Stem Cell Reports, Volume *11*

Supplemental Information

Hsp83/Hsp90 Physically Associates with Insulin Receptor to Promote Neural Stem Cell Reactivation

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SUPPLEMENTAL TABLES

Table S1. Knockdown of hsp70 did not result in defects in NSC reactivation

S/N	Isoforms of Hsp70	RNAi stock number	NSC reactivation defects
1	Hsp70Aa	BDSC#42639	No
2	Hsp70Ab	BDSC#35663	No
3	Hsp70Ba	BDSC#35672	No
4		BDSC#43289	No
5	Hsp70Bb	BDSC#32997	No
6		BDSC#33948	No
7	Hsp70Bbb	BDSC#28787	No
8		BDSC#33000	No
9		BDSC#33916	No
10	Hsp70Bc	BDSC#35697	No
11		BDSC#42626	No
12	Hsc70-4	BDSC#28709	No
13		BDSC#35684	No
14		BDSC#54810	No
15	Hop	BDSC#32979	No
16		BDSC#34002	No

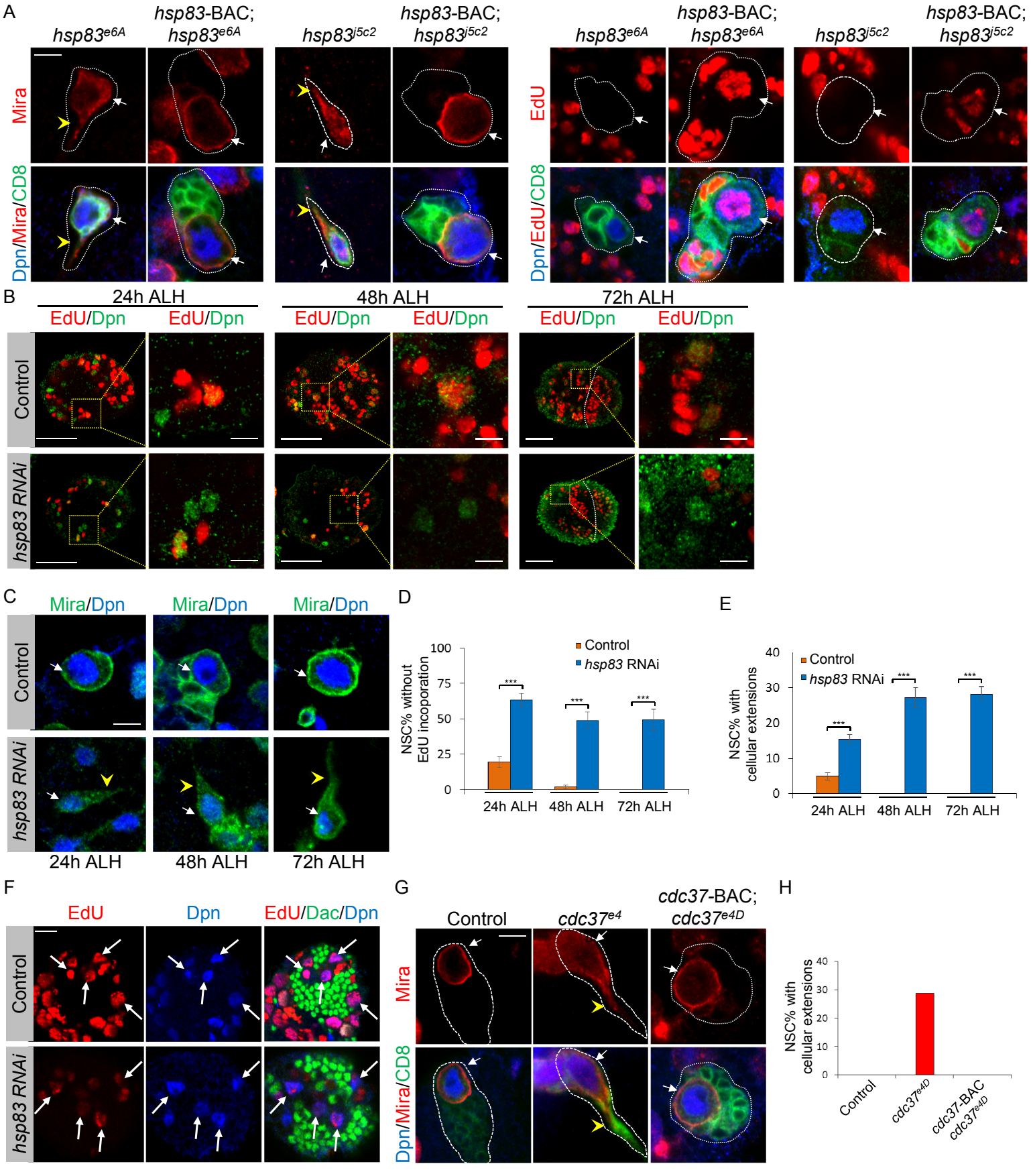
Table S2: Lists of primers used to generate generate entry clones for Hsp83, InR^{intra} and Cdc37 and proper controls

Primer name	Primer Sequence (5'-3')	Purpose
BP.Hsp83.F	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC ATG CCA GAA GAA GCA GAG ACC	To generate pDONR211-Hsp83
BP.Hsp83.stop.R	GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC TTA ATC GAC CTC CTC CAT GTG GGA A	
BP.Hsp83.no-stop.R	GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC ATC GAC CTC CTC CAT GTG GGA AGC G	To generate pDONR211-Hsp83 without stop codon
BP.Hsp83C.F	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC GAG GAT GAG AGC GAG AAG AAG	To generate pDONR211-Hsp83C without stop codon
BP.InRintra.F	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC ATG ATT CAG TTG GCT CCA CTA	To generate pDONR211-InR^{intra}
BP.InRintra.stop.R	GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC TTA CGC CTC CCT TCC GAT G	
BP.InRintra.no-stop.R	GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC TTT CGC CTC CCT TCC GAT G	To generate pDONR211-InR^{intra} without stop codon
BP.Cdc37.F	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC ATG GTG GAC TAC AGC AAG TGG	To generate pDONR211-Cdc37
BP.Cdc37.stop.R	GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC TCA GTC AAC GTC CTC GGT GCT GAC G	
BP.Cdc37.no-stop.R	GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC GTC AAC GTC CTC GGT GCT GAC GCC A	To generate pDONR211-Cdc37 without stop codon
BP.2HA.F	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT GGA TAT CCG T	To generate pDONR221-2xHA without stop codon
BP.2HA.no-stop.R	GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC TGC ATA GTC AGG CAC GTC ATA CGG ATA TCC AGC GTA ATC T	
BP.3Myc.F	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC ATG GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG GGA GAG CAG AAG CTA ATA TCT GAG GAA G	To generate pDONR221-3xMyc without stop codon
BP.3Myc.no-stop.R	GGGG AC CAC TTT GTA CAA GAA AGC TGG GTC TAG ATC TTC TTC CGA AAT AAG TTT CTG TTC TCC GAG GTC TTC CTC AGA TAT TAG CTT C	
Modifier pD221 F	GGGG ACAAGTTTGTACAAAAAAGCAGGCTTC GGTACCGCTGAAACGAAGTTAAACTTTGAGGT GTACGGGTAAGTATTAGAAAGCAGGACTAAAC G	To generate pDONR221-Modifier to remove RfB cassette from BiFC, PAHW and PAMW constructs
Modifier pD221 R	GGGG ACCACTTTGTACAAGAAAGCTGGGTC AAGCTTCTATTCAATGGTCCGGCGGCCGACGAC ATGAGGATATGGTCGTTTAGTCCTGCTTTCTAA	

F: forward primer; R: reverse primer

Table S3: List of primers used for qPCR

Genes	**Primer sequence (5' to 3')**	
Hsp83-1	Forward	TCTACAAATCCCTGACCAAC
	Reverse	GTTCTTGCTGAACTGGTCAT
Hsp83-2	Forward	GGTGTGCGTCGTAACAACAAGC
	Reverse	CATCAGCTGAGCAATCTCAGCCT
Cdc37	Forward	CACTCACCCAAACATAGACA
	Reverse	TTTTCGAGCTCTTTCTTCAG
InR-1	Forward	GAATGGATCGTCTGACAAAT
	Reverse	GCGACTTCTTAAAACTGGTG
InR-2	Forward	CTCAGCCATACCAGGGACTTT
	Reverse	CTCTCCATAACACCGCCATC
InR-3	Forward	GTCACAATATTTTGCGAACA
	Reverse	TTGGCCAGTAGGATAAAGAG
Akt	Forward	ACCTACCGTTTGTTCTTCAG
	Reverse	TAACCCATCAGTCTTCCATC
4E-BP	Forward	CTCCTGGAGGCACCAAACTTATC
	Reverse	TTCCCCTCAGCAAGCAACTG
Actin5C	Forward	CAGATCATGTTCGAGACCTTCA
	Reverse	TCATGATGGAGTTGTAGGTGGT
GADPH1	Forward	ATGACGAAATCAAGGCTAAG
	Reverse	GAGTAACCGAACTCGTTGTC
GADPH2	Forward	ATGAAATTAAGGCCAAGGTT
	Reverse	GAGTAGCCAAACTCGTTGTC
G6DPH	Forward	ACGAGCAGAAGAAGTACGAG
	Reverse	GATGTTGACAGTCACCTCCT
RPII215	Forward	ACGAGCGATTAATGAAAAAG
	Reverse	TCTGCACATTCCAGATCATA


A
hsp83e6A
hsp83-BAC; hsp83e6A
hsp83j5c2
hsp83-BAC; hsp83j5c2
Mira
Dpn/Mira/CD8
EdU
Dpn/EdU/CD8
B
24h ALH
48h ALH
72h ALH
EdU/Dpn
Control
hsp83 RNAi
C
Mira/Dpn
Control
hsp83 RNAi
24h ALH
48h ALH
72h ALH
D
NSC% without EdU incoporation
Control
hsp83 RNAi
100
75
50
25
0
***
24h ALH
48h ALH
72h ALH
E
NSC% with cellular extensions
40
30
20
10
0
F
EdU
Dpn
EdU/Dac/Dpn
Control
hsp83 RNAi
G
Control
cdc37e4
cdc37-BAC; cdc37e4D
Mira
Dpn/Mira/CD8
H
NSC% with cellular extensions
Control
cdc37e4D
cdc37-BAC cdc37e4D

SUPPLEMENTAL FIGURE LEGNEDS

Figure S1. Hsp83 and its co-chaperone Cdc37 contribute to NSC reactivation

(A) Larval NSC clones of *hsp83*^{e6A}, *hsp83*^{e6A} with *hsp83*-BAC, *hsp83*^{j5C2}, *hsp83*^{j5c2} with *hsp83*-BAC, at 96h ALH were stained for GFP (green), Dpn (blue) and Mira (red) or labeled with GFP (green), Dpn (blue) and EdU (red). NSC lineages were marked with CD8-GFP. White arrows point to NSCs and yellow arrowheads point to cellular extensions of NSCs. Clone outline is indicated by white dotted lines. Scale bars, 5 μm. (B) Larval brains of control (UAS-*dicer2*) and *hsp83* RNAi (VDRC#108568) under the control of *insc*-Gal4 at 24h, 48h and 72h ALH were labeled with EdU (red) and Dpn (green). Yellow dotted boxes indicate the region of zoomed-in images. Central brain is to the left of white dotted lines. Scale bars, 30 μm for whole brain lobe and 5 μm for single cell. (C) Larval brains of control (UAS-*dicer2*) and *hsp83* RNAi (VDRC#108568) induced with *insc*-Gal4 at 24h, 48h and 72h ALH were stained for Mira (green) and Dpn (blue). White arrows point to NSCs and yellow arrowheads point to cellular extensions of NSC. Scale bars, 5 μm. (D, E) Quantification of EdU incorporation (D) and cellular extensions (E) for control and *hsp83* RNAi in (C). Data are presented as mean ± SD. (F) Larval brains of control (UAS-*dicer2*) and *hsp83* RNAi (VDRC#108568) induced with *insc*-Gal4 at 24h ALH were stained for Dac (green), EdU (red) and Dpn (blue). Arrows point to MB NSCs. Scale bars, 10 μm. (G) Larval NSC clones of wild-type control, *cdc37*^{e4D}, and *cdc37*^{e4D} with *cdc37*-BAC at 96h ALH were stained for GFP (green), Dpn (blue) and Mira (red). NSC lineages were marked with CD8-GFP. White arrows point to NSCs and yellow arrowheads point to cellular extensions of NSC. Clone outline is indicated by white dotted lines. Scale bars, 5 μm. (H) Quantification of cellular extensions for control, *cdc37*^{e4D}, and *cdc37*^{e4D} with *cdc37*-BAC in (G). Data are presented as mean ± SD (D, E). Statistical analyses were done comparing between two different genotypes using two-tailed Student's *t*-test (D, E). ***$P < 0.001$.

Figure S2

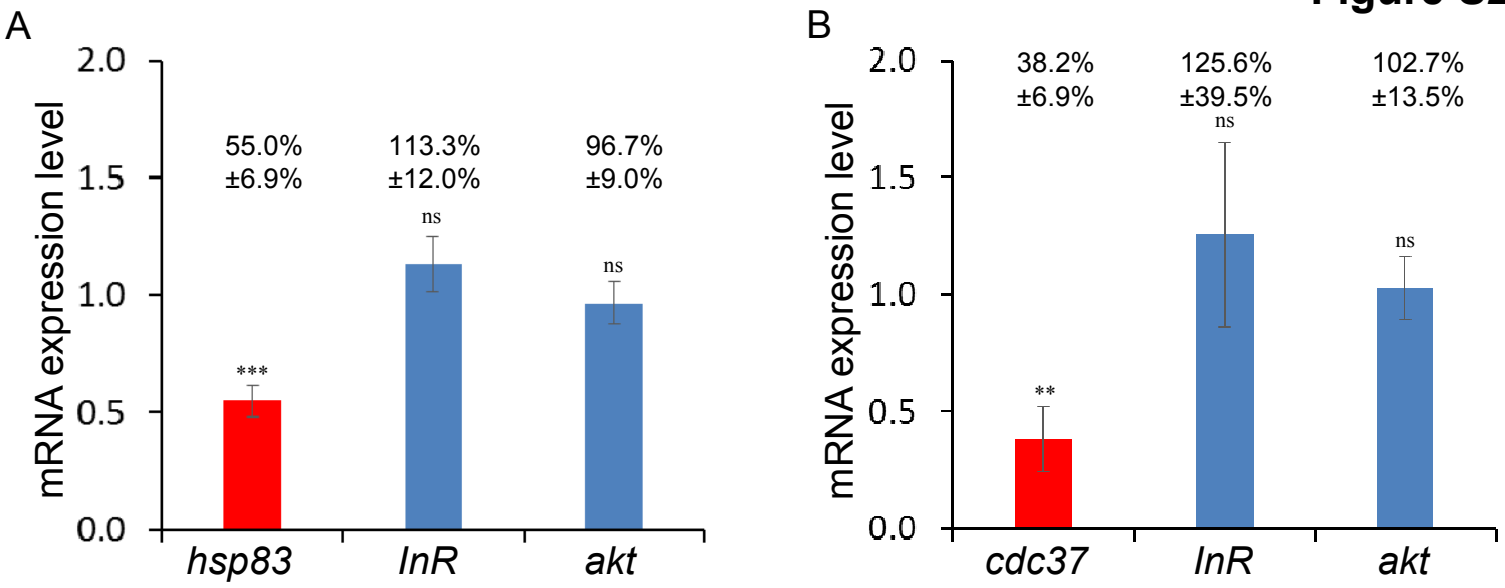
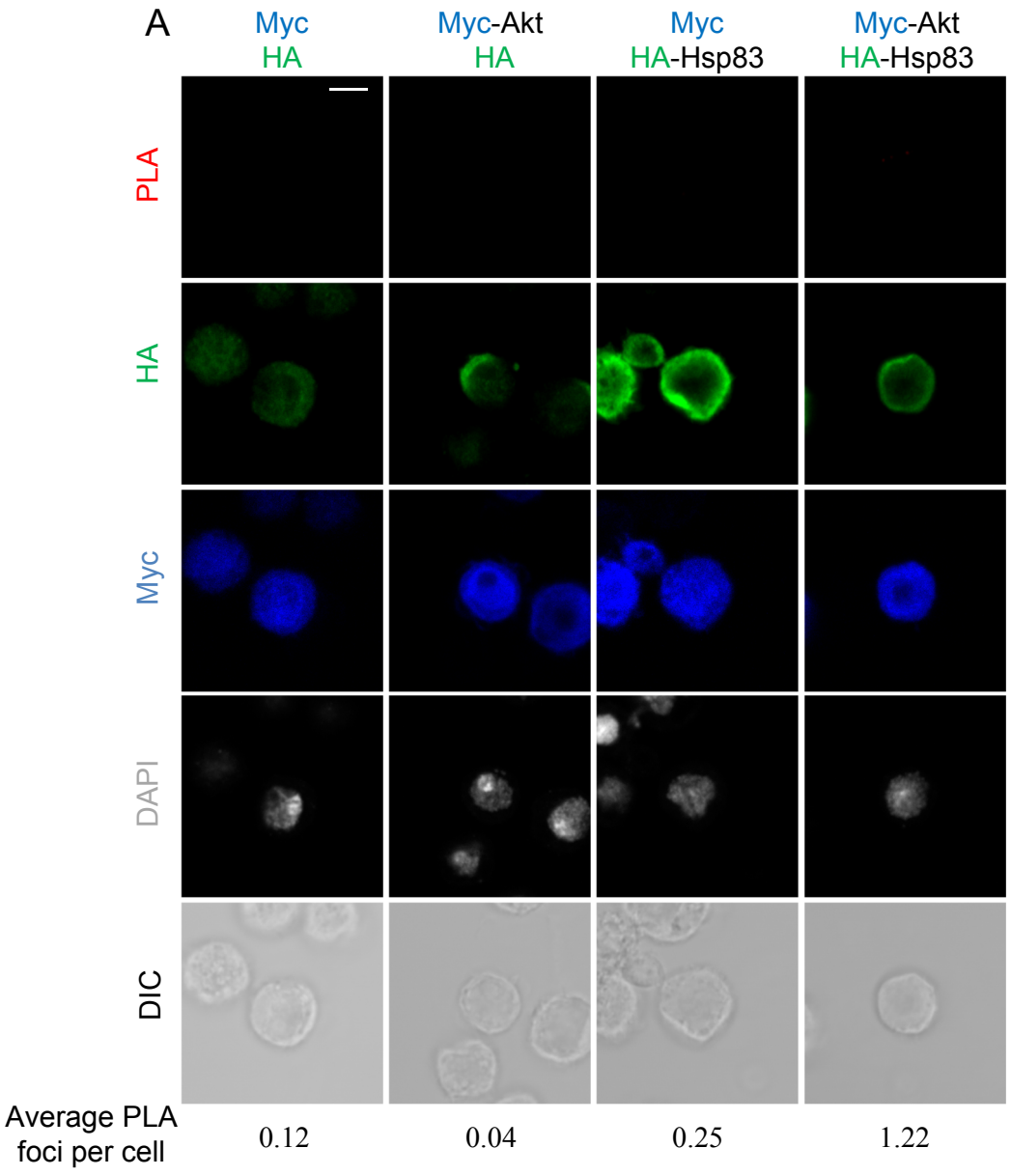

A
mRNA expression level
2.0
1.5
1.0
0.5
0.0
55.0%
±6.9%
113.3%
±12.0%
96.7%
±9.0%
***
ns
ns
hsp83
lnR
akt
B
mRNA expression level
2.0
1.5
1.0
0.5
0.0
38.2%
±6.9%
125.6%
±39.5%
102.7%
±13.5%
**
ns
ns
cdc37
lnR
akt

Figure S2. mRNA levels of InR and akt remain unaltered upon *hsp83* and *cdc37* knockdown in larval brains

(A) qPCR analysis of *hsp83*, *InR* and *akt* in larval brains of control (UAS-*dicer2*) and *hsp83* RNAi (VDRC#108568) under *insc*-Gal4 at 72h ALH. n=3. Statistical analyses were done comparing between control (actin5C and GADPH1) and *hsp83* knockdown, using two-tailed Student's *t*-test. The mRNA levels of control were normalized to 1. ns: non-significant; ***$P < 0.001$. (B) qPCR analysis of *cdc37*, *InR* and *akt* in larval brains of control (UAS-*dicer2*) and *cdc37* RNAi (VDRC#110727; induced with *insc*-Gal4) at 72h ALH. n=3. The mRNA levels of internal control (actin5C and GADPH1) was normalized to 1. Statistical analyses were done comparing between control and *cdc37* knockdown using two-tailed Student's *t*-test. ns: non-significant; **$P<0.01$.

Figure S3


A
Myc
HA
Myc-Akt
HA
Myc
HA-Hsp83
Myc-Akt
HA-Hsp83
PLA
HA
Myc
DAPI
DIC
Average PLA foci per cell
0.12
0.04
0.25
1.22

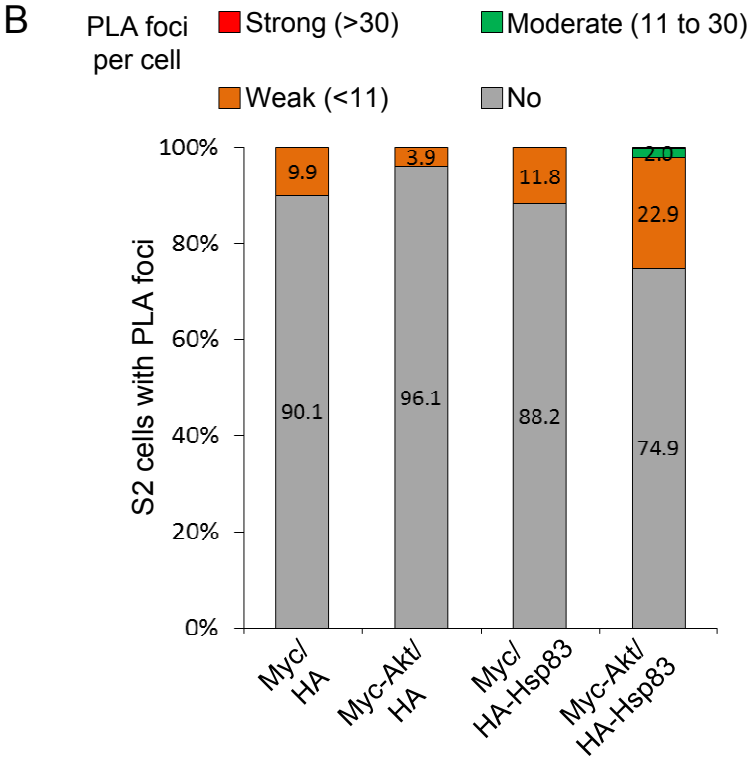
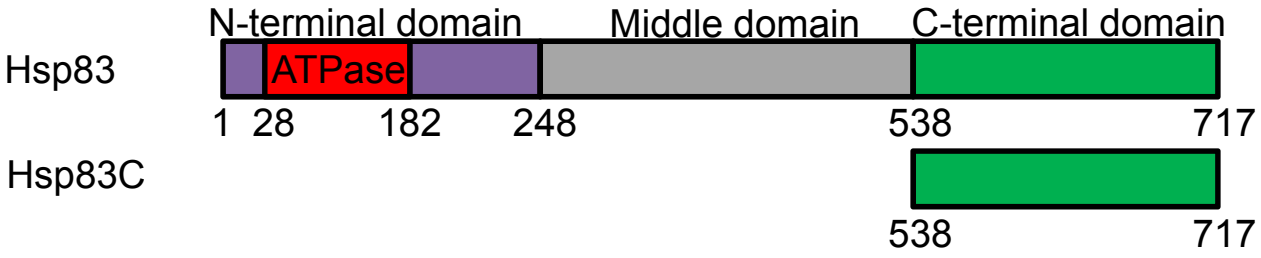

B
PLA foci per cell
Strong (>30)
Moderate (11 to 30)
Weak (<11)
No
S2 cells with PLA foci
100%
80%
60%
40%
20%
0%
9.9
90.1
3.9
96.1
11.8
88.2
2.0
22.9
74.9
Myc/ HA
Myc-Akt/ HA
Myc/ HA-Hsp83
Myc-Akt/ HA-Hsp83

Figure S3. Hsp83 does not interact with Akt

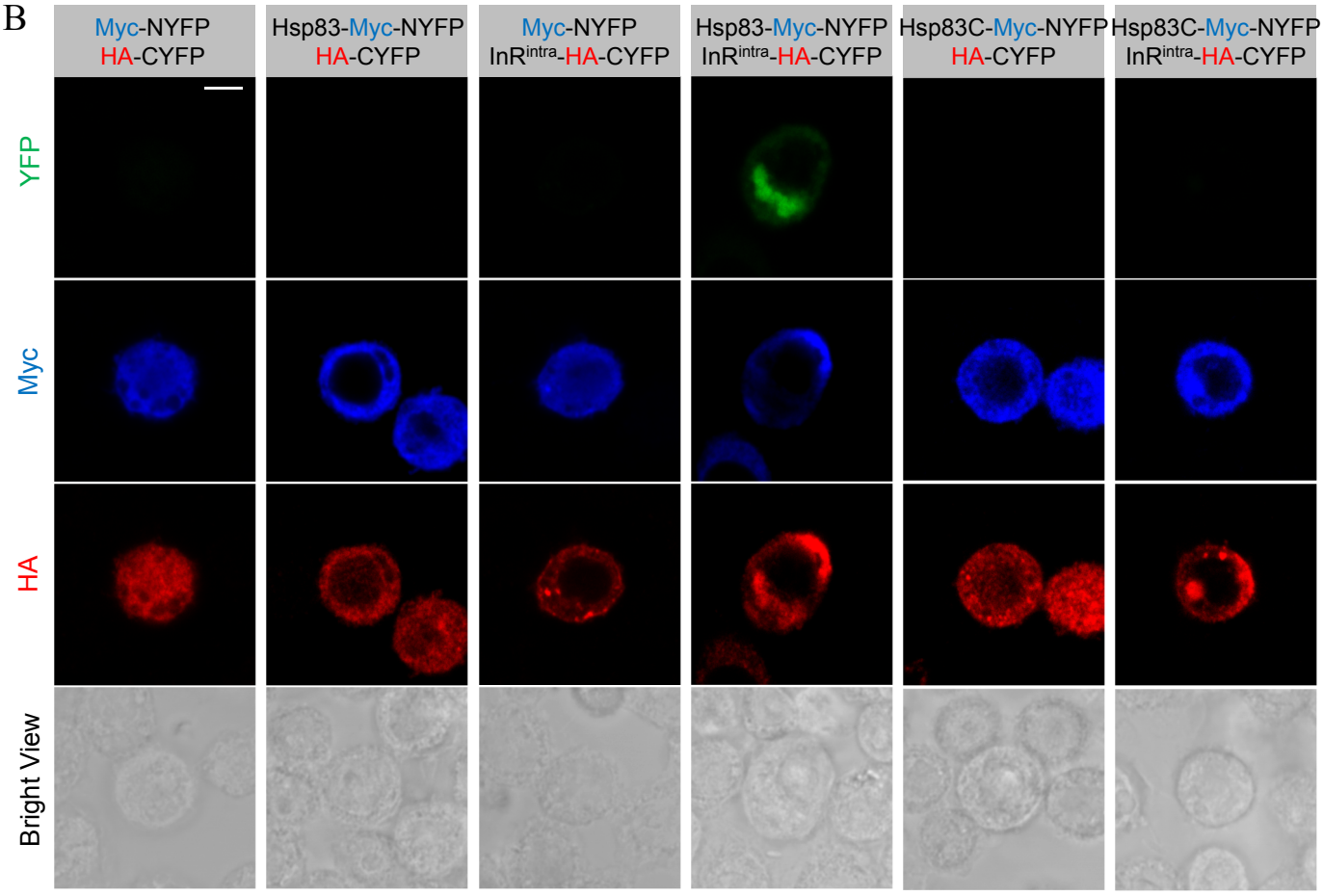
(A) *In situ* PLA assay between Hsp83 and Akt in S2 cells. S2 cells transfected with two of the indicated plasmids (Myc, HA, Myc-Akt and HA-Hsp83) were stained for HA (green), Myc (blue) and DNA (grey) and detected for PLA signal (red). Cell outline was shown by differential interference contrast (DIC) images. Scale bars, 4 μm. (B) Graphs showing the percentage of S2 cells with no PLA signal (grey), weak (≤10 foci, orange), moderate (11-30 foci, green) and strong (>30 foci, red) PLA signals for (A).

Figure S4

A


N-terminal domain
Middle domain
C-terminal domain
Hsp83
ATPase
1
28
182
248
538
717
Hsp83C
538
717

B


Myc-NYFP
HA-CYFP
Hsp83-Myc-NYFP
HA-CYFP
Myc-NYFP
InR^intra-HA-CYFP
Hsp83-Myc-NYFP
InR^intra-HA-CYFP
Hsp83C-Myc-NYFP
HA-CYFP
Hsp83C-Myc-NYFP
InR^intra-HA-CYFP
YFP
Myc
HA
Bright View

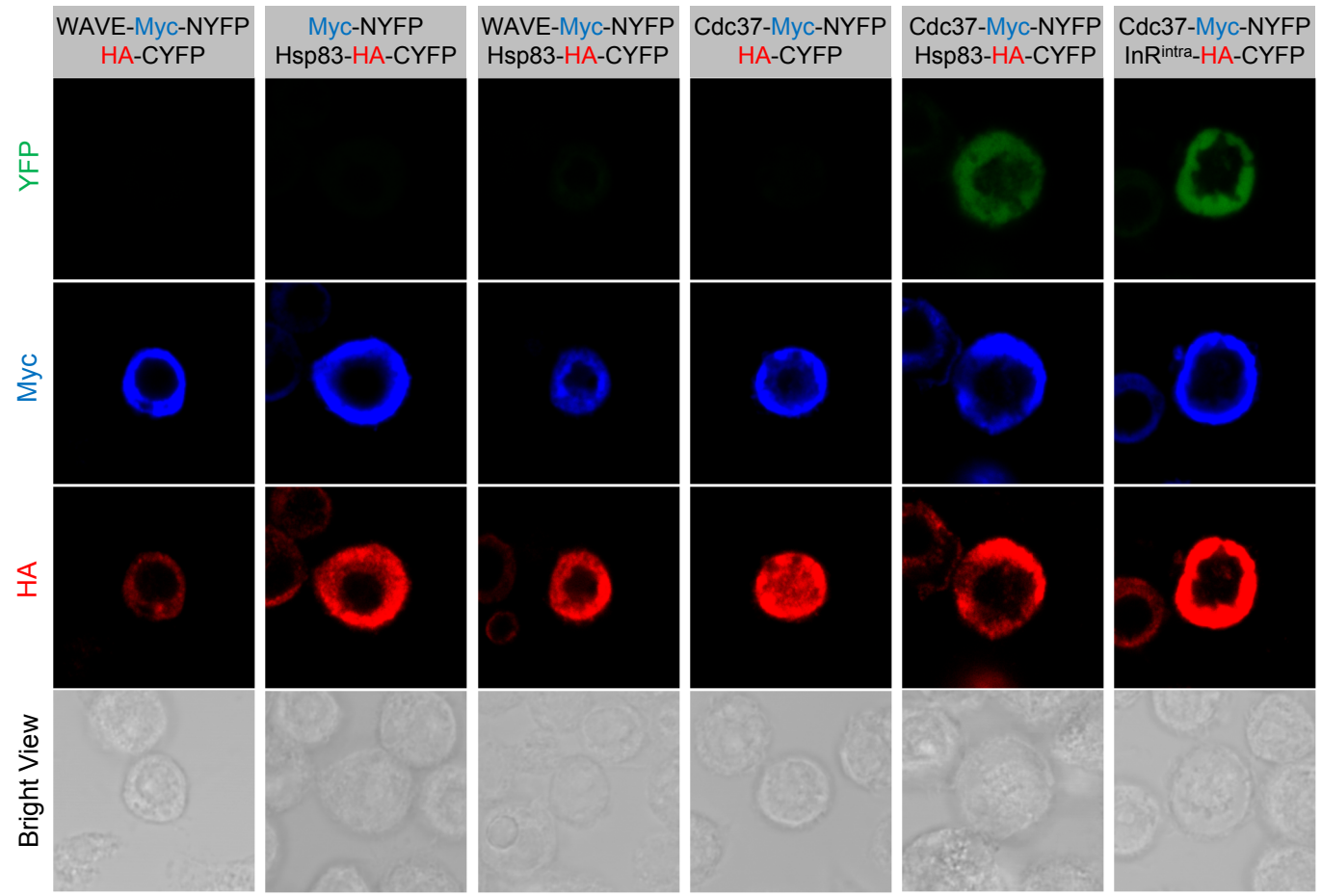
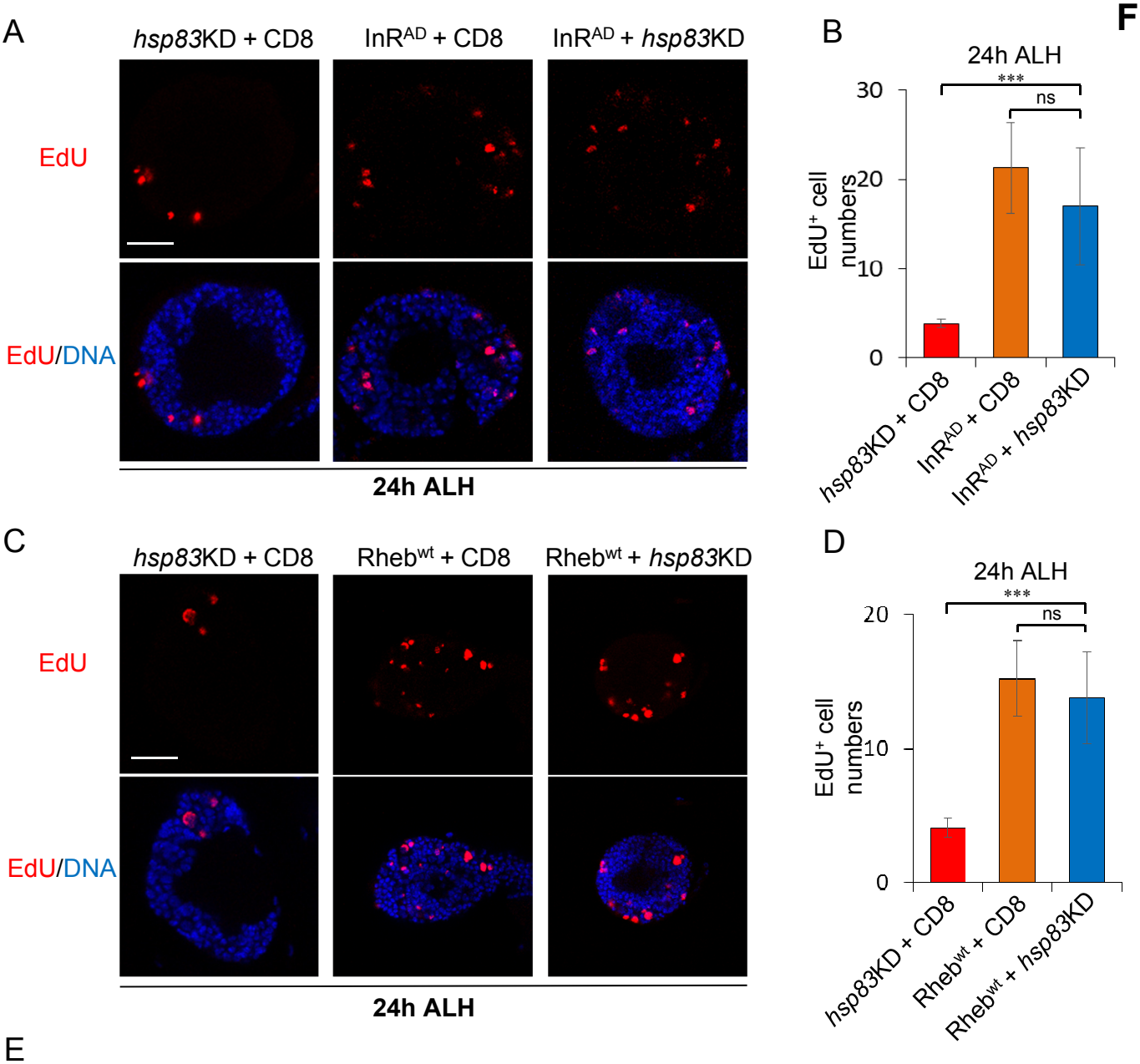

WAVE-Myc-NYFP
HA-CYFP
Myc-NYFP
Hsp83-HA-CYFP
WAVE-Myc-NYFP
Hsp83-HA-CYFP
Cdc37-Myc-NYFP
HA-CYFP
Cdc37-Myc-NYFP
Hsp83-HA-CYFP
Cdc37-Myc-NYFP
InR^intra-HA-CYFP
YFP
Myc
HA
Bright View

Figure S4. BiFC assays for Hsp83, Cdc37 and InR^{intra} interaction in S2 cells

(A) A schematic representation of the Hsp83C fragment. Hsp83C contains only the C-terminal domain of Hsp83 (538-717 amino acids). (B) Hsp83, Cdc37 and InR^{intra} interact in BiFC assays. To test the interaction between Hsp83 and InR^{intra}, UAS-Hsp83-Myc-NYFP and UAS-InR^{intra}-HA-CYFP were co-transfected into S2 cells, stained for Myc (blue) and HA (red) and detected for YFP fluorescence (green). Plasmids used for negative controls were UAS-Hsp83C-Myc-NYFP, UAS-HA-CYFP, UAS-Myc-NYFP and UAS-WAVE-Myc-NYFP. UAS-Cdc37-Myc-NYFP and UAS-Hsp83-HA-CYFP were used to test interaction between Cdc37 and Hsp83. Expression was induced by *actin*-Gal4. Scale bars, 4 μm.

Figure S5


A
hsp83KD + CD8
InR^AD + CD8
InR^AD + hsp83KD
EdU
EdU/DNA
24h ALH
B
24h ALH
***
ns
EdU+ cell numbers
30
20
10
0
hsp83KD + CD8
InR^AD + CD8
InR^AD + hsp83KD
C
hsp83KD + CD8
Rheb^wt + CD8
Rheb^wt + hsp83KD
EdU
EdU/DNA
24h ALH
D
24h ALH
***
ns
EdU+ cell numbers
20
10
0
hsp83KD + CD8
Rheb^wt + CD8
Rheb^wt + hsp83KD

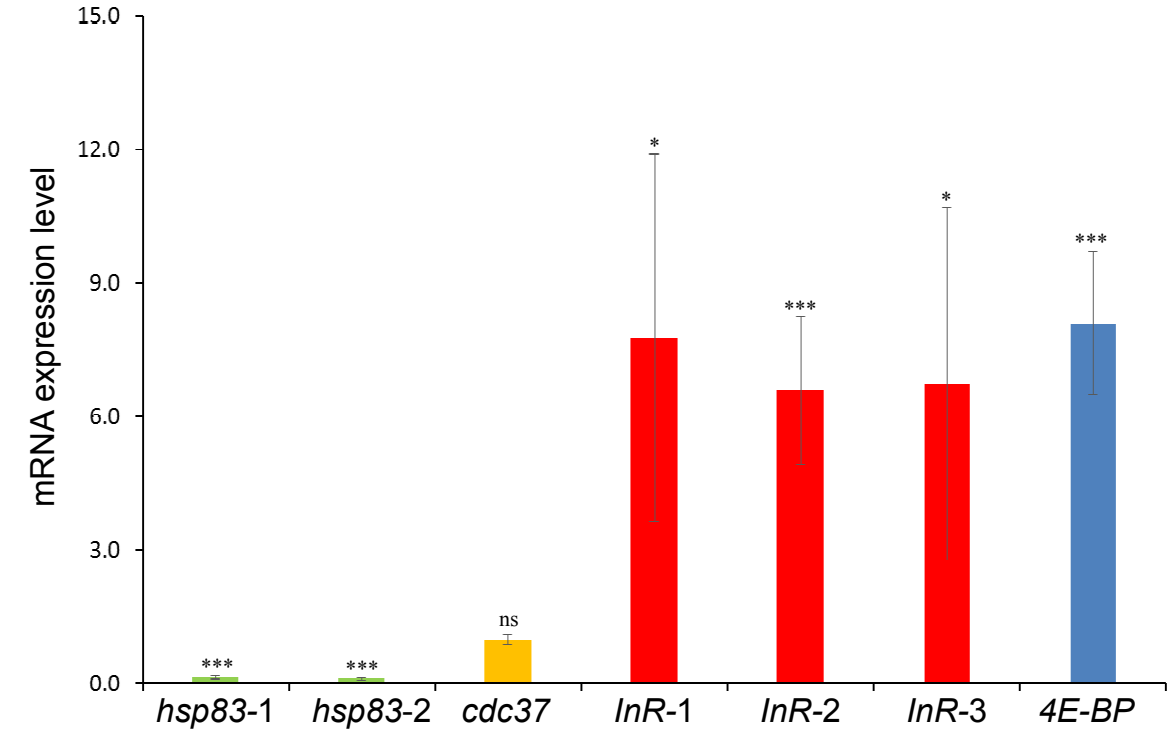

E
mRNA expression level
15.0
12.0
9.0
6.0
3.0
0.0
***
***
ns
*
***
*
***
hsp83-1
hsp83-2
cdc37
InR-1
InR-2
InR-3
4E-BP

Figure S5. Hsp83 functions upstream of InR/PI3K/Akt pathway during NSC reactivation

(A, B) Larval brains overexpressing InR^{AD} (BDSC#8440) with *hsp83* knockdown (VDRC#108568) under *insc*-Gal4 at 24h ALH in sucrose-only food were labeled with EdU (red) and DNA (blue). The control used was InR^{AD} overexpression with UAS-CD8-GFP. Quantifications are presented in (B) as mean ± SD. *hsp83* knockdown, 3.9 ± 0.5 (n=14) EdU^+ cells per brain hemisphere at 24h ALH. Statistical analyses were done comparing between InR^{AD} overexpression and InR^{AD} overexpression with *hsp83* knockdown using two-tailed Student's *t*-test. ns: non-significant. Scale bars, 15 μm. (C, D) Larval brains overexpressing $Rheb^{wt}$ (BDSC#9689) with *hsp83* knockdown (VDRC#108568) or with UAS-CD8-GFP (control) under *insc*-Gal4 at 24h ALH in sucrose-only food were labeled with EdU (red) and DNA (blue). Data are presented in (D) as mean ± SD. *hsp83* knockdown, 4.1 ± 0.7 (n=13) EdU^+ cells per brain hemisphere at 24h ALH. Statistical analyses were done comparing between $Rheb^{wt}$ overexpression and $Rheb^{wt}$ overexpression with *hsp83* knockdown using two-tailed Student's *t*-test. ns: non-significant. Scale bars, 15 μm. (E) qPCR analysis of *hsp83*, *cdc37*, *InR* and *4EBP* in whole larvae of control (*yellow-white*, *yw*, fed with normal food) and nutritional restricted condition (*yw* in sucrose-only) at 24h ALH. The mRNA levels of *hsp83* were accessed by two different pairs of primers, while *InR* mRNA level were measured by three different pairs of primers. In sucrose food, the mRNA level of *cdc37* changes to 97.3%±10.6% (n=4) compared to control, while the mRNA level of *4E-BP* was 808.7%±161.8% (n=4). Three different pairs of primers showed the mRNA levels of *InR* were 776.3%±413.5% (n=4), 658.8%±167.3% (n=4) and 673.7%±395.4% (n=4) respectively compared with control. Statistical analyses were done comparing between control (actin5C, GADPH1, GADPH2, G6DPH and RPII215) and nutritional restricted condition, using two-tailed Student's *t*-test. The mRNA levels of control were normalized to 1. ns: non-significant; $*P<0.05$; $***P < 0.001$.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Immunochemistry

Larval brains were dissected in phosphate-buffered saline (PBS) and fixed for 22 minutes in 0.3% PBS-Triton (PBT) with 4% EM grade formaldehyde (methanol-free). Fixed brains were then washed three times with 0.3% PBT for 10 minutes each. After washing, brains were blocked with 3% bovine serum albumin (BSA) in 0.3% PBT for 45 minutes and then incubated with primary antibody diluted in 3% BSA overnight at 4°C. Following two more washings (10 minutes each), larval brains were incubated with secondary antibody diluted in 0.3% PBT for 90 minutes. DNA was labelled by ToPro-3 (1:5000, Invitrogen, Cat#: T3605) or DAPI (1:1500, Molecular Probes, Cat#: D1306). Larval brains were mounted in Vector shield (Vector Laboratory) for Confocal microscopy. Samples were analyzed with a Zeiss LSM710 Confocal microscope and processed with Adobe Photoshop CS6.

Primary antibodies used were: guinea pig anti-Dpn (1:1000, J Skeath), rat anti-CD8 (1:250, Invitrogen, Cat#: MCD0800), rabbit anti-GFP (1:500, Molecular Probes, Cat#: A21311), mouse anti-Mira (1:40, F. Matsuzaki), mouse anti-Dac (1:5, DSHB, Cat#: mAbdac2-3), rabbit anti-Akt (1:100; Cell signaling, Cat#: 4691), mouse anti-Myc (1:200, abcam, Cat#: ab32) and rabbit anti-HA (1:100, Sigma, Cat#: H6908).

EdU pulse-chase analysis

Larvae were fed with standard food supplemented with 0.2mM EdU from *Click*-iT® EdU Alexa Fluor® 555 *Imaging Kit* (Invitrogen) for 4 hours prior to dissection. The dissected larval brains were then fixed with 4% EM grade formaldehyde (in 0.3% PBT) for 22 minutes. The brains were washed three times and blocked with 3% BSA in PBT for 1 hour. Following blocking, EdU was detected by Alexa Fluor azide according to the Click-iT EdU protocol (Invitrogen). The brains were then washed twice and blocked with 3% BSA again for 30 minutes, and processed by immunohistochemistry.

Deprivation of dietary amino acids in larvae

To deprive larvae of dietary amino acids, larvae were transferred to 5% sucrose, 1% Agar in PBS within 2 hours after larval hatching. Larvae were raised at appropriate temperature at various time points prior to further analysis.

Quantification of cellular extensions and EdU incorporation

Following immunostaining, *Drosophila* larval brains were placed on microscope slides with their dorsal side up. The confocal z-stacks were taken from the dorsal surface to the deep layers of the larval brains (24-30 slides per z-stack with 3 µm intervals). Quantification was carried out using ImageJ or Zen software.

Generation of plasmids and transgenic flies

Plasmid constructs were generated using Gateway® BP Clonase® II Enzyme mix (Invitrogen). cDNA clones used in this study were AT20544 (*hsp83*) and FI07667 (*cdc37*) (Drosophila Genomics Resource Centre [DGRC]). pENTR-InR containing the full length of InR cDNA was kindly provided by Dr. H. Stocker. InR^{intra} domain was referred from Dr. H. Stocker's published research (Almudi et al., 2013). Hsp83C contains only the C-terminal domain of Hsp83, from amino acid 538-717. Briefly, desired regions of genes were amplified by polymerase chain reaction (PCR) and inserted into a Gateway entry vector pDONR221 (Invitrogen) using Gateway BP Clonase II Enzyme mix. They were subsequently inserted into Gateway destination vectors (PAMW, PAHW, pUAST-CYFP-HA-RfB, pUAST-NYFP-Myc-RfB, pUAST-RfB-HA-CYFP and pUAST-RfB-Myc-NYFP) by LR recombination using Gateway LR Clonase II enzyme mix. Primers used for generating entry clones were listed in Table S2.

Gateway destination vectors pUAST-CYFP-HA-RfB (RfB, reading frame cassette B, a Gateway recombination cassette for LR recombination), pUAST-NYFP-Myc-RfB, pUAST-RfB-HA-CYFP and pUAST-RfB-Myc-NYFP were kindly provided by Dr S. Bogdan. To remove the Gateway recombination cassettes that contains multiple STOP codons and allow the expression of half-YFP in the control vectors, 2xHA and 3xMyc sequence were inserted into pDONR221 to generate Gateway entry clones pDONR221-2xHA and pDONR221-3xMyc respectively. Next, 2xHA and 3xMyc from entry clones were inserted into destination vectors pUAST-RfB-HA-CYFP and pUAST-RfB-Myc-NYFP respectively to generate C-terminal tagging control plasmids pUAST-HA-CYFP and pUAST-Myc-NYFP (primers listed in Table S2). For N-terminal tagging controls, a 99base pair sequence containing multiple stop codons was inserted (Koe C., unpublished data; Table S2) into destination vectors to remove Gateway recombination cassettes. Sequences inserted were:

GCTGAAACGAAGTTAAACTTTGAGGTGTACGGGTAAGTATTAGAAAGCAGGACTAAACGACCATA

TCCTCATGTCGTCGGCCGCCGGACCATTGAATAG.

In brief, the sequences were inserted into pDONR211 to generate entry clones first (Modifier pDONR211) and then cloned into destination vectors PAMW, PAHW, pUAST-CYPF-HA-RfB and pUAST-NYFP-Myc-RfB. The plasmids generated were N-terminal tag control Myc (PAMW-*actin5C*-6xMyc), HA (PAHW-*actin5C*-3xHA), pUAST-CYFP-HA and pUAST-NYFP-Myc. To generate tagged constructs of Hsp83, Hsp83C, Cdc37, and InR^{intra}, the following entry clones were generated pDONR211-Hsp83, pDONR211-Cdc37, pDONR-InR^{intra} (with or without stop codon), and pDONR211-Hsp83C (without stop codon). Next, cDNA from entry clones was inserted into destination vectors (PAMW, PAHW, pUAST-RfB-HA-CYFP, and pUAST-RfB-Myc-NYFP). The plasmids generated were pUAST-Hsp83-Myc-NYFP, pUAST-Hsp83-HA-CYFP, pUAST-Hsp83C-Myc-NYFP, pUAST-Cdc37-Myc-NYFP, pUAST-Cdc37-HA-CYFP, pUAST-InR^{intra}-HA-CYFP, Myc-Hsp83, HA-Hsp83, Myc-Cdc37 and HA-InR^{intra}.

UAS-CYFP-HA, UAS-NYFP-Myc, UAS-Hsp83-Myc-NYFP, UAS-Cdc37-Myc-NYFP, UAS-Cdc37-HA-CYFP and UAS-InR^{intra}-HA-CYFP were generated by ΦC31-integrase system by BestGene, Inc. The stocks injected were BDSC#8622 yw; P{y[+t7.7]=CaryP}attP2 (chromosome III, 68A4).

Drosophila Hsp83 domain analysis

Full length of amino acid sequence of Hsp83 was obtained from Flybase and analyzed by Simple Modular Architecture Research Tool (SMART).

Drosophila genomic constructs and genomic rescue

Bacterial artificial chromosomes (BACs), CH322-129N17 for *hsp83* and CH322-35F18 for *cdc37*, covering from 10,000 bp upstream to 5,000 bp downstream of the genomic region of the genes, were obtained from the BACPAC Resources Center. Transgenic flies expressing the BAC clone were generated by ΦC31-integrase system by BestGene, Inc. The stocks injected were BDSC#8621 yw; P{y[+t7.7]=CaryP}attP1 (chromosome II, 55C4).

S2 cell culture and transient transfection

Drosophila S2 cells were cultured in Express Five serum free medium (Gibco), and supplemented with 2mM glutamine (Thermo Fisher Scientific). For *in vitro* bimolecular fluorescence complementation (BiFC), $1x10^6$ cells S2 cells were seeded directly to Poly-L-lysine (PLL)-coated cover slips (Iwaki). For PLA assay, $5x10^4$ cells cells were seeded to 8-well chamber slides (Lab-Tek, Cat#: 154941). Plasmids were transfected into S2 cells together using Effectene Transfection Reagent (QIAGEN). For *in vitro* bimolecular fluorescence complementation (BiFC), S2 cells were transfected with *actinC5*-Gal4 and the respectively BiFC constructs each at 200 ng per well. For PLA, the transfection amount for each plasmid is 50 ng.

Bimolecular fluorescence complementation (BiFC)

For *in vitro* BiFC, the expression of BiFC constructs was under the control of *actinC5*-Gal4, which was co-transfected with the plasmids. Coverslips coated with S2 cells were collected after 48 hours of transfection, S2 cells were rinsed with PBS and fixed with 4% EM-grade formaldehyde in 0.1% PBS-Triton (PBT) for 15 minutes followed by three rinses with 0.1% PBT. Fixed cells were blocked with 5% BSA (in 0.1% PBT) for 1 hour and incubated with primary antibodies diluted in 5% BSA for 2 hours at room temperature. Antibodies used for BiFC were mouse anti-Myc (1:2000, abcam, Cat#: ab32) and rabbit anti-HA (1:2000, Sigma, Cat#: H6908). Cells were then rinsed twice with 0.1% PBT and incubated with secondary antibody diluted in 0.1% PBT for 1 hour. Cells were rinsed with 0.1% PBT again for two times. Coverslips were then mounted onto glass slides using Vector shield (Vector Laboratory) for Confocal microscopy. pUAST-HA-CYFP, pUAST-Myc-NYFP, pUAST-Hsp83-Myc-NYFP, pUAST-Hsp83-HA-CYFP, pUAST-Hsp83C-Myc-NYFP, pUAST-Cdc37-Myc-NYFP, pUAST-InR^{intra}-HA-CYFP and pUAST-WAVE-HA-CYFP (Bogdan, S) were used for *in vitro* bimolecular fluorescence complementation (BiFC). For *in vivo* BiFC, the constructs were expressed under *insc*-Gal4 and followed by standard immunohistochemistry.

Proximity ligation assay (PLA)

The basis of PLA is as follows: primary antibodies bind to tagged proteins of interest are recognized by secondary antibodies conjugated with PLA probe PLUS and PLA probe MINUS; if two proteins of interest are present in close proximity (<40 nm), the connector oligos hybridize to PLA probes and are ligated by T4 ligases. Ligated oligos form a circular template and can be amplified with DNA polymerase. Amplified DNA is bound by

fluorescent-labeled complementary oligos, resulting fluorescent spots *in situ*. For proximity ligation assay (PLA), plasmids used were control Myc (PAMW-*actin5C*-6xMyc), control HA (PAHW-*actin5C*-3xHA), Myc-Hsp83, HA-Hsp83, Myc-Cdc37, HA-InR^{intra} and Myc-Akt (Li et al., 2014).

After 48 hours of transfection, chamber slides with S2 cells were rinsed with cold PBS three times and fixed with 4% EM-grade formaldehyde in PBS for 15 minutes. Cells were rinsed with cold PBS three times (2 minutes each time), blocked with 5% BSA (in 0.1% PBT) for 1 hour and incubated with primary antibodies diluted in 5% BSA (in 0.1% PBT) for 2 hours. Cells were then incubated with PLA probes followed by ligation and amplification according to manufacturer's protocol (Sigma-Aldrich). Cells were then incubated with the same primary antibodies again for 2 hours (diluted in 5% BSA in PBS). After rinses with PBS once, cells were incubated with secondary antibody diluted in PBS for 90 minutes. Cells were mounted in *in situ* Mounting Medium with DAPI (Sigma-Aldrich) for Confocal microscopy.

Protein extraction and immunoblotting

Larval brains dissected in PBS or whole larvae at 6h ALH were homogenized in RIPA buffer (50 mM Tris pH7.5, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS). Proteins were separated by SDS-PAGE and analyzed by western blotting.

Blots were probed with the following antibodies: mouse anti-Actin (1:5000; MP Biomedicals, Cat#: 08691001), rabbit anti-Akt (1:1500; Cell signaling, Cat#: 4691), rat anti-Hsp83 (1:1000; Abcam, Cat#: 13492) and rabbit anti-pInR (1:1000; Cell signaling, Cat#: 3021).

RNA extraction and qPCR analysis

RNA from larval brains or whole larvae was extracted using TRI reagent (Sigma-Aldrich) following the manufacturer's protocol. Reverse transcription was carried out using ProtoScript First Strand cDNA Synthesis kit (New England Biolabs, Inc.).

qPCR was performed using SsoFastTM EvaGreen® supermix (Bio-Rad)with different primer pairs. Primers were picked using Primer3 with default setting, except the following: Hsp83-2 (Sawarkar et al., 2012), InR-2 (Danielsen et al., 2014) and 4E-BP (Owusu-Ansah et al., 2013). The primers used were listed in Table S3.

The experiments were ran using Bio-Rad CFX96™ Real-Time PCR system. Program used was: 95°C for 3 second, 56°C for 30 second and plate read for 40 cycles. Melt curve: 65°C - 95°C with 0.5°C increment for 5 second followed with plate read. Results were analyzed using REST excel file.

Statistical analysis

Statistical analysis among different groups was performed by two-tailed unpaired Student's t-test, and a value of $P < 0.05$ was considered as statistical significant. In this work, ns (non-significant) indicates $P > 0.05$, * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$. All quantification data were shown as mean ± SD, except for MARCM clones that only percentages were calculated.

SUPPLEMENTAL REFERENCES

Almudi, I., Poernbacher, I., Hafen, E., and Stocker, H. (2013). The Lnk/SH2B adaptor provides a fail-safe mechanism to establish the Insulin receptor-Chico interaction. Cell communication and signaling : CCS *11*, 26.
Danielsen, E.T., Moeller, M.E., Dorry, E., Komura-Kawa, T., Fujimoto, Y., Troelsen, J.T., Herder, R., O'Connor, M.B., Niwa, R., and Rewitz, K.F. (2014). Transcriptional control of steroid biosynthesis genes in the Drosophila prothoracic gland by ventral veins lacking and knirps. PLoS genetics *10*, e1004343.
Li, S., Wang, C., Sandanaraj, E., Aw, S.S., Koe, C.T., Wong, J.J., Yu, F., Ang, B.T., Tang, C., and Wang, H. (2014). The SCFSlimb E3 ligase complex regulates asymmetric division to inhibit neuroblast overgrowth. EMBO reports *15*, 165-174.
Owusu-Ansah, E., Song, W., and Perrimon, N. (2013). Muscle mitohormesis promotes longevity via systemic repression of insulin signaling. Cell *155*, 699-712.
Sawarkar, R., Sievers, C., and Paro, R. (2012). Hsp90 globally targets paused RNA polymerase to regulate gene expression in response to environmental stimuli. Cell *149*, 807-818.