Cell Stem Cell, Volume *1*

Supplemental Data

Stage-Specific Differences in the Requirements for Germline Stem Cell Maintenance in the *Drosophila* Ovary

Halyna R. Shcherbata, Ellen J. Ward, Karin A. Fischer, Jenn-Yah Yu, Steven H. Reynolds, Chun-Hong Chen, Peizhang Xu, Bruce A. Hay, and Hannele Ruohola-Baker

Supplemental Results

Loss of *dicer-1* and *Mad* mutant adult GSCs

Since both Dicer-1 and Mad are required for adult GSC maintenance, we analyzed the process of GSC loss in these two mutant backgrounds. Interestingly, the extreme phenotypes for *Mad* and *dcr-1* GSC clones observed in late timepoints (S 3B,D) are somewhat different for the two mutants. While the extreme *dicer-1* mutant phenotypes show germaria with either very few germline cells (Figure 1D, S 1A-1C) or no germline cells (S 3B), in the *Mad* extreme phenotypes, large cysts often develop in the niche (S 3C-E). To further dissect the cause of these differences, we analyzed the earliest events that take place in GSC loss in each mutant. The *dicer-1* mutant GSCs leave the niche moving posteriorly while undergoing a stereotypic division pattern (S 3A). While *Mad* GSCs also undergo a stereotypic division pattern, they can initiate this differentiation process in an abnormal location, while still in the niche (S 3C-E). For example, in SC a four cell *Mad* mutant cyst is observed in the niche five days after adult clonal induction. Therefore, while both *dicer-1* and *Mad* GSCs have a maintenance problem, the manifestation is different. *dicer-1* GSCs leave the niche and differentiate outside the niche. While *Mad* germline stem cells can also leave the niche, 40% of the time GSCs initiate the differentiation process while still in the niche.

The target for TGF-β pathway control in GSCs is the transcriptional repression of Bam. We tested whether Bam protein was upregulated in *dicer-1* mutant GSCs; a potential cause for the observed maintenance defect. However, no obvious upregulation of BamC was observed in adult *dicer-1* GSC clones (S 1B), again supporting the notion that *dicer-1* and *Mad* adult GSC maintenance defects manifest themselves somewhat differently.

Supplementary Materials and Methods

QPCR analysis of microRNA levels in *dcr-2* mutants

Total RNA was prepared from 10 to 20 female whole flies using TRIzol (Invitrogen) and treated with DNaseI (Fermentas). 0.5μg of the extracted RNA was reversed transcribed into cDNA with Omniscript reverse transcriptase (Qiagen) and oligo dT primer. mRNA levels of RP49, a ribosomal protein, were tested by QPCR with SyberGreen master mix (Applied Biosystems) on ABI 7300 Real-time PCR system to

evaluate the total RNA levels in the sample with the following primers: Forward, ATGACCATCCGCCCAGCA; Reverse, TTGGGGTTGGTGAGGCGGAC (PCR fragment=436bp). The reaction were incubated in a 96 well plate at 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 sec and 60^{o}C for 60 sec. miRNA levels were quantified using TaqMan MicroRNA Assays (dme-miR-8 and dme-bantam, Applied Biosystems) according to the manufacturer's instruction using 10ng of total RNA on ABI 7300 Real-time PCR system.

We used following fly lines in the QPCR assays: *w-, +/+;Ly/TM3, FRT42Ddcr-2;Ly/TM3, +/+;FRT82Bdcr-1/TM3, FRT42Ddcr-2;FRT82Bdcr-1/TM3, FRT42D miR-8*$^{\Delta 1}$.

Table S1.
GSC maintenance depends on clonal induction stage in *dcr-1* and *Mad* mutants

Genotype	Clone induction stage		% germaria with clonal GSC			%GSC loss/day[1]
			Time point 1	Time point 2	Time point 3	
Control hsFLP;;FRT82B GFP/ FRT82B	Larval/Pupal	Exp.I	27.3%(5d) n=252	25.4%(8d) n=315	11.4%(14d) n=157	4.2±0.7
		Exp.II	24.4%(5d) n=41	18.8%(8d) n=32	15.0%(14d) n=40	
		Exp.III	23.1%(5d) n=117	22.0%(8d) n=83		
		Exp.IV	40.0%(5d) n=105	38.0%(8d) n=50	35.0%(21d) n=57	
dcr-1 hsFLP;;FRT82B GFP/ FRT82B dcr-1^{Q1147x}	Larval/Pupal	Exp.I	27.7%(5d) n=224	19.4%(8d) n=165	14.5%(14d) n=96	4.4±0.7
		Exp.II	27.0%(5d) n=48	24.2%(8d) n=33	20.0%(14d) n=54	
		Exp.III	19.6%(5d) n=112	19.0%(8d) n=57		
		Exp.IV	31.0%(5d) n=77	29.0%(8d) n=42	22.0%(21d) n=134	
Control hsFLP;GFP FRT40A/ FRT40A	Larval/Pupal	Exp.I	34.4%(7d) n=93		31.3%(14d) n=83	1.3
Mad^{12} hsFLP;GFP FRT40A/ Mad^{12} FRT40A	Larval/Pupal	Exp.I	32.7%(7d) n=107		24.8%(14d) n=129	2.7±0.6
		Exp.II	38.9%(7d) n=79		36.6%(14d) n=134	
		Exp.III	20.1%(7d) n=214		14.3%(14d) n=140	
		Exp.IV	17.4%(7d) n=132		14.4%(12d) n=178	
Mad^{12}; UASDl hsFLP;GFP FRT40A/FRT40A Mad^{12}; UASpDl/nanos Gal4	Larval/Pupal	Exp.I	19.1%(7d) n=68		14.6%(14d) n=41	0.2±1.8
		Exp.II	18.6%(7d) n=97		22.4%(14d) n=76	
Control *hsFLP;FRT42D GFP/ FRT42D*	Larval/Pupal	Exp.I		18.3%(8d) n=224	13.7%(15d) n=300	2.8±0.5
		Exp.II		19.2%(8d) n=245	16.4%(15d) n=329	
miR-8$^{\Delta 1}$ hsFLP;FRT42D GFP/ FRT42D miR-8$^{\Delta 1}$	Larval/Pupal	Exp.I		18.9%(8d) n=175	15.9%(15d) n=301	1.6±0.5
		Exp.II		20.2%(8d) n=233	18.8%(15d) n=425	
ban$^{\Delta 1}$ hsFLP;;GFP FRT80B / ban$^{\Delta 1}$ FRT80B	Larval/Pupal	Exp.I	11.3%(5d) n=115	8.7%(10d) n=126	6.5%(14d) n=170	6.1±0.3
		Exp.II		7.4%(10d) n=135	5.4%(14d) n=147	
		Exp.III		8.7%(8d) n=115	4.9%(15d) n=163	
		Exp.IV		11.1%(8d) n=99	5.6%(15d) n=107	
iswi hsFLP;FRT42B GFP/ FRT42D iswi^{2}	Larval/Pupal	Exp.I	11.9%(7d) n=188		2.4%(14d) n=168	10.3±0.3
		Exp.II	17.0%(7d) n=182		4.7%(14d) n=170	

Control ***hsFLP;;FRT82B GFP/ FRT82B***	**Adult**	**Exp.I**	**23.1%(4d) n=248**	**21.2%(7d) n=170**	**20.9%(10d) n=144**	**2.0±0.7**
		Exp.II	**31.4%(3d) n=137**	**31.2%(5d) n=231**	**34.1%(10d) n=231**	
		Exp.III	**27.6%(5d) n=182**		**26.5%(10d) n=215**	
		Exp.IV	**32.5% (6d) n=123**		**26.3% (9d) n=156**	
dcr-1 hsFLP;;FRT82B GFP/ FRT82B dcr-1^{Q1147x}	**Adult**	**Exp.I**	**30.4%(4d) n=125**	**15.5%(7d) n=58**		**11.9±1.1**
		Exp.II	**23.2%(3d) n=289**	**16.8%(5d) n=190**	**12.7%(10d) n=189**	
		Exp.III	**22.8%(5d) n=281**		**8.3%(10d) n=319**	
		Exp.IV	**20.2%(3d) n=79**	**13.9%(6d) n=79**	**8.3%(9d) n=121**	
Control ***hsFLP;GFP FRT40A/ FRT40A***	**Adult**	**Exp.I**	**31.0%(7d) n=84**		**29.9%(14d) n=67**	**0.5**
Mad^{I2} hsFLP;GFP FRT40A/ Mad^{I2}FRT40A	**Adult**	**Exp.I**	**40.4%(5d) n=94**	**21.2%(9d) n=108**	**14.5%(14d) n=145**	**11±1.0**
		Exp.II	**21.9%(5d) n=155**		**1.6%(12d) n=62**	
		Exp.III	**32.4%(7d) n=102**		**6.8%(14d) n=29**	
Control hsFLP;;GFP FRT80B / FRT80B **or** ***hsFLP;;arm lacZ FRT80B/ GFP FRT80B***	**Adult**	**Exp.I**	**9.9%(4d) n=91**	**10.8%(9d) n=65**		**-1.6±2.3**
		Exp.II	**7.1%(4d) n=70**	**8.2%(6d) n=98**		
		Exp.III	**21.1%(5d) n=95**		**13.0%(14d) n=92**	
ban$^{\Delta 1}$ hsFLP;;GFP FRT80B / ban$^{\Delta 1}$ FRT80B	**Adult**	**Exp.I**	**17.1%(4d) n=88**	**3.0%(9d) n=66**		**14.1±2.8**
		Exp.II	**13.2%(4d) n=121**	**6.7%(6d) n=89**		
		Exp.III	**6.9%(5d) n=72**		**2.5%(14d) n=80**	
		Exp.IV		**5.5%(7d) n=128**	**1.9%(15d) n=162**	
Control ***hsFLP;FRT42D GFP/ FRT42D***	**Adult**	**Exp.I**		**18.1%(8d) n=198**	**18.6%(15d) n=313**	**1.1±1.1**
		Exp.II		**16.1%(8d) n=174**	**13.1%(15d) n=275**	
miR-8$^{\Delta 1}$ hsFLP;FRT42D GFP/ FRT42D miR-8$^{\Delta 1}$	**Adult**	**Exp.I**		**28.6%(8d) n=256**	**27.5%(15d) n=335**	**1.3±0.6**
		Exp.II		**27.8%(8d) n=266**	**23.7%(15d) n=354**	
yorkie hsFLP;FRT42D GFP/ FRT42D yki^{b5}	**Adult**	**Exp.I**	**33.0%(5d) n=297**	**33.6%(9d) n=345**	**31.9%(15d) n=345**	**0.2±0.5**

n = number of germaria analyzed

( ) - days after heat shock induction in parentheses

[1] – average germline stem cell loss per day ± standard error

GSC loss per day = (% of clonal GSC at timepoint 1 - % of clonal GSC at timepoint 2)^{x}100%
% of clonal GSC at timepoint 1/elapsed time

Table S2.

The microRNA and TGF-ß pathways interact to maintain GSCs during development

Genotype	Clonal induction stage		% germaria with clonal GSC			
			Time point 1	Time point 2	Time point 3	%GSC loss/day[1]
Control dcr-1 hsFLP;; FRT82B dcr-1^{Q1147X}/ FRT82B GFP	Larval/ Pupal	Exp.I	27.0%(5d) n=48	24.2%(8d) n=33	20.0%(14d) n=54	3.2±0.3
Control CyO/+;dcr-1 hsFLP; CyO/+; FRT82B dcr-1^{Q1147X}/ FRT82B GFP	Larval/ Pupal	Exp.I	25.8%(6d) n=116		22.6%(12d) n=106	2.5±0.4
		Exp.II	22.0%(6d) n=36	20.0%(9d) n=15		
Control iswi/+;dcr-1 hsFLP; iswi^{2}/+; FRT82B dcr-1^{Q1147X}/ FRT82B GFP	Larval/ Pupal	Exp.I	11.5%(7d) n=227		7.6%(14d) n=173	4.9
Mad/+;dcr-1 hsFLP; Mad^{12} FRT40A/+; FRT82B dcr-1^{Q1147X}/ FRT82B GFP	Larval/ Pupal	Exp.I	23.9%(6d) n=142		9.7%(12d) n=113	14.1±1.1
		Exp.II	28.8%(6d) n=118	31.3%(9d) n=76	0%(12d) n=15	
		Exp.III		6.5%(12d) n=31	1.1%(18d) n=89	
		Exp.IV		10.4%(9d) n=134	0.0%(15d) n=144	
Control Mad; TM6/+ hsFLP; Mad^{12} FRT40 / GFP FRT40A; TM6/+	Larval/ Pupal	Exp.I	16.9%(6d) n=77	16.7%(9d) n=24		0.2
Mad;dcr-1/+ hsFLP; Mad^{12}FRT40A/ GFP FRT40A; FRT82B dcr-1^{Q1147X}/+	Larval/ Pupal	Exp.I	17.4%(6d) n=167	11.1%(9d) n=89		12.3±2.6
		Exp.II	17.2%(5d) n=29		3.8%(14d) n=26	

Table S3.

Preadult GSC lacking Dicer-1 and Dicer-2 activities are lost

Genotype	Clonal induction stage		% germaria with clonal GSC			
			Time point 1	Time point 2	Time point 3	%GSC loss/day[1]
dcr-2 hsFLP; FRT42D dcr2^{L811X}/ FRT 42D Ubi-GFP	Larval/ Pupal	Exp.I	34.3%(7d) n=137		27.9%(14d) n=136	2.7
dcr-2;dcr-1 hsFLP; dcr-2^{L811X}; FRT82B dcr-1^{Q1147X}/ FRT82B GFP	Larval/ Pupal	Exp.I	15.4%(7d) n=117	15.6%(10d) n=90	1.01%(13d) n=99	16.1±6.5
		Exp.II	12.0%(7d) n=31		3.6%(11d) n=89	
dcr-2 hsFLP; FRT42D dcr2^{L811X}/ FRT 42D Ubi-GFP	Adult	Exp.I	22.2%(5d) n=180	21.8%(9d) n=55		1.8±0.9
		Exp.II	28.3%(5d) n=113		22.2%(12d) n=45	
dcr-2;dcr-1 hsFLP; dcr-2^{L811X}; FRT82B dcr-1^{Q1147X}/ FRT82B GFP	Adult	Exp.I	5.9%(5d) n=186	3.2%(8d) n=193		15.3

n = number of germaria analyzed

( ) - days after heat shock induction in parentheses

[1] – average germline stem cell loss per day ± standard error

GSC loss per day = (% of clonal GSC at timepoint 1 - % of clonal GSC at timepoint 2)^{x}100%

% of clonal GSC at timepoint 1/elapsed time

Table S4.
GSCs require Dicer-1 for proper division during all developmental stages, whereas preadult GSCs lacking Mad activity divide normally

Genotype	Clonal induction stage	Time point	Average number of cysts generated heterozygous GSCs	Average number of cysts generated by mutant *GFP*$^-$ GSCs	Relative Division Index[1] ± AD
Mad^{I2} *hsFLP; GFP FRT40A / Mad*^{I2} *FRT40A*	Larval/ Pupal	7d	4.73 n=11	4.55 n=11	0.96±0.22
		12d	3.82 n=17	3.17 n=23	0.87±0.25
		14d	4.57 n=7	3.67 n=18	0.80±0.13
dcr-1 hsFLP;;FRT82B GFP/ FRT82B dcr-1^{Q1147x}	Adult	3d	3.50 n=4	2.16 n=6	0.61±0.07
		6d	4.60 n=15	2.20 n=10	0.48±0.21
		7d	4.75 n=12	1.55 n=17	0.34±0.14
		9d	4.83 n=6	1.75 n=28	0.39±0.25
Mad^{I2} *hsFLP; GFP FRT40A/ Mad*^{I2} *FRT40A*	Adult	5d	4.15 n=13	1.64 n=14	0.50±0.24
		9d	5.81 n=16	3.13 n=22	0.51±0.25
		14d	3.91 n=23	1.95 n=20	0.40±0.17
ban$^{\Delta 1}$ *hsFLP;;GFP FRT80B / ban*$^{\Delta 1}$ *FRT80B*	Adult	5d	3.0 n=6	1.3 n=4	0.43
		9d*	2.8 n=5	1.07 n=13	0.38

[1] - The Relative Division Rate or Division Index for a mutant GSC is determined by the average number of GFP$^-$ mutant cysts generated by a GFP$^-$ GSC divided by the average number of GFP/+ mutant cysts generated by a GFP/+ GSC. n= the number of total GSC examined. * - few examples of *ban*$^{\Delta 1}$ GSCs analyzed 12 days after adult heat shock produced no progeny.

Figure S1. Adult induced *dicer-1* GSC clones

(A-C) show tissue from females with adult-induced *dicer-1* GSC clones, (D) shows a germarium with control clones. Germaria oriented anterior right, GSCs outlined with dashes, cap cells identified with pink asterisks

(A) Example of an extreme phenotype observed when both GSCs are mutant for *dicer-1*. This germarium contains only a single germline stem cell (white dashes). Due to reduced cell divisions, the germarium is smaller and adjacent to an older egg chamber.

(B) Although germline stem cells lacking *dicer-1* are lost from the niche at a high frequency (see Figure 3A), *dicer-1* mutant germline stem cells do not express BamC.

(C) GSCs lacking Dicer-1 activity from adulthood onwards divide slower which results in smaller germaria in comparison to control (D). (D) Germarium with two control (parental) clonal GSCs in the niche. Red=Adducin (A), BamC (B) or Adducin+Lamin C (C-D), Blue=DAPI, Green=GFP.

Figure S2. Larva/pupal induced GSC clones.

When GSCs lose Mad activity during larval/pupal development, the Mad^{12} mutant GSCs are maintained in the niche, divide normally and produce normal cysts (A,B). However, when Mad levels are reduced in *dcr-1* mutant background, GSC are lost even after larval/pupal clone induction (C,D).

(A) Upper ovariole shows larval/pupal-induced mutant GSCs in the niche producing normal cysts and egg chambers. Lower ovariole shows that larval/pupal-induced Mad^{12} mutant follicle cells are defective, leading to encapsulation defects (Jordan et al., 2000).

(B) A germarium with an all clonal germline showing that larval/pupal-induced Mad^{12} mutant germline stem cells divide normally and are maintained in the niche.

(C,D) When Mad levels are reduced in GSCs lacking Dicer-1 during larval/pupal stages (*hsFlp; Mad^{12} FRT40A/+; FRT82B dcr-1^{Q1147X}/FRT82B GFP),* mutant GSCs leave the niche (C,C') and have division defects typical for *dcr-1* mutant GSCs, resulting in smaller germaria (D). Red=Adducin (A,C-D) or Cyclin E (B), Blue=DAPI, Green=GFP, mutant GSCs outlined with white dashed lines, control GSCs with yellow dashes.

Jordan, K.C., Clegg, N.J., Blasi, J.A., Morimoto, A.M., Sen, J., Stein, D., McNeill, H., Deng, W.M., Tworoger, M., and Ruohola-Baker, H. (2000). The homeobox gene mirror links EGF signalling to embryonic dorso-ventral axis formation through notch activation. Nat. Genet. 24, 429–433.

Figure S3. Different defects are observed when GSCs lack either *dicer-1* or *Mad*

dicer-1^{Q1147X} mutant germline stem cells generated during adulthood leave the niche by differentiation (A; 6 days after adult clone induction *dicer* GSC has left the niche and produced a 2-cell *dicer-1* cyst marked with turquoise dashes; Figure 6A). (B) Occasionally, when both germline stem cells lack *dicer-1* activity, both germline stem cells leave the niche, ultimately resulting in an empty niche. Mad^{12}

mutant GSCs are lost by two different mechanisms. Most *Mad* mutant GSCs leave the niche. However, 43% of *Mad* mutant GSCs stay in the niche and differentiate while still being in contact with the niche cells (C-E). Mad cysts outlined with turquoise dashes; the niche/cap cells marked with pink asterisks, Red=Adducin (A-C, E) or Adducin+Lamin C (D), Blue=DAPI, Green=GFP.

Figure S4. Germaria from *bantam* hetereoallelic mutants exhibit the same mutant characteristics associated with *bantam* clones

(A-E) Show tissue from (A) control and (B-E) listed bantam heteroallelic mutants.

(A) Normal size germarium from a control fly with two GSCs. (B) An example of abnormally small germarium from a *bantam* heteroallelic mutant (ban^{L1170}/ban^{EP3622}). The single GSC is dividing, but must be dividing very slowly since there are very few cysts and the overall size of the germarium is small. Note the *bantam* alleles were isolated independently. (C-D) Two examples from another *bantam* heteroallelic combination ($ban^{L1170}/ban^{\Delta 1}$) showing a germarium reduced in size with a single GSC (C) and an empty germarium (D). (E) Another example of a germarium reduced in size with a single GSC from a $ban^{EP3622}/ ban^{\Delta 1}$ heteroallelic fly. Germaria oriented anterior right, GSCs outlined with white dashes, Red=Adducin, Blue=DAPI.

Figure S5. Germline stem cell maintenance depends on developmental stage during mutation induction

Exponential curves are fitted to a time-course of germline stem cells maintenance, where the numbers of clonal germline SCs at 5 days after preadult heat shock or 3 days after adult heat shock are normalized to 1. (A) Larval-pupal induced *dcr-1, Mad,* and *Mad, UAS Dl* GSCs show no maintenance defects, suggesting that Dcr -1 and Mad are not required for GSC maintenance in developmental stages. However, chromatin remodeling factor ISWI is required for GSC maintenance regardless which stage of development it is lost. (B) In contrast GSCs are lost in exponential order when components of Dcr-1 and Mad *(dcr-1, bantam* miRNA and *Mad)* were mutated in GSC during adulthood, showing that Mad and miRNA pathway, and specifically *bantam* miRNA, are essential for adult germline stem cell maintenance.

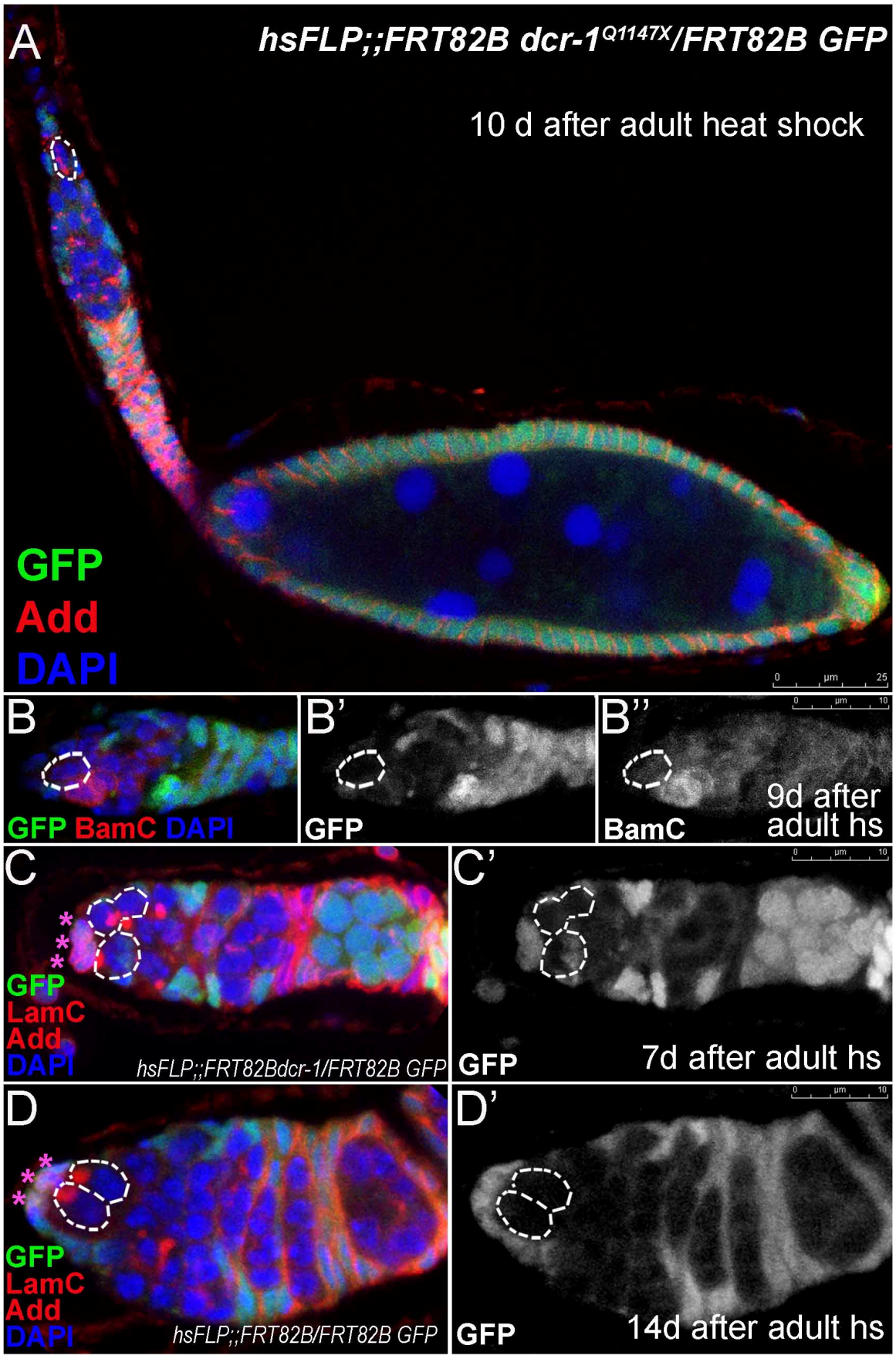

A
hsFLP;;FRT82B dcr-1Q1147X/FRT82B GFP
10 d after adult heat shock
GFP
Add
DAPI
0 µm 25
B
GFP BamC DAPI
B'
GFP
B''
BamC
9d after adult hs
C
GFP
LamC
Add
DAPI
hsFLP;;FRT82Bdcr-1/FRT82B GFP
C'
GFP
7d after adult hs
D
GFP
LamC
Add
DAPI
hsFLP;;FRT82B/FRT82B GFP
D'
GFP
14d after adult hs

Figure S1

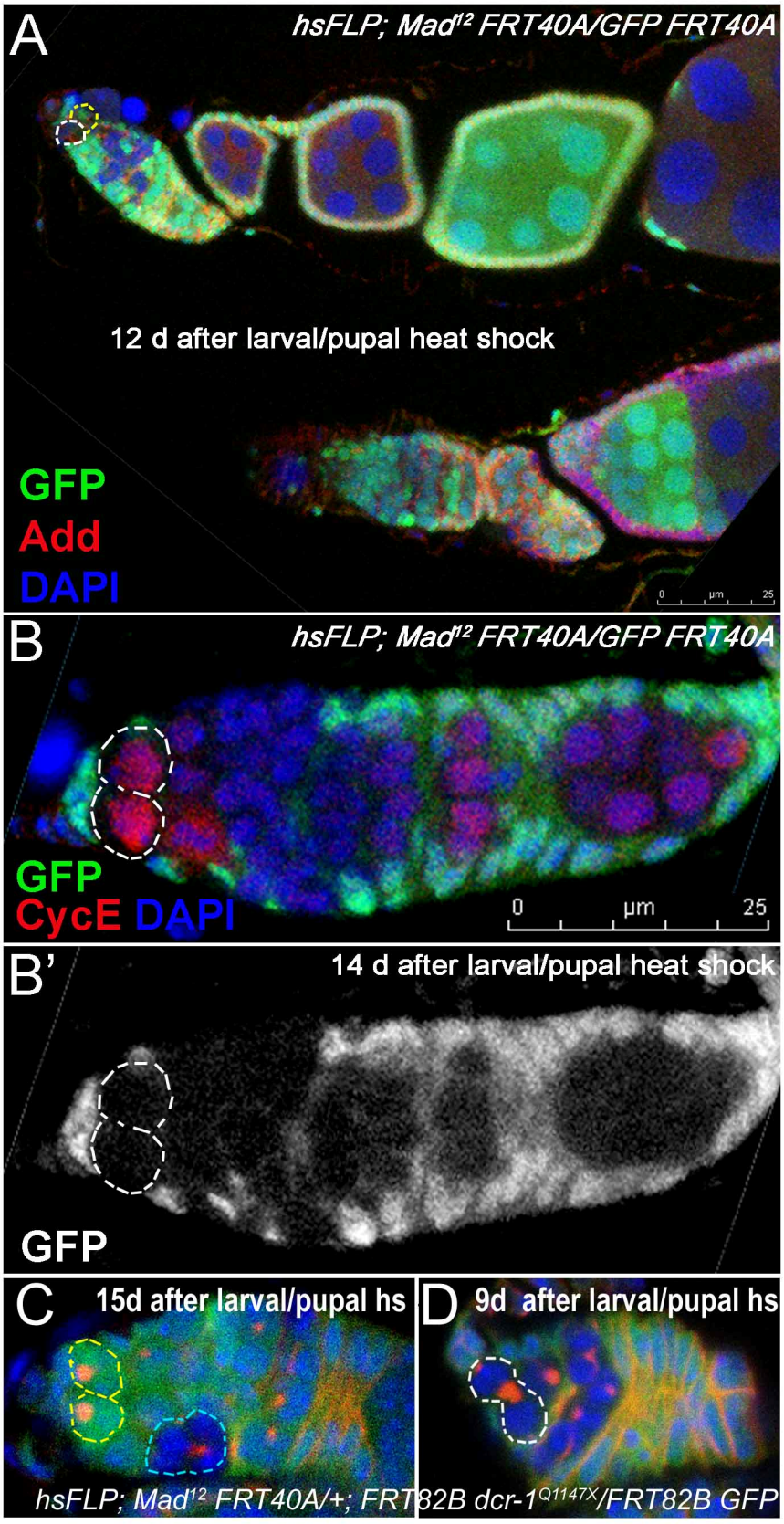

A
hsFLP; Mad12 FRT40A/GFP FRT40A
12 d after larval/pupal heat shock
GFP
Add
DAPI
0 µm 25
B
hsFLP; Mad12 FRT40A/GFP FRT40A
GFP
CycE DAPI
0 µm 25
B'
14 d after larval/pupal heat shock
GFP
C
15d after larval/pupal hs
D
9d after larval/pupal hs
hsFLP; Mad12 FRT40A/+; FRT82B dcr-1Q1147X/FRT82B GFP

Figure S2

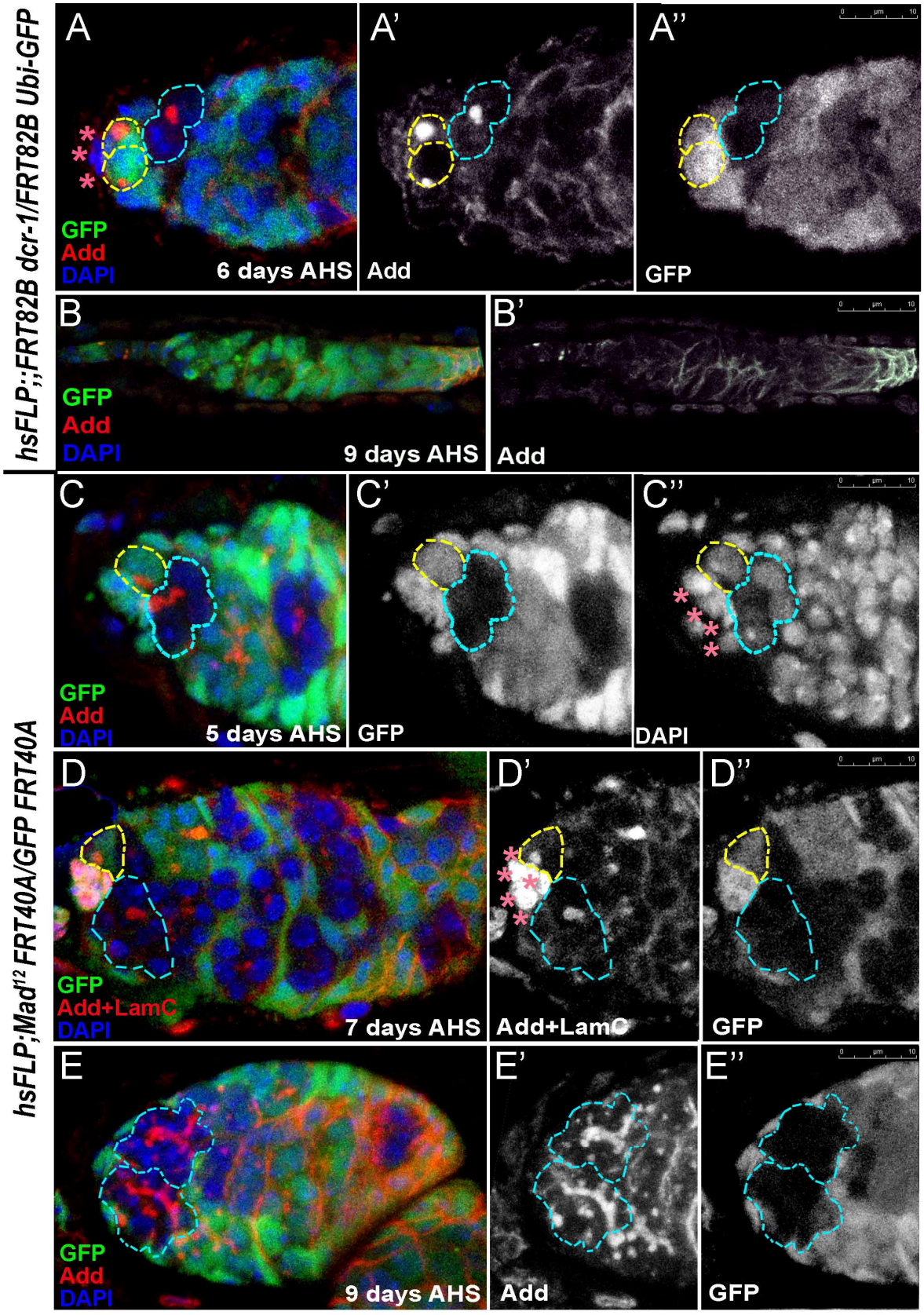

hsFLP;;FRT82B dcr-1/FRT82B Ubi-GFP
hsFLP;Mad12 FRT40A/GFP FRT40A
A
A'
A''
GFP
Add
DAPI
6 days AHS
Add
GFP
B
B'
GFP
Add
DAPI
9 days AHS
Add
C
C'
C''
GFP
Add
DAPI
5 days AHS
GFP
DAPI
D
D'
D''
GFP
Add+LamC
DAPI
7 days AHS
Add+LamC
GFP
E
E'
E''
GFP
Add
DAPI
9 days AHS
Add
GFP

Figure S3

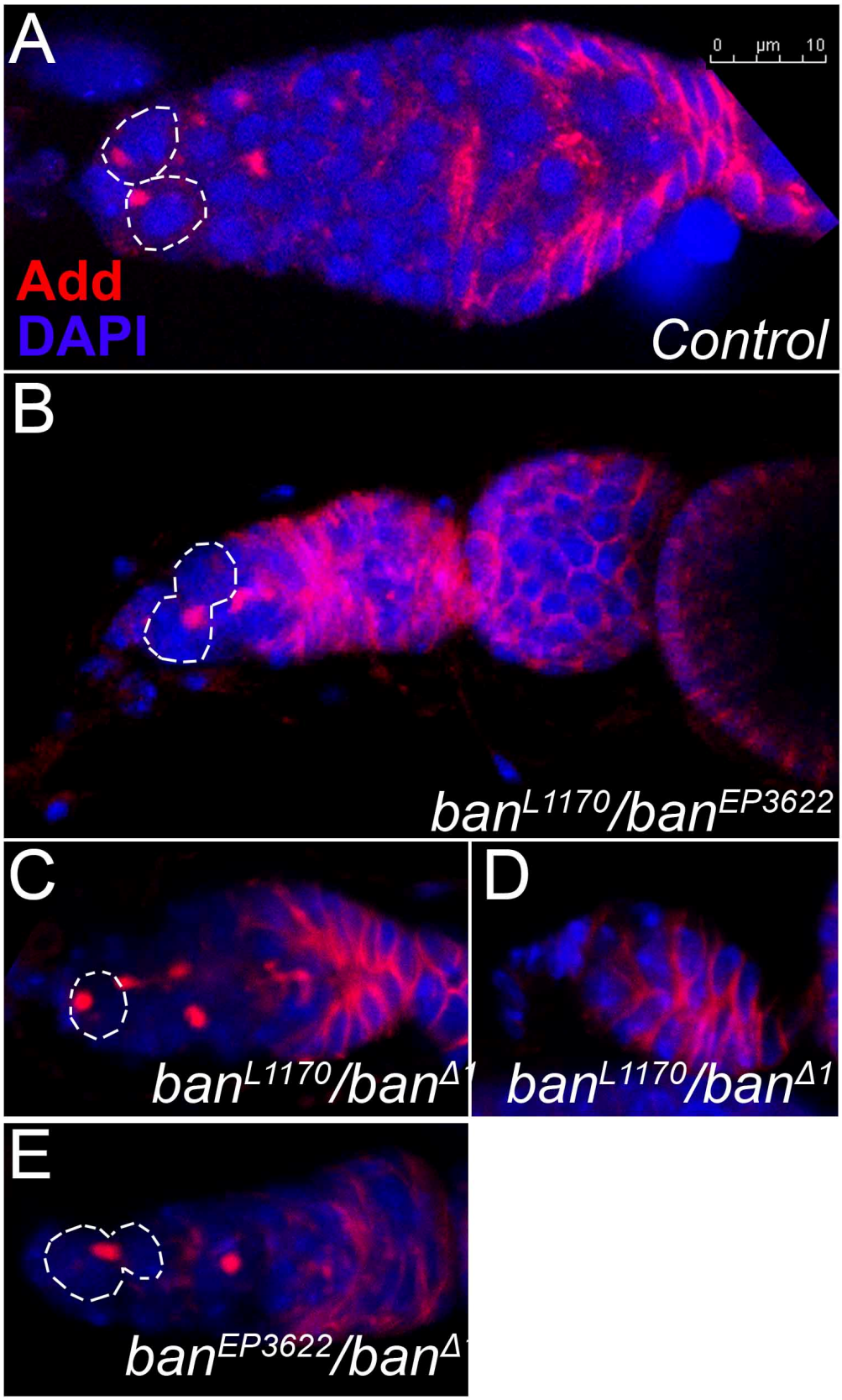
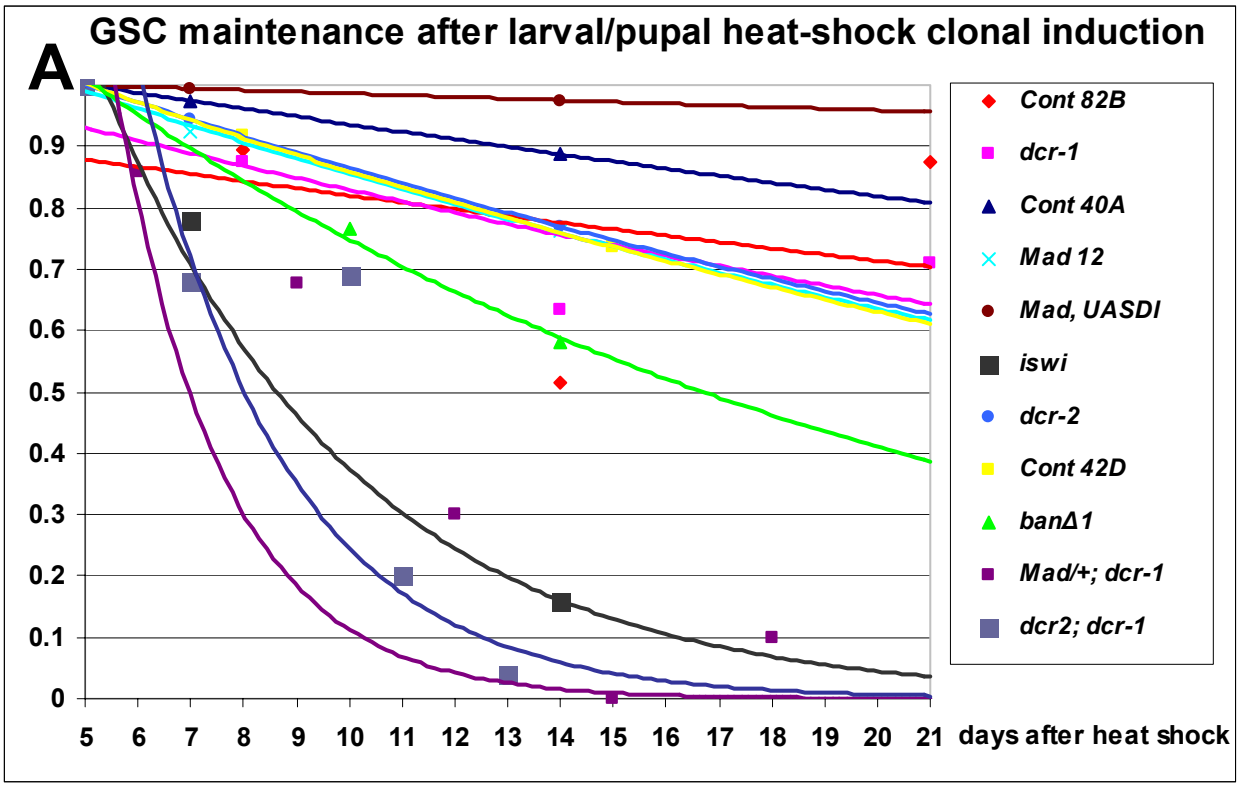

A
0 µm 10
Add
DAPI
Control
B
ban^L1170/ban^EP3622
C
ban^L1170/ban^Δ1
D
ban^L1170/ban^Δ1
E
ban^EP3622/ban^Δ

Figure S4


A
GSC maintenance after larval/pupal heat-shock clonal induction
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
days after heat shock
Cont 82B
dcr-1
Cont 40A
Mad 12
Mad, UASDI
iswi
dcr-2
Cont 42D
banΔ1
Mad/+; dcr-1
dcr2; dcr-1

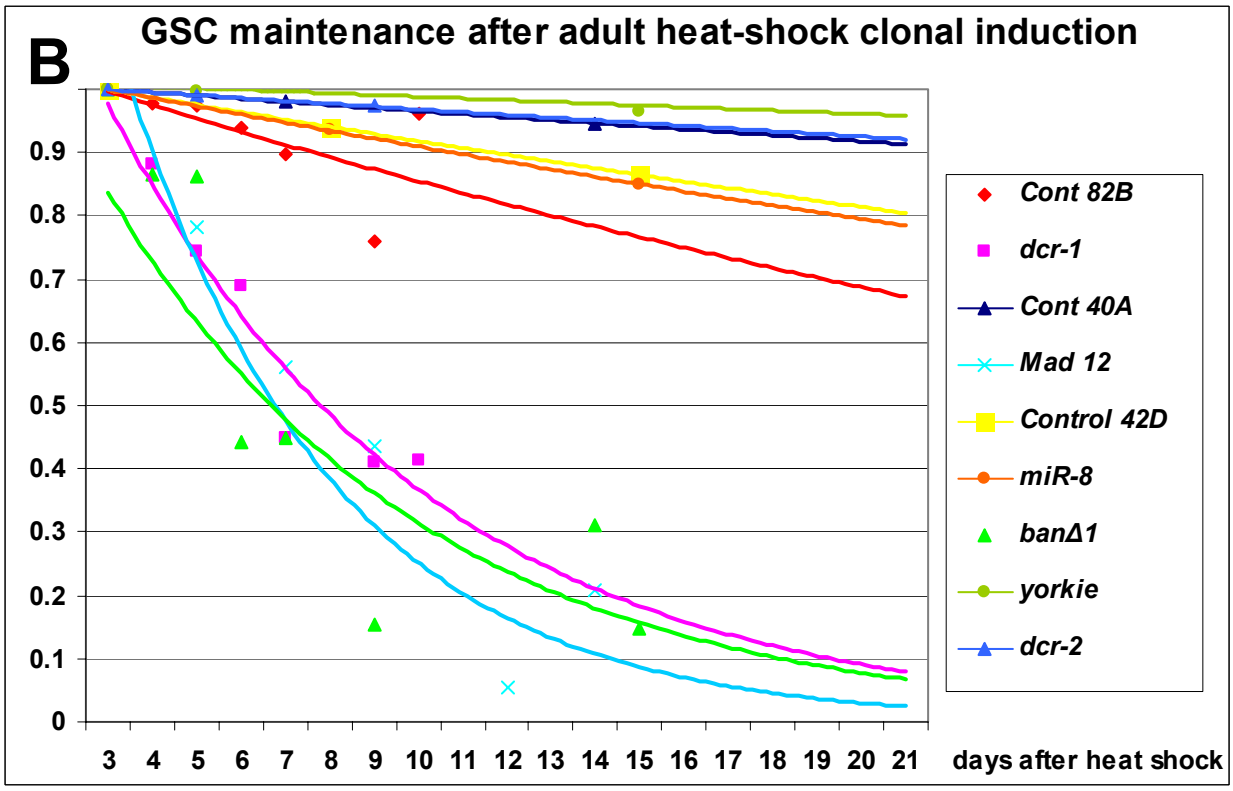

B
GSC maintenance after adult heat-shock clonal induction
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
days after heat shock
Cont 82B
dcr-1
Cont 40A
Mad 12
Control 42D
miR-8
banΔ1
yorkie
dcr-2

Figure S5