGGS	None	None	None	None	None
K373	SQQPIIMQSVKSTQVQKPVLQ	None	None	None	None	None
K470	PPSYAKSMQAKAATVVQQQQQ	None	None	None	None	None
K561	QSNNNNNSEIKPPSCNNNNIQ	High	None	None	Consensus inv	High
K630	IKHASPIPERKKISKEKEEER	Low	None	None	None	Low
K636	IPERKKISKEKEEERKEFRIR	None	None	None	None	None
K666	FMEQHIENVIKSYRQRTYRKN	None	None	None	None	None
K710	NQKESNYIRLKRAKMDKSMFV	None	None	None	None	None
K713	ESNYIRLKRAKMDKSMFVKLK	Low	Consensus	Low	None	None
K721	RAKMDKSMFVKLKPIGVGAFG	None	None	None	None	None
K738	GAFGEVTLVSKIDTSNHLYAM	None	None	None	None	None
K766	VLKRNQVAHVKAERDILAEAD	High	NDSM	High	None	None
K811	PGGDLMSLLIKLGIFEEELAR	None	None	None	None	None
K845	HKMGFIHRDIKPDNILIDRDG	Low	NDSM	Low	Consensus inv	Low
K1015	KRLGKSVDEVKSHDFFKGIDF	None	None	None	None	None
K1040	KQKAPYIPEIKHPTDTSNFDP	None	None	None	None	None
K1091	EFTFRRFFDDKQPPDMTDDQA	None	None	None	None	None

b

SUMOylation sites	NDSM	[Ψ]-[K]-[x]-[α]-[x]-[α]$_6$	Ψ = A,F,I,L,M,P,V or W; 2 out of 6 α must be D or E
	Consensus inverted	[α]-[x]-[K]-[Ψ]	Ψ = A,F,I,L,M,P,V or W; α = D or E

c

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                                                  Wts K766/Lats1 K751
Warts_DROME GVGAFGEVTLVSKIDTSNHLYAMKTLRKADVLKRNQVAHVKAERDILAEADNNWVVKLYY 785
LATS1_HUMAN GIGAFGEVCLARKVDT-KALYATKTLRKKDVLLRNQVAHVKAERDILAEADNEWVVRLYY 770
LATS2_HUMAN GIGAFGEVCLACKVDT-HALYAMKTLRKKDVLNRNQVAHVKAERDILAEADNEWVVKLYY 733
            *:****** *. *:** : *** ***** *** **********************:***:***

                        Wts K561
Warts_DROME PPPPYQSNNNNNSEIKPPSCNNNNIQISNSNLATTPPIPPAKYNNNSSNTGANSSGGSNG 605
LATS1_HUMAN PPPPYPKHLLHQNPSVPP-YESISK----PSKEDQPSLPKE---------------DESE 594
LATS2_HUMAN PPPPYPKHLLLRSKSEQYDLDSLCAGME-QSLRAGPNEPEG---------------GDKS 557
            ***** .:   ..        :.        .    * *                  ...
```

(a) The results for Wts putative SUMOylation sites predicted by the Joined Advanced SUMOylation Site and Sim Analyser (JASSA). **(b)** SUMOylation sites of NDSM and consensus inverted. **(c)** Alignment of the amino acid among *Drosophila wts*, human LATS1 and LATS2 in the region encompassing the Wts K766 and K561 sites.