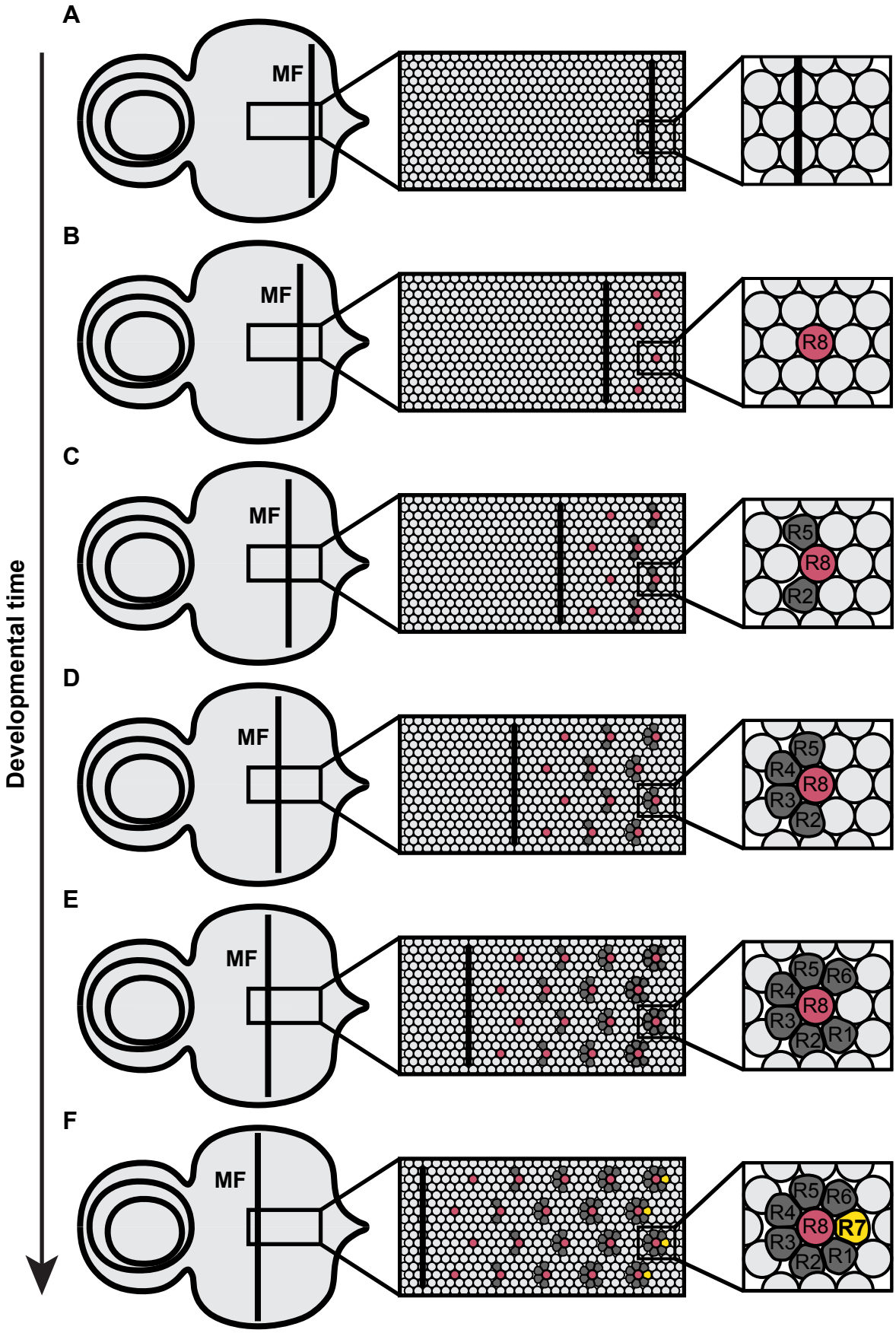
A
MF
B
MF
R8
C
MF
R5
R8
R2
D
MF
R5
R4
R8
R3
R2
E
MF
R5
R6
R4
R8
R3
R2
R1
F
MF
R5
R6
R4
R8
R7
R3
R2
R1
Developmental time

FIGURE S1

Figure S1. Schematic of photoreceptor specification in the eye/antennal disc. Related to Figure 1.

A-F) Photoreceptor specification and morphogenetic furrow during eye development. Light gray circle=unspecified cell; maroon circle=R8 cell; dark gray circle=outer photoreceptor cell (R1-R6); yellow circle=R7 cell.

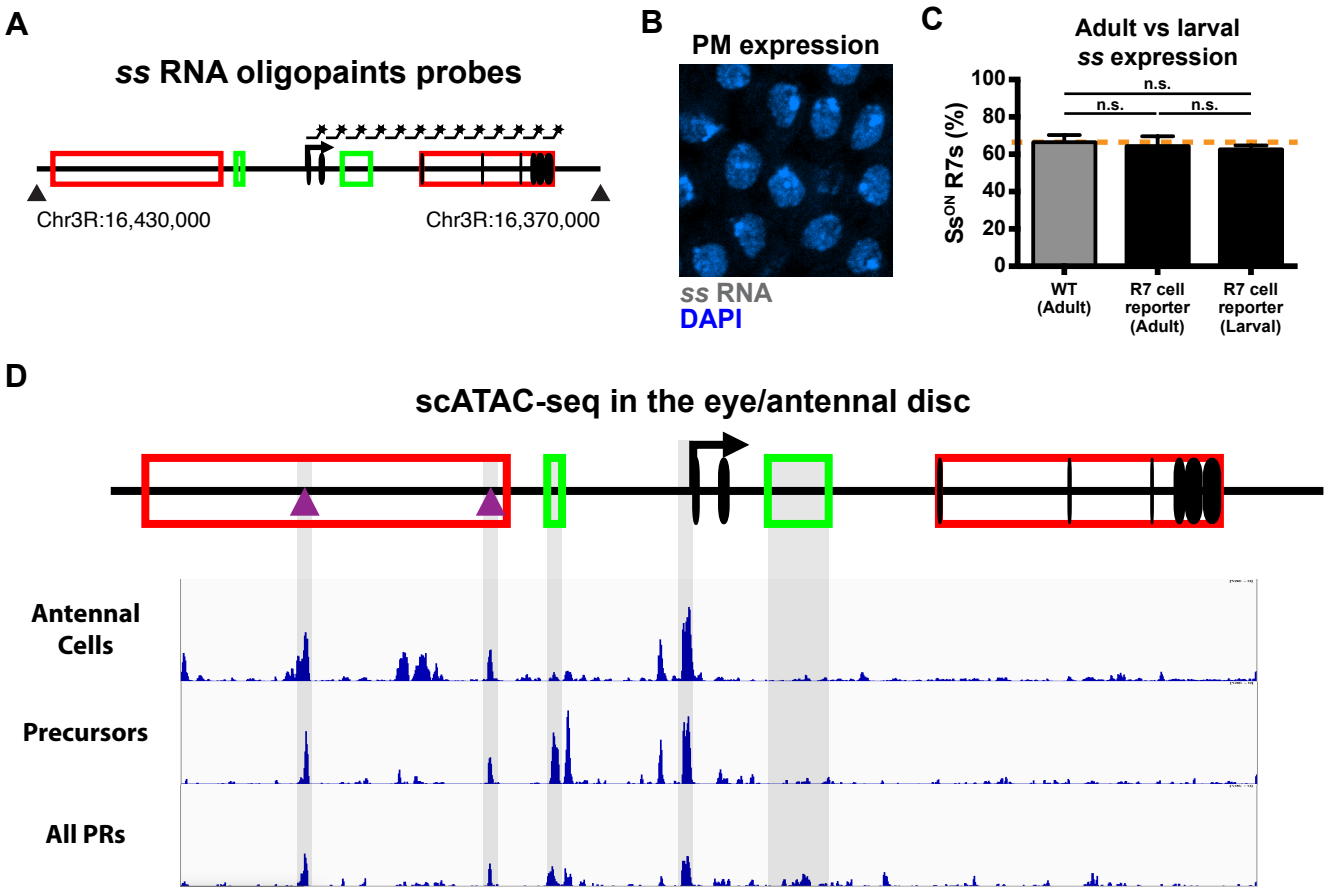
A
ss RNA oligopaints probes
Chr3R:16,430,000
Chr3R:16,370,000
B
PM expression
ss RNA
DAPI
C
Adult vs larval
ss expression
n.s.
n.s.
n.s.
SsON R7s (%)
100
80
60
40
20
0
WT (Adult)
R7 cell reporter (Adult)
R7 cell reporter (Larval)
D
scATAC-seq in the eye/antennal disc
Antennal Cells
Precursors
All PRs

FIGURE S2

Figure S2. *ss* expression analysis; scATAC-seq analysis at the *ss* locus. Related to Figure 2.

A) Schematic of probes used to label *ss* RNA.

B) *ss* is not expressed in peripodial membrane cells (ss^{OFF} control). Gray=*ss* RNA; blue=DAPI.

C) Percent Ss^{ON} R7s is similar in adult R7s in WT flies, in adult R7s in the R7 cell reporter line, and in larval R7s in the R7 cell reporter line. Orange line indicates mean percent Ss^{ON} R7s in adults. n.s. denotes $p > 0.05$.

D) scATAC-seq reads at the *ss* locus by region of the eye/antennal disc. scATAC-seq data were integrated with independently generated scRNA-seq data to produce a virtual map enabling cell identification based on gene expression and enhancer identification based on chromatin accessibility. Gray rectangles=peaks matching *cis* regulatory elements; purple triangles=putative PREs; PRs=photoreceptors.

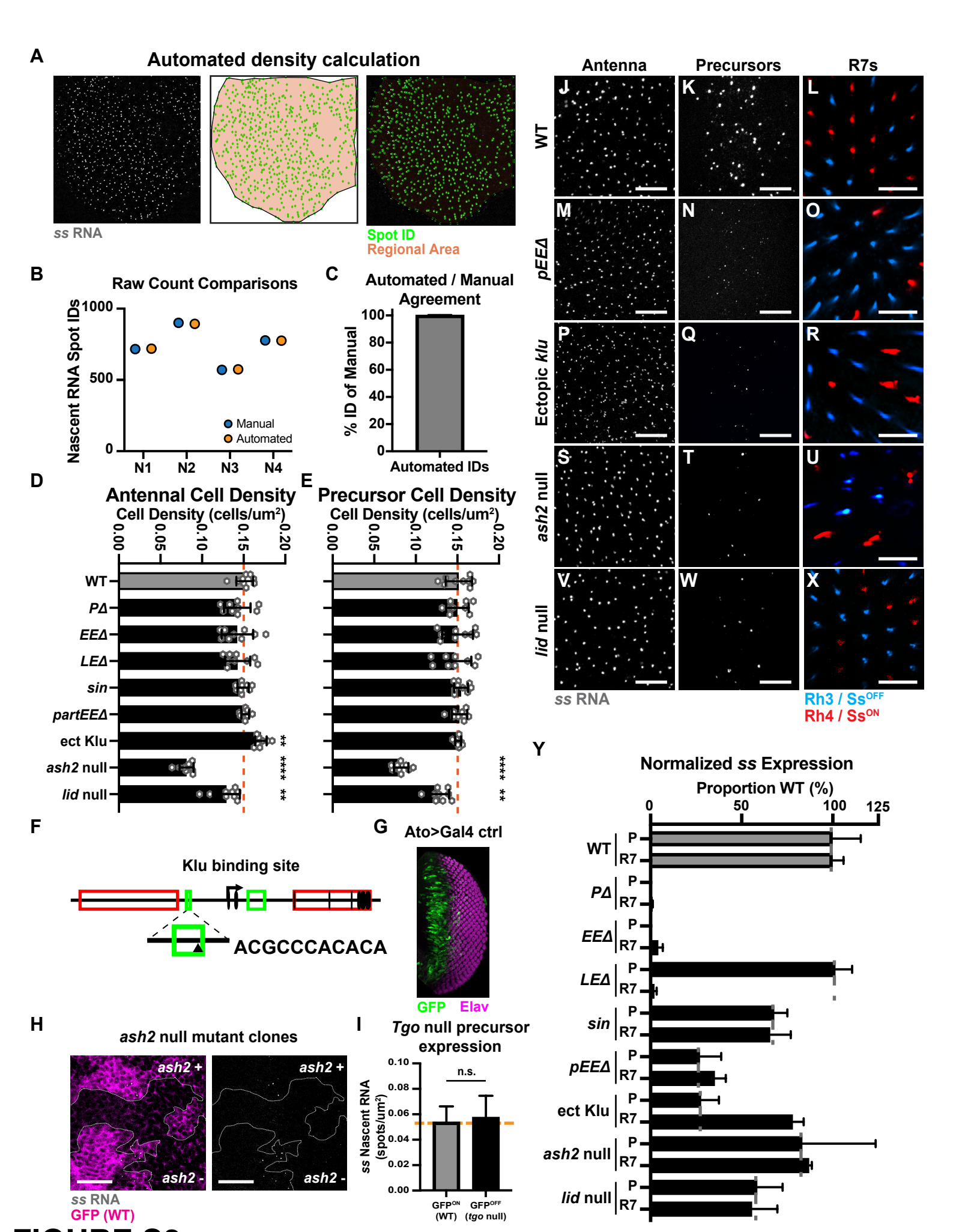
A
Automated density calculation
ss RNA
Spot ID
Regional Area
B
Raw Count Comparisons
Nascent RNA Spot IDs
1000
500
0
Manual
Automated
N1
N2
N3
N4
C
Automated / Manual Agreement
% ID of Manual
100
80
60
40
20
0
Automated IDs
D
Antennal Cell Density
Cell Density (cells/um²)
0.00
0.05
0.10
0.15
0.20
WT
PΔ
EEΔ
LEΔ
sin
partEEΔ
ect Klu
ash2 null
lid null
**
****
**
E
Precursor Cell Density
Cell Density (cells/um²)
0.00
0.05
0.10
0.15
0.20
**
****
Antenna
Precursors
R7s
WT
pEEΔ
Ectopic klu
ash2 null
lid null
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
ss RNA
Rh3 / Ss^OFF
Rh4 / Ss^ON
F
Klu binding site
ACGCCCACACA
G
Ato>Gal4 ctrl
GFP
Elav
H
ash2 null mutant clones
ash2 +
ash2 -
ash2 +
ash2 -
ss RNA
GFP (WT)
I
Tgo null precursor expression
ss Nascent RNA (spots/um²)
0.10
0.08
0.06
0.04
0.02
0.00
n.s.
GFP^ON (WT)
GFP^OFF (tgo null)
Y
Normalized ss Expression
Proportion WT (%)
0
50
100
125
WT
PΔ
EEΔ
LEΔ
sin
pEEΔ
ect Klu
ash2 null
lid null
P
R7

FIGURE S3

Figure S3. Automated density calculation; decreasing early *ss* expression decreases the proportion of Ss^{ON} R7s. Related to Figure 3.

For A-C) Automated identification and calculation of *ss* nascent RNA expression and density.

A) Example of identification of nascent ss RNA spots and determination of boundary region. Gray=ss RNA; green=spot ID; orange=boundary and area.

B) Comparison between the automated and manual quantification of 4 images to confirm high fidelity automated quantification.

C) Percent agreement between automated and by hand quantification. N=4.

D-E) Quantification of cell density within the antenna (**D**) and precursor (**E**) regions to ensure changes in expression are not derived from tissue differences. N=3, n=9. Orange dashed line=WT cell density. ** denotes $p < 0.005$; **** denotes $p < 0.0001$.

F) Schematic of the Klu binding site and binding motif in the *early enhancer* of the *ss* locus.

G) Ato>Gal4 drives GFP in precursors. Green=GFP; magenta=Elav.

H) Early *ss* RNA expression is reduced in precursors in *ash2* null mutant clones. GFP- indicates *ash2* mutant clones; GFP+ indicates wild type clones. Magenta=GFP; dashed line=clone boundary. Gray=*ss* RNA; green=spot ID; orange=regional area.

I) Quantification of expression for Tgo null mutant clones in **3Y**. Orange line indicates mean WT *ss* expression. n.s. denotes $p > 0.05$.

For J, K, M, N, P, Q, S, T, V, W) Scalebar=10 μm.

For J, M, P, S, V) *ss* RNA expression in the antenna. Gray=*ss* RNA.

For K, N, Q, T, W) Early *ss* RNA expression in precursors. Gray=*ss* RNA.

For L, O, R, U, X) Adult $Rh3/Ss^{OFF}$ and $Rh4/Ss^{ON}$ expression in R7s. Blue=Rh3; red=Rh4.

J-L) In wild type (WT) animals, *ss* is expressed in antennal cells, precursors, and a subset of R7s.

M-O) *pEE∆/def* mutants display normal *ss* expression in antennal cells, reduced *ss* expression in precursors, and a decrease in % $Rh4/Ss^{ON}$ R7s.

P-R) Animals with ectopic expression of Klu display normal *ss* expression in antennal cells, reduced *ss* expression in precursors, and a decrease in % $Rh4/Ss^{ON}$ R7s.

S-U) *ash2* null mutants display normal *ss* expression in antennal cells, reduced *ss* expression in precursors, and a decrease in % $Rh4/Ss^{ON}$ R7s.

V-X) *lid* null mutant*s* display normal *ss* expression in antennal cells, reduced *ss* expression in precursors, and a decrease in % $Rh4/Ss^{ON}$ R7s.

Y) Quantification of the proportional changes from WT in precursors and R7s normalized to cell density. P=precursors; R7=R7s; gray solid line=WT precursor and R7 agreement; gray dashed lines=precursor and R7 agreement by genotype.

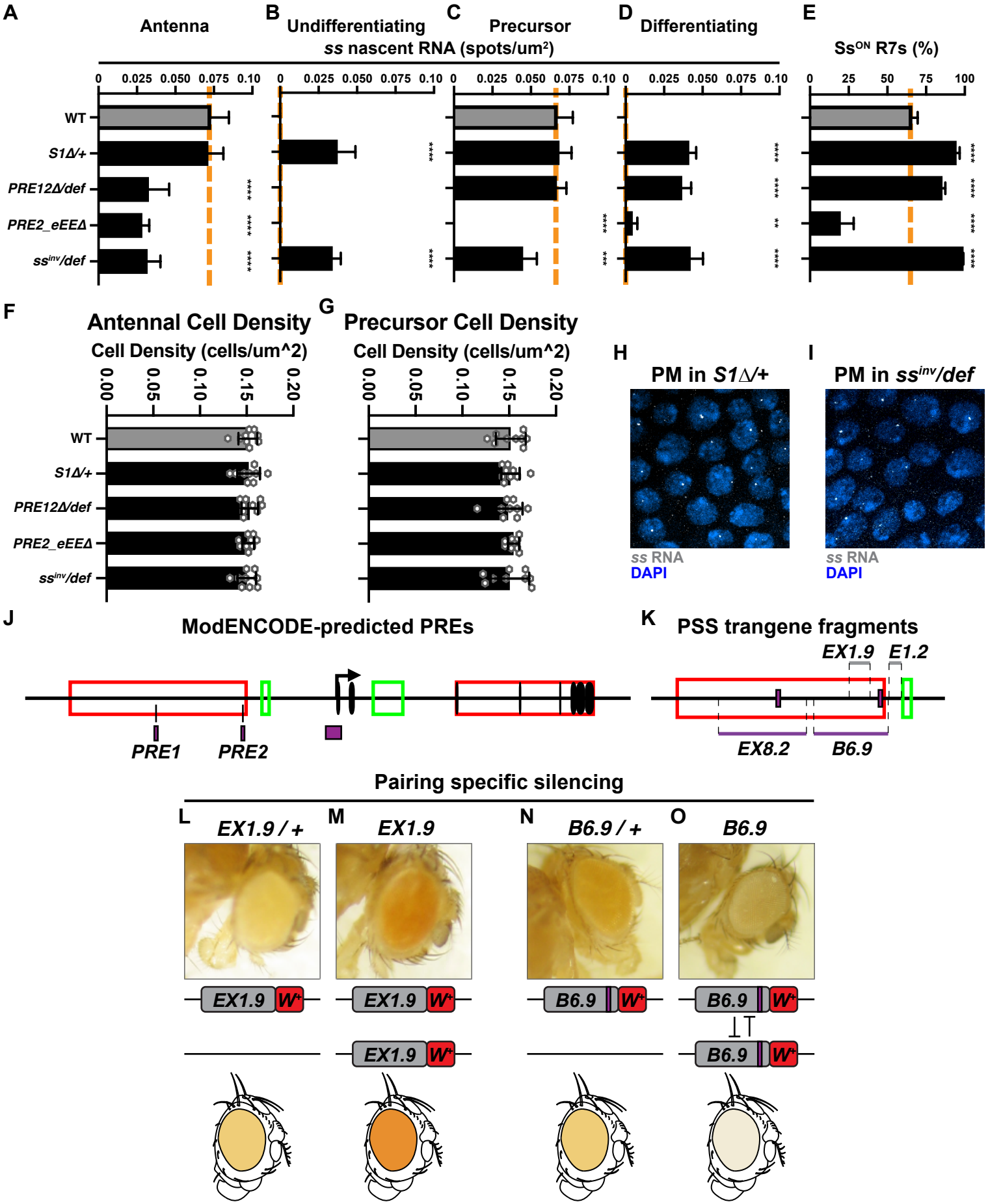
A
Antenna
B
Undifferentiating
C
Precursor
D
Differentiating
E
ss nascent RNA (spots/um²)
Ss^ON R7s (%)
WT
S1Δ/+
PRE12Δ/def
PRE2_eEEΔ
ss^inv/def
F
Antennal Cell Density
Cell Density (cells/um^2)
G
Precursor Cell Density
H
PM in S1Δ/+
I
PM in ss^inv/def
ss RNA
DAPI
J
ModENCODE-predicted PREs
PRE1
PRE2
K
PSS trangene fragments
EX1.9
E1.2
EX8.2
B6.9
Pairing specific silencing
L
EX1.9 / +
M
EX1.9
N
B6.9 / +
O
B6.9
W+

FIGURE S4

Figure S4. Derepressing early *ss* expression increases the proportion of Ss^{ON} R7s; analyzing PREs in the *ss* locus. Related to Figure 4.

For A-E) Quantification of *ss* expression. Orange line indicates mean WT *ss* expression. ** denotes p < 0.005; *** denotes p < 0.0005; **** denotes p < 0.0001.

A) Antennal cells.

B) Undifferentiated cells.

C) Precursors

D) Differentiating cells.

E) R7s

F-G) Quantification of cell density within the antenna cell (**F**) and precursor cell (**G**) regions to ensure changes in expression are not derived from tissue differences. N=3, n=9.

H-I) *ss* is expressed in peripodial membrane cells in *S1Δ* and *ss^{inv}* mutants. Gray=*ss* RNA; blue=DAPI.

J) Predicted PREs in the *ss* locus. Data adapted from FlyBase Jbrowse modENCODE plug-in. Purple rectangles indicate putative PREs.

For K-O) We tested the activity of PRE1 and PRE2 using pairing sensitive silencing assays (Kassis, 1994; Kassis et al., 1991). In this assay, flies that are homozygous for a transgene carrying the *white* gene, but not a PRE, have darker red eyes than heterozygotes. In contrast, flies that are homozygous for a transgene carrying the *white* gene linked to a PRE have lighter eye color than heterozygotes. For flies carrying the EX8.2 transgene that contained PRE1 or the B6.9 transgene that contained PRE2, homozygotes had lighter eye color than heterozygotes (**Fig. S4K, N-O**), consistent with pairing sensitive silencing and the presence of functional PREs. In contrast, for flies carrying the EX1.9 transgene or the E1.2 transgene that neighbored PRE2, homozygotes had darker eye color than heterozygotes (**Fig. S4K-M**), consistent with the absence of functional PREs (Emmons et al., 2007). Together, the ChIP peaks for Polycomb group Proteins, the scATAC-seq peaks, and the pairing sensitive silencing assays suggest that PRE1 and PRE2 are functional PREs.

K) Schematic for regions of DNA used to generate transgenes. Purple line=DNA fragment containing a PRE; gray line=DNA fragment lacking a PRE.

For L-O) Gray oval=DNA fragment denoted in K; purple line=PRE site; red oval=white gene. Schematic for genetic background and cartoon for eye color below.

L) Flies heterozygous for the EX1.9 transgene display light orange eyes.

M) Flies homozygous for the EX1.9 transgene display dark orange eyes.

N) Flies heterozygous for the E6.9 transgene display orange eyes.

O) Flies homozygous for the E6.9 transgene display very light orange eyes.

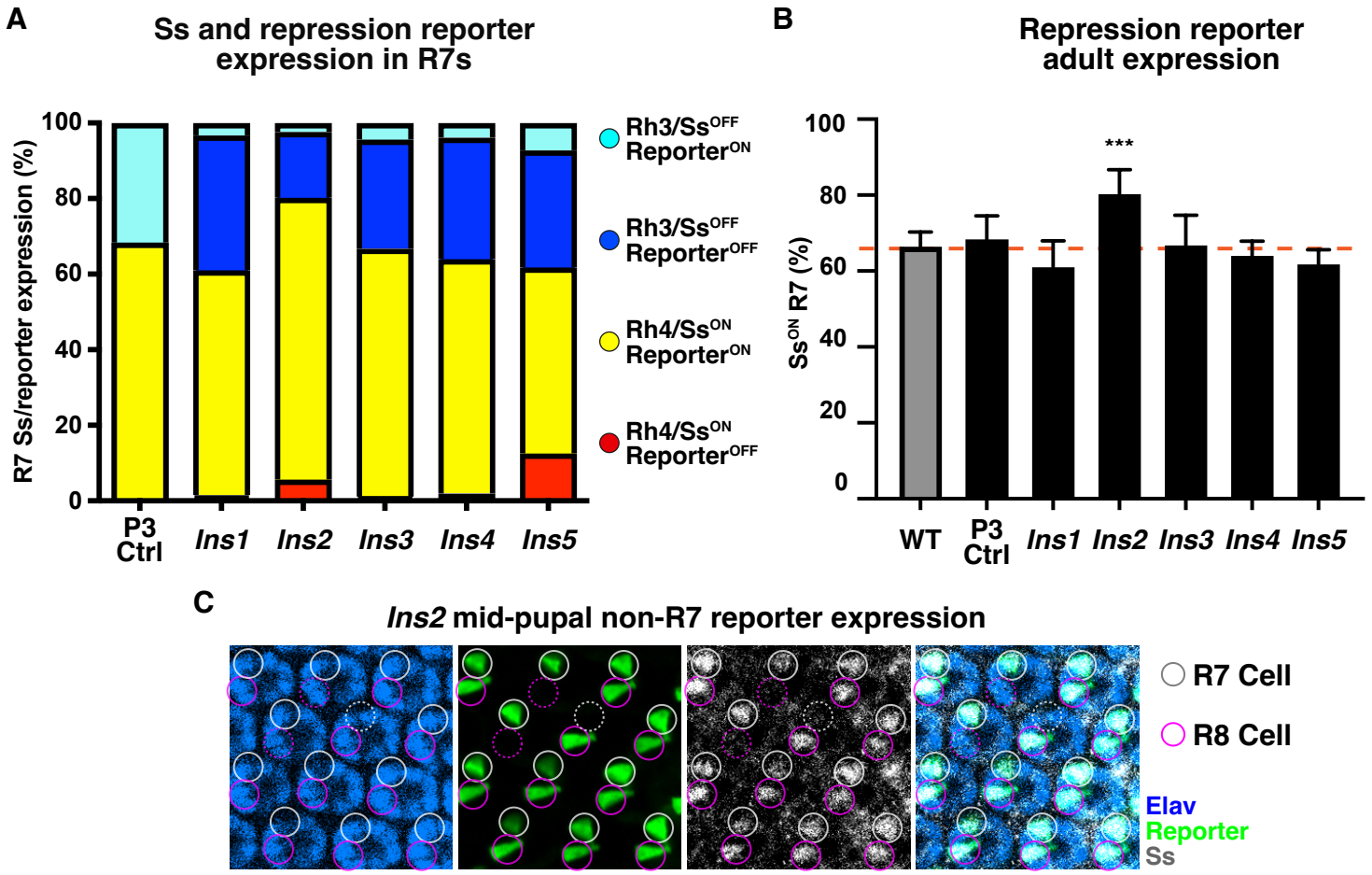
A
Ss and repression reporter expression in R7s
R7 Ss/reporter expression (%)
100
80
60
40
20
0
P3 Ctrl
Ins1
Ins2
Ins3
Ins4
Ins5
Rh3/Ss^OFF Reporter^ON
Rh3/Ss^OFF Reporter^OFF
Rh4/Ss^ON Reporter^ON
Rh4/Ss^ON Reporter^OFF
B
Repression reporter adult expression
Ss^ON R7 (%)
***
WT
C
Ins2 mid-pupal non-R7 reporter expression
R7 Cell
R8 Cell
Elav
Reporter
Ss

FIGURE S5

Figure S5. Repression by the *ss* locus limits expression to a subset of R7s; reporter and Ss are expressed in R8s in flies carrying *Ins2.* Related to Figure 5.

A) Quantification of rhodopsin and reporter expression. Cyan=Rh3/Ss^{OFF}, reporter^{ON}; blue=Rh3/Ss^{OFF}, reporter^{OFF}; yellow=Rh4/Ss^{ON}, reporter^{ON}; red=Rh4/Ss^{ON}, reporter^{OFF}.

B) % Rh4/Ss^{ON} R7s in control flies or flies carrying the repression reporter. Orange line indicates mean WT Ss expression.

C) The reporter and Ss are expressed in R8s in flies carrying *Ins2* in mid-pupal retinas. Gray circles=R7s; maroon circles=R8s; blue=Elav; green=reporter; gray=Ss. Solid lines indicate reporter expressing cell; dotted lines indicate no reporter expression.

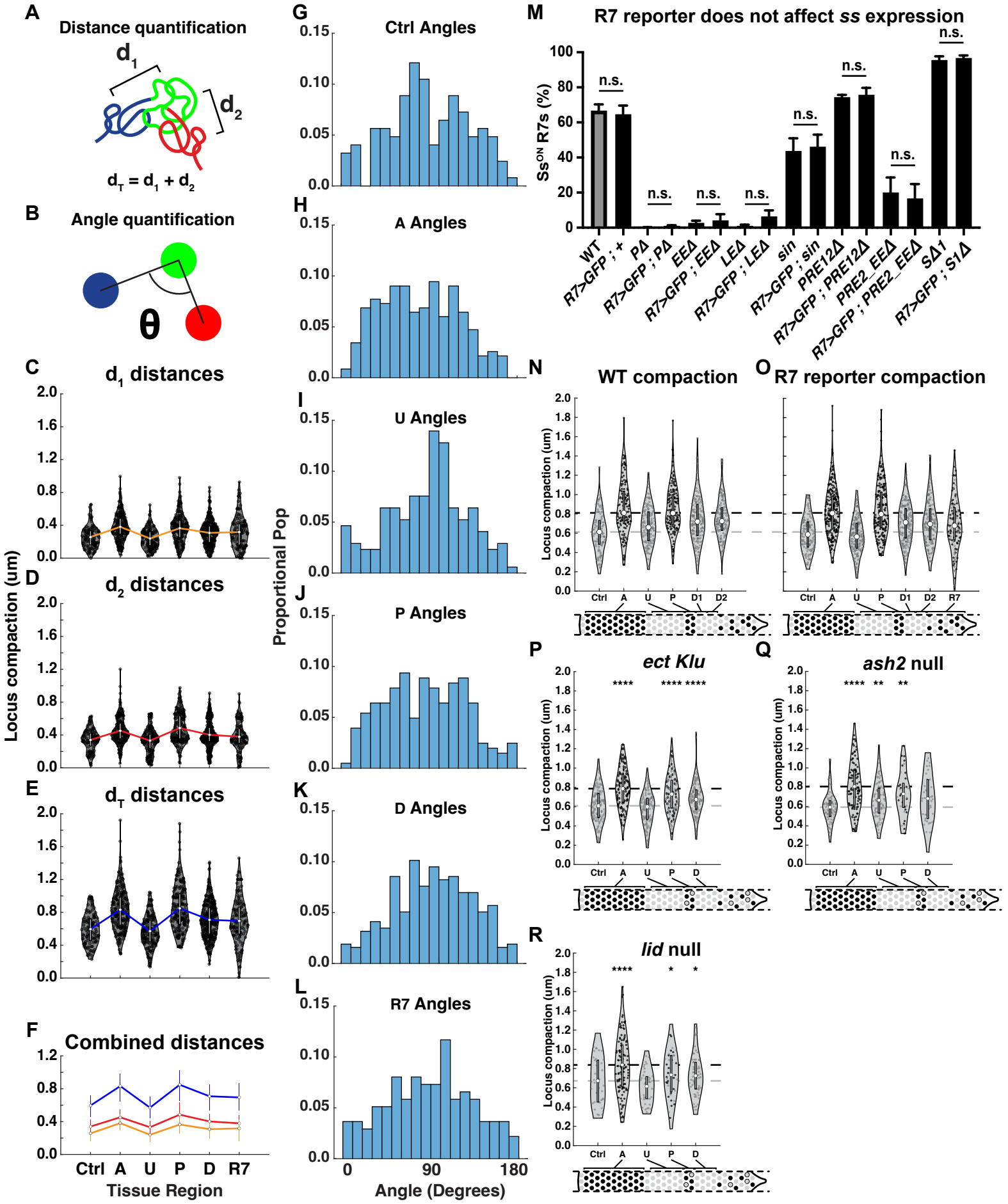
A
Distance quantification
d1
d2
dT = d1 + d2
B
Angle quantification
θ
C
d1 distances
D
d2 distances
E
dT distances
F
Combined distances
Ctrl A U P D R7
Tissue Region
Locus compaction (um)
G
Ctrl Angles
H
A Angles
I
U Angles
J
P Angles
K
D Angles
L
R7 Angles
Proportional Pop
0 90 180
Angle (Degrees)
M
R7 reporter does not affect ss expression
SsON R7s (%)
n.s.
WT
R7>GFP ; +
PΔ
R7>GFP ; PΔ
EEΔ
R7>GFP ; EEΔ
LEΔ
R7>GFP ; LEΔ
sin
R7>GFP ; sin
PRE12Δ
R7>GFP ; PRE12Δ
PRE2_EEΔ
R7>GFP ; PRE2_EEΔ
SΔ1
R7>GFP ; S1Δ
N
WT compaction
O
R7 reporter compaction
Ctrl A U P D1 D2 R7
P
ect Klu
Q
ash2 null
R
lid null
Ctrl A U P D

FIGURE S6

Figure S6. Analysis of compaction comparing d_1, d_2, and d_t and angles; R7 reporter does not affect *ss* expression; compaction in additional mutants. Related to Figure 6.

For A-B) Blue=*ss* upstream DNA; green=*ss* locus DNA; red=*ss* downstream DNA.

A) Schematic for compaction distance calculations.

B) Schematic for compaction angle calculations.

For C-L) Ctrl=peripodial membrane cells; A=antennal cells; U=undifferentiated cells; P=precursors; D=differentiating cells; R7=R7s.

C) d_1 compaction distances. Orange line=compaction changes by region.

D) d_2 compaction distances. Red line=compaction changes by region.

E) d_T compaction distances. Blue line=compaction changes by region.

F) Compaction changes as in **C-E**.

For G-L) Quantification of compaction angles by tissue region.

G) Ctrl cell angles.

H) Antennal cell angles.

I) Undifferentiated cell angles.

J) Precursor cell angles.

K) Differentiating cell angles.

L) R7 cell angles.

M) Adult Rh3/Ss^{OFF} and Rh4/Ss^{ON} expression in R7s with and without the R7 reporter show no effect on ss expression. n.s=p> 0.05.

For N-R) Quantification of compaction. Ctrl=peripodial membrane cells; A=antennal cells; U=undifferentiated cells; P=precursors; D=differentiating cells; R7=R7s. Black circle=ss^{ON} cell; gray circle=ss^{OFF} cell; white rectangle=quartile; white circle=median; gray dashed line=ss^{OFF} control/peripodial membrane median; black dashed line=ss^{ON} control/antennal cells median. * denotes $p < 0.05$; ** denotes $p < 0.005$; **** denotes $p < 0.0001$.

N-O) WT (**N**) and R7 reporter **(O)** lines show no differences in compaction dynamics. Differentiating cells have been subdivided into early differentiating/D1 and late differentiating/D2. D1 and D2 cells display no difference in compaction.

P) *ectopic klu* mutants. n > 106 cells for each region.

Q) *ash2* null mutants. n > 38 cells for each region.

R) *lid* null mutants. n > 40 cells for each region.

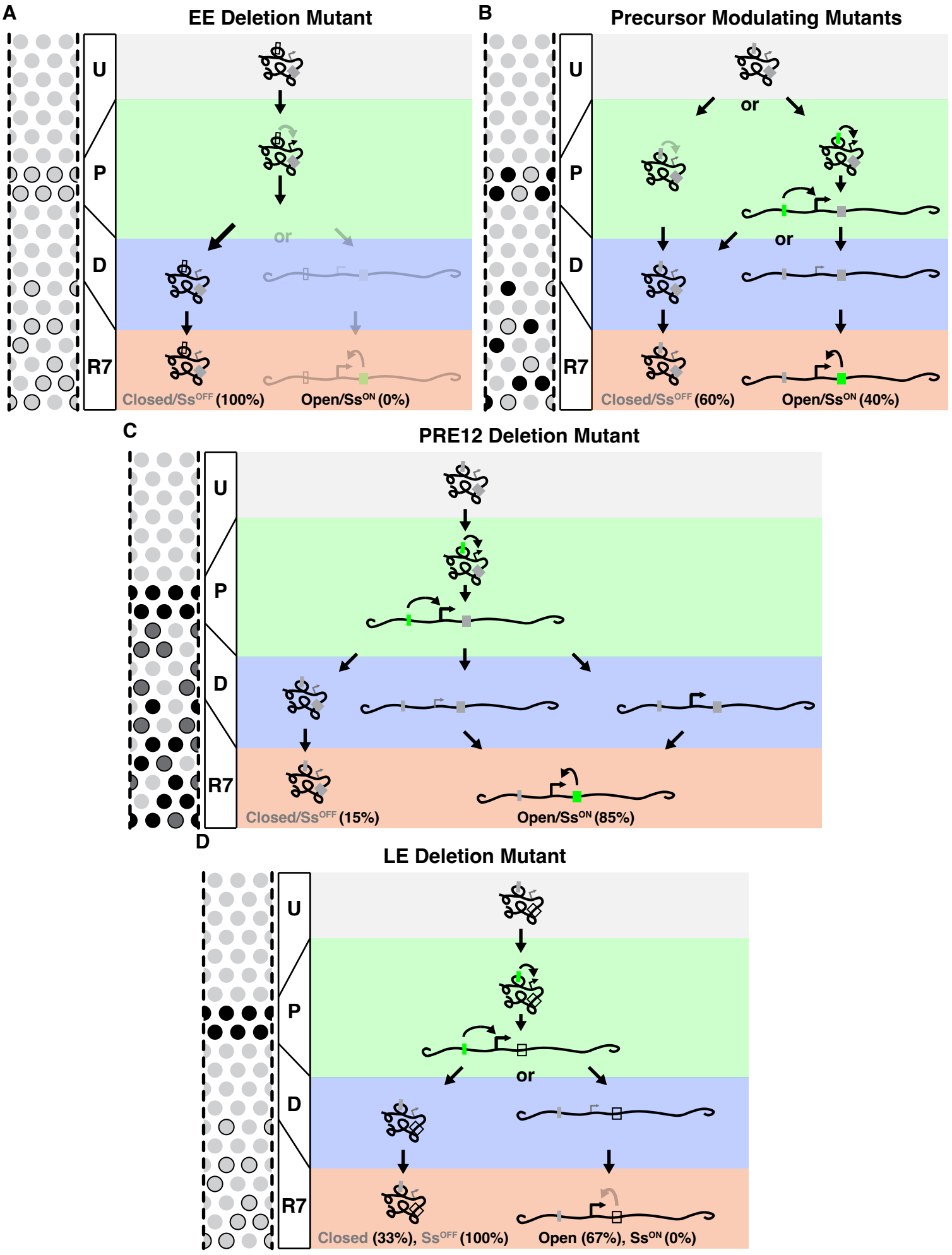
A
EE Deletion Mutant
U
P
D
R7
or
Closed/Ss^OFF (100%)
Open/Ss^ON (0%)
B
Precursor Modulating Mutants
U
P
D
R7
or
or
Closed/Ss^OFF (60%)
Open/Ss^ON (40%)
C
PRE12 Deletion Mutant
U
P
D
R7
Closed/Ss^OFF (15%)
Open/Ss^ON (85%)
D
LE Deletion Mutant
U
P
D
R7
or
Closed (33%), Ss^OFF (100%)
Open (67%), Ss^ON (0%)

FIGURE S7

Figure S7. Relating the proposed mechanism for stochastic R7 subtype specification to *ss* mutant phenotypes. Related to Figure 7.

We briefly review four key experiments and how they fit with this model. In *EE∆* mutants, *ss* is not expressed in precursors and the *ss* locus remains compact as cells mature into Ss^{OFF} R7s (**Fig. S7A**). In mutants with reduced *ss* expression in precursors, *ss* is expressed in a subset of precursors and the *ss* locus opens. In some of these cells, *ss* remains open as cells differentiate into Ss^{ON} R7s whereas in others, *ss* compacts as cells mature into Ss^{OFF} R7s. For the subset of precursors that do not express ss in precursors, the *ss* locus remains compact as these cells mature into Ss^{OFF} R7s (**Fig. S7B**). In *PRE12∆* mutants, *ss* is expressed in all precursors and the *ss* locus opens. In some differentiating cells, *ss* expression ceases and the *ss* locus remains open as cells mature into Ss^{ON} R7s or closes as cells mature into Ss^{OFF} R7s. In the other differentiating cells, *ss* expression continues and the *ss* locus remains open as cells mature into Ss^{ON} R7s (**Fig. S7C**). In *LE∆* mutants, *ss* is not expressed in R7s and *ss* locus compaction is unaffected (**Fig. S7D**).

For A-D) U=undifferentiated cells; P=precursors; D=differentiating cells; R7=R7s. Gray box=inactive enhancer; green box=active enhancer.

A) *EE∆* mutants.

B) Precursor modulating mutants.

C) *PRE12∆* mutants.

D) *LE∆* mutants.

Table S1. CRISPR deletion screen, Related to Figure 2

Deletion Name	Deletion Size (bp)	Rh4/Ss^{ON} (%)
R1Δ	6,229	67.68 ± 2.4
R2Δ	6,863	65.84 ± 4.7
EEΔ	1,278	0
pEEΔ	4,089	24.9 ± 5.8
extEEΔ	5,367	0
R3Δ	1,540	53.63 ± 4.3
PΔ	1,993	0
R4Δ	1,543	63.38 ± 3.68
LEΔ	3,181	0
R5Δ	1,645	51.25 ± 5.78
R6Δ	3,848	65.56 ± 4.56
R7Δ	2,393	55.78 ± 7.8
R8Δ	3,933	50.147 ± 2.1
R9Δ	4,871	61.01 ± 2.2

Table S2. Trans factor regulator screen, Related to Figure 3

Category	Gene	Mutant allele	Rh4/Ss^{ON} (%)
Wildtype	yw	yw^{67}	61 ± 6
RNAi Control	GFP	RNAi	52 ± 2
Insulator	CP190	RNAi	67 ± 5 ↑
	Su(HW)	RNAi	55 ±4
	Mdg4	RNAi	51± 7
TRX complex	Trx	Trx^{E2}	79 ± 9 ↑
		RNAi	55 ± 6
	Ash2	RNAi v20	37 ± 9 ↓
		RNAi v22	17 ± 8 ↓
	Mnn1	Mnn1^{e173}	33 ± 5 ↓
		Mnn1^{e30}	40 ± 5 ↓
		RNAi	61 ± 12
		CRISPR Deletion	59 ± 12
TRR complex	Trl	RNAi	57 ± 6
PRC1	Pc	RNAi	55 ± 7
		Pc^{4}	94 ± 3
		Pc^{XT}	No R7s
	Ph-d Ph-p	Ph-d^{410} Ph-p^{401}	43 ± 8 ↓
Polycomb Recruitment	Cg	Cg^{A22}	55 ± 9
	Pho	Pho^{1}	32 ± 6 ↓
		Pho^{b}	61 ± 5
		RNAi	72 ± 4 ↑
	Phol	Phol^{81A}	63 ± 11
	Spps	Spps^{1}	No retinas
PRC2	E(z)	E(z)^{61} 18°C control	68 ± 4
		E(z)^{61} 29°C hs 3rd instar 4hrs	57 ± 3
		E(z)^{61} 29°C hs 3rd instar 24hrs	65 ± 7
		E(z)^{61} 29°C hs 2nd instar to late pupation 120 hrs	55 ± 3 ↓
	Su(z)12	Su(z)12^{5}	44 ± 8 ↓
	Psc	Psc^{h28}	65 ± 9
Histone	H3.3	H3.3K27M	69 ± 4
Eye development	Lid	RNAi	No R7s
		Lid^{140}	36.5 ± 7 ↓
	Osa	Osa^{306}	57 ± 3

Table S3. Fly lines, Related to STAR Methods

Short Genotype	Full Genotype	Figures	Source
WT	*yw ; + ; +*	1, 2, 3, S1, S2, S3, S4, S5, T2	N/A
ss Def	*yw ; + ; Df(3R)Exel6269*	2, 3, S3	Bloomington (7736), (Parks et al., 2004)
ss Protein Null	*yw ; + ; ss $^{d115.7}$/ Df(3R)Exel6269*	1	(Duncan et al., 1998)
R7 Reporter	*yw ; pm181>Gal4,UAS>mCD8 GFP/CyO ; +*	1, 5, S5	(Lee et al., 2001)
EE Reporter	*yw ;; EE>GFP ; +*	1	N/A
LE Reporter	*yw ; LE>GFP ; +*	1	N/A
PΔ	*yw ; + ; ss$^{P\Delta}$*	1, 2, 5, S5	N/A
eEEΔ	*yw ; + ; ss$^{eEE\Delta}$/ Df(3R)Exel6269*	1	N/A
pEEΔ	*yw ; + ; ss$^{pEE\Delta}$/ Df(3R)Exel6269*	1, 2, S2	N/A
EEΔ	*yw ; + ; ss$^{EE\Delta}$*	1, 2, 5, S5	N/A
LEΔ	*yw ; + ; ss$^{LE\Delta}$*	1, 2, 5, S5	N/A
tgo Null Clones	*ey>flp/yw ; +/CyO ; FRT82B tgo^{del6}/FRT82B ubi>GFP*	S1	(Thanawala et al., 2013)
sin	*yw ; + ; ss^{sin}*	2	Anderson 2017
Ectopic *klu*	*Ato(384)>Gal4/yw ; UAS>klu/+ : +*	2, S2	(Klein and Campos-Ortega, 1997)
ash2 Null	*yw ; ey>Gal4, UAS>flp/+ ; FRT82B ash2^{1}, e, red/GMR>hid*	2, S2	Bloomington (5253)
lid Null	*yw ; FRT40A lid^{140} ; FRT40A GMR>hid ; ey>Gal4, UAS>flp/+*	2, S2	Bloomington (76954)
ash2 Null Clones	*ey>flp; +; FRT82B Ubi GFP/FRT82B ash2^{1}, e, red*	2, S2	
Ectopic *klu* Ctrl	*Ato(384)>Gal4/yw ; UAS>klu/+ ; P{y[+t7.7] w[+mC]=20XUAS-6XGFP}attP2/+*	S2	Bloomington (52262), (Klein and Campos-Ortega, 1997)
klu Null Clones	*ey>flp ; +/CyO ; klu^{R51} FRT2A/ubi>GFP FRT2A*	3	(Klein and Campos-Ortega, 1997; Stowers and Schwarz, 1999)
klu Null	*yw ; ey>Gal4, UAS>flp/+ ; GMR>hid,cl FRT2A/klu^{R51} FRT2A*	3, S3	(Klein and Campos-Ortega, 1997; Stowers and Schwarz, 1999)
S1Δ	*yw ; + ; S1Δ/+*	3, S3	(Johnston and Desplan, 2014)

PRE12Δ	*yw ; + ; PRE12Δ/Df(3R)Exel6269*	3, S3	N/A
PRE2-EEΔ	*yw ; + ; PRE2-EEΔ*	3, S3	N/A
ss^{inv}	*yw ; + ; ss^{inv}/Df(3R)Exel6269*	3, S3	
X 3xP3 Ctrl	*yw, M{3xP3-RFP.attP}ZH-2A ; + ; + ; M{RFP[3xP3.PB] GFP[E.3xP3]=vas-int.Dm}ZH-102D*	4, S4	Bloomington (24480)
Ins1	*yw ; + ; 3xP3>RFP*	4, S4	N/A
Ins2	*yw ; + ; 3xP3>RFP*	4, S4	N/A
Ins3	*yw ; + ; 3xP3>RFP*	4, S4	N/A
Ins4	*yw ; + ; 3xP3>RFP*	4, S4	N/A
Ins5	*yw ; + ; GMR>GFP/+*	4, S4, 5	(Thanawala et al., 2013)
WT R7 Reporter	*yw ; pm181>Gal4,UAS>mCD8 GFP/CyO ; +*	5, S5	N/A
PΔ R7 Reporter	*yw ; pm181>Gal4,UAS>mCD8 GFP/CyO ; PΔ*	5, S5	N/A
EEΔ R7 Reporter	*yw ; pm181>Gal4,UAS>mCD8 GFP/CyO ; EEΔ*	5, S5	N/A
LEΔ R7 Reporter	*yw ; pm181>Gal4,UAS>mCD8 GFP/CyO ; LEΔ*	5, S5	N/A
R1Δ	*yw ; + ; R1Δ/Df(3R)Exel6269*	1, T1	N/A
R2Δ	*yw ; + ; R2Δ/Df(3R)Exel6269*	1, T1	N/A
R3Δ	*yw ; + ; R3Δ/Df(3R)Exel6269*	1, T1	N/A
R4Δ	*yw ; + ; R4Δ/Df(3R)Exel6269*	1, T1	N/A
R5Δ	*yw ; + ; R5Δ/Df(3R)Exel6269*	1, T1	N/A
R6Δ	*yw ; + ; R6Δ/Df(3R)Exel6269*	1, T1	N/A
R7Δ	*yw ; + ; R7Δ/Df(3R)Exel6269*	1, T1	N/A
R8Δ	*yw ; + ; R8Δ/Df(3R)Exel6269*	1, T1	N/A
R9Δ	*yw ; + ; R9Δ/Df(3R)Exel6269*	1, T1	N/A
PΔ	*yw ; + ; $ss^{PΔ}$/Df(3R)Exel6269*	1, T1	N/A
EEΔ	*yw ; + ; $ss^{EEΔ}$/Df(3R)Exel6269*	1, T1	N/A
LEΔ	*yw ; + ; $ss^{LEΔ}$/Df(3R)Exel6269*	1, T1	N/A
GFP RNAi	*elav>Gal4/w ; UAS>Dcr2/+ ; UAS>GFP RNAi/+*	T2	Bloomington (35786)
Cp190 RNAi	*elav>Gal4/w ; UAS>Dcr2/+ ; UAS>Cp190 RNAi/+*	T2	Bloomington (35078)
Su(HW) RNAi	*elav>Gal4/w ; UAS>Dcr2/+ ; UAS>su(Hw) RNAi/+*	T2	Bloomington (33906)
Mdg4 RNAi	*elav>Gal4/w ; UAS>Dcr2/+ ; UAS>mdg4 RNAi/+*	T2	Bloomington (33907)
Trx^{E2}	*yw ; ey>Gal4, UAS>flp/+ ; FRT82B trx E2/FRT82B GMR>hid*	T2	Gift from Jessica Treisman
Trx RNAi	*elav>Gal4/w ; UAS>Dcr2/+ ;*	T2	Bloomington

	UAS>trx RNAi/+		(33703)
Ash2 RNAi	*elav>Gal4/w ; UAS>Dcr2/+ ; UAS>ash2 RNAi v20/+*	T2	Bloomington (64942)
Ash2 RNAi	*elav>Gal4/w ; UAS>Dcr2/+ ; UAS>ash2 RNAi v22/+*	T2	Bloomington (35388)
Mnn1^{e173}	*w; Mnn1^{e173} ; +*	T2	Gift from Andre Bedard
Mnn1^{e30}	*w; Mnn1^{e30} ; +*	T2	Gift from Andre Bedard
Mnn1 RNAi	*elav>Gal4/w ; UAS>Dcr2/+ ; UAS>Mnn1 RNAi/+*	T2	Bloomington (35150)
Mnn1Δ	*w ; MnnΔ ; +*	T2	N/A
Trl RNAi	*elav>Gal4/w ; UAS>Dcr2/+ ; UAS>Trl RNAi/+*	T2	Bloomington (67265)
Pc RNAi	*elav>Gal4/w ; UAS>Dcr2/+ ; UAS>Pc RNAi/+*	T2	Bloomington (36070)
Pc^{4}	*ey>flp ; +/CyO ; FRT2A Pc^{4}/ FRT2A ubi>GFP*	T2	Gift from Judith Kassis
Pc^{XT}	*ey>flp ; +/CyO ; FRT2A Pc^{XT}/ FRT2A ubi>GFP*	T2	Gift from Judith Kassis
Ph-d^{410} Ph-p^{401}	*Ph-d^{410} Ph-p^{401}; +; +*	T2	Gift from Judith Kassis
Cg^{A22}	*w; FRT42D cg^{A22}/FRT42D GMR>hid; ey>Gal4, UAS>flp/+*	T2	Gift from Judith Kassis
Pho^{1}	*w; +; +; pho^{1}*	T2	Gift from Judith Kassis
Pho^{b}	*w; +; +; pho^{b}*	T2	Gift from Judith Kassis
Pho RNAi	*elav>Gal4/w ; UAS>Dcr2/+ ; UAS>pho RNAi/+*	T2	Bloomington (35206)
Phol^{81A}	*w; +; phol^{81A}*	T2	Gift from Judith Kassis
Spps^{1}	*w; +; Spps^{1}*	T2	Gift from Judith Kassis
E(z)^{61} 18°C	*w; +; E(z)^{61}*	T2	Gift from Judith Kassis
E(z)^{61} 29°C hs 3rd instar 4hrs		T2	Gift from Judith Kassis
E(z)^{61} 29°C hs 3rd instar 4hrs		T2	Gift from Judith Kassis
E(z)^{61} 29°C hs 2nd instar to late pupation 120		T2	Gift from Judith Kassis

hrs			
Psc^{h28}	*w ; Psc^{h28} ; +*	T2	Gift from Judith Kassis
H3.3K27M	*w ; GMR>Gal4/UAS>H3.3K27M ; +*	T2	Gift from Kami Ahmed
Lid RNAi	*elav>Gal4/w ; UAS>Dcr2/+ ; UAS>lid RNAi/+*	T2	Bloomington (28944)
Lid^{140}	*yw; FRT40A lid^{140} /FRT40A GMR>hid; UAS>flp/+*	T2	Gift from Judith Kassis
Osa^{306}	*yw ; ey>Gal4, UAS>flp/+ ; FRT82B osa^{308}/FRT82B GMR>hid*	T2	Gift from Jessica Treisman

Table S4. CRISPR Deletion Mutants, Related to STAR Methods

Deletion Name	Homologous Bridge	Deletion Size
R1Δ	GAGTTGATTGAAGGCTGTAAGAGCAGATTACAGTGGGGCGGAGGCCCAA GTCTGGATCTCGGTACCAACTCTCCATTGTCGATAATAATTGATTTGATT GATGCTTCGACGGCTGCCGATGCTGCT	6,229 bp
R2Δ	CACAATTTACCAATCAAATAACAAATGCGCCACCAAAGATGCTTATTAA TGGCGGCCAACGGTACCTCTGGGTATTCATTTTTTTCGACTTGGCAATTG CAAATGCAAAACCATTTCATTTGCCGA	6,863 bp
EEΔ	AAAATAGATGGCTATTAACTATAGTTATTGAAGTGGGTCCATCAATCCAT CTTCTCCATTGGATCCCATGGCCTCAGAGCGAAAAAAAAACGAGGGGGC AGAGGCGTAGTTTTCGGAATTAATCCT	1,278 bp
pEEΔ	ATCGGGTTACGTACTCCCTCGCAACGTGAGAACCTCGGCCACTGTCAGGT GAAGTCCACTGAATTCTCTCATAAGAATCTTAAAAATGATAAAGATATTTT TCTTCGATAGCTTTATTTCAGATATC	4,089 bp
extEEΔ	AAATAGATGGCTATTAACTATAGTTATTGAAGTGGGTCCATCAATCCATC TTCTCCATTAGAATTCCTCATAAGAATCTTAAAAATGATAAAGATATTTT CTTCGATAGCTTTATTTCAGATATCA	5,367 bp
R3Δ	TCACATAAAGGCAGCCATTTTTGGGCCGCCACAAACGGCAATTATAATT GCGGCTACTTAGAATTCGATAAACCAGAGCCCACGAGCAACAACACTAC CAACACAAACGGCAAAAGTGCAAGTGAA	1,540 bp
PΔ	CTTCCACTTTGCTACACTTCACTCCACTCCACTCGACTCAGCTCACTTATT AGTGCCACCGAATTCGATAAACCAGAGCCCACGAGCAACAACACTACCA ACACAAACGGCAAAAGTGCAAGTGAA	1,993 bp
R4Δ	CTTGGGTGCTCTGTTATTACTGAAAGATTTCAATCAATGCGATTGGCTGC ACTTACCACTGGTACCGTCCTAGCGAATATTACGCATACGACGCATAGAC TTACTGCACATTTGGCCAAGTTCATT	1,543 bp
LEΔ	CAATTTAATTGAGCTCCCAAGTGCTGGGAAGCAGCTGCCCTTTGAATTGG GCTTCTCACCGAATTCTGGCCTGGCTTTGGAGCTCCTTTTGGTGAGAGAC CAAAAGAGATTCCGCTGCGCGAATCG	3,181 bp
R5Δ	GGAGGTGGTATCTGGCCCGGGCAGGTGATACTTTCAGTTACCTTTTCCCT TACCCAGAACGAATTCGAGGGCCGTGAACCTGAGCCTGCGACCGATTCT TCCGAGATTCTTAGTCGCAGCCTTCGT	1,645 bp
R6Δ	TCCCGAAAGGCGAAAAGGACCAACCGACCGACAGACAGACAAACACAC GCACGTGCTGTTGAATTCTACACATTACATATAATGTGTCTATTATTTCCA TCTACACATCCTTATGCCAATCTATCC	3,848 bp
R7Δ	GGATATAAATAGTTATTCTAATGACTTTGGTTTTTCCAAGCTTAATAAAA GTTTTCCTATGAATTCACAGGCGCACATACCCATCACTGTATATATATAT ATATCTATGATATATTGGGTTTTTAT	2,393 bp
R8Δ	CCTCTCAATCACCCCGATTTGTCTTCATCAGTGTTTTGTTTGATGGCTTCA TTGGCCAGGGAATTCATTCATTCATCTAGCTATTGATTCTTGGATCTATCC ACTTGTCTGAGTGGCACACCGTAA	3,933 bp
R9Δ	TTCAGCAGGAGGAGCATCGGATATTCCATACCAGCAATTGGCCATTGTGT CCGCTCCTCTGGATCCGGCAAGCTACCGAGTGATGATACTGCTGGTACGG TAATGCTGCTTCTGTGGCATCGGCAA	4,871 bp
PRE12Δ	TGAGTTGATTGAAGGCTGTAAGAGCAGATTACAGTGGGGCGGAGGCCCA AGTCTGGATCTGCCGGCCTCTGGGTATTCATTTTTTTCGACTTGGCAATTG CAAATGCAAAACCATTTCATTTGCCG	10,285 bp Deletion
MnnΔ	GAGAAAAAAAAACAAGAAAACAAAAACAAAACGCGCAGCCGAAAATCG GCAACAACAAAAGCGAGGAAGAGCTAAATGTGTGGGAGGACAACTGGG ACGACGACAACGTGGAGGACGACTTC	7,656 bp Deletion

Table S5. CRISPR Insertion Mutants, Related to STAR Methods

Mutant Name	Homologous Bridge	Mutation
Ins1	ACACAAGATACCAGGATCCAAAAATGAATGCTGCCAAATTCACTTTAGGTGTAT ATTCTTAACAAGGTCTGTTGAATTCATGATGGTTTATAAAATGAAAGTAAACTAC CTCCTGTAAGATGTAAATTAAAAGCACCCTTATAAATTTGTAAATGCTTTTAAAC ACTAAAGTTAGTTTTTTTCTAAGTATAACCCCATTGTCCTTGCTCGTCATTAACCG TAGAAATTGTTTATGACAAACAATTCAAATTGGGAAACTTGAAAGACAAAGTTA TCATAAAATATAATTGCCAGGGCGGACAAGTATTAATTCAAAAAAAAATTCCAT CACTTATTTATTTAGTAATTTCGAGAATTTGTCAGGTCGTGGAACTTGTTTTTTCT TCAGACTGTTTAAGTAATATTTCATTTTAATTAATATAATTTGTTTAAGATAATTG GTCAACAGACTAAAGTGTTTTATGGATCAATTGGAAATTTGCGTGCCAGTTTTGG TAACAGTTTAGTTAATGGCTTTATTGATTTGGTTAAGAGGGGGGAGGGCCCTAAA GCTGGGATAGGGGTGTTTCAGTAACTTCACTATTTTTGTGAACACACTTTTCCTTT TCCTTTTCAGCTAAGTAATAACTCTGGTAGTATATACTAAAATTTTATTTAACATG CATTTTGCATTTGCATATGTAATGAGAGAAGTGACTTATATACTTATTTATTGCG GCTATTCGGTGAGACAATTAAAACATAATAAAGTACTATGCATTATACAATTTTA AATTATTTTTATAGGGAGACCCACCTGAAACTCGAGGCACACCTCTTCTGGCTAA CACTCATCGTCAGTAAATAATATTCGTATAAAAATGCGCAAATAAAAAAAAAAT GAAGAAGCAGAAGAAGAAAAGGAGGGAAAATGTTTAAAAATAAAGTCATAATT TCTGGAATGATTTATGAATTGCACCGCGGAGGGGGAACAGGCCAGCAGACAGGT GAAGAGGAGGTTGGGGAGGACTTTGGACCCAACAGGGTCTCTCTCCATTATTAC ACATTTTATTTAATTTCTTTTTGTGGCCCGCTCTTATTGCAACGCTTTTTAAATTA ATTTCTTCGCTGAATTGCATTTTTAGCTCCAGTTCGAGTTGGCTTCAATTCGCATT TCTCAGTCGGGCGGACGGGGGTGGGTGTGTGAAAGGGGGGAAATCGGGGATCTC AGATCGGCATTAGCATAACCCGACACGCATGCCGTCTGCTGTCTGCCCGAGGGC AGGGTTGCCCGCGGTTGGGGAAGGGGGTAAGGTGAATTCTCCTACCCAGAGAGC TTGTTAAATTTCGACATGGCCTCTTATGATTAGCTGAATTATGCTTTTAAAATTTG CATTGCGATAAGATCGAGCTTAGCGCCTAGCCAGCTTCGATGCCTCCGACGCAG CGGCATTATCATTTGAATGGGATATGCATAAAGAAGCTTGTGAGAGAGGGCCAA TGGCTTGGCAAAGGGTTAACCTGATAATCAAACCATTAAGCTGTCTTAAAATGCT CCGACTAATTGCAGACAGCAGGGTTTCCCAGCCCTGTCTAAAGCACAAAGCCTG TTCAGTCGGAGTTTGATAGTGAAAACATGATCCTAATCTATGCAAAG	40 bp Deletion, ~2,200 bp Insert
Ins2	GTGAGTGTCCCCCGAATAAGGCAATACAACAACAATAGAGGCGAAAACAGAAA CAAAAGCGAAACGCAGCACGCGATACCCTGTAACTGGAGGTTCTGTAAAGGCTT CGAGGAGTCACGTGTGCGAATTGCAGACCGCCGGCGGTCATTCACTTCTATCTAT TTTACTATTCCAACACACCCACGCGCGCGTGTGTGTGTGCGTGCCTGAAAAATGT CACTTTGACAACTCGCCTCTGGTTGCTCTGCCGGCGGATCTGCCTACGTCGACGT CGATCGTGCCGCAAAATGAAAATGATTTGCGTGCCGTTCTTGCGTTCGGAAAAG CGAAGAACCAGGATCTATTTAGGATAAGCACGCAGTTGATAGATTGGTTATCCA ACTCATCTAATATGGCGTGTAATGTTCGTCTACTTAGTTGTAGTCGGATTAGTAA ACCAGTTATTAACAGTATAGCTCCATTTCAAAGATACTCTACGGGGAATATTTAC TATTAACCGCTTAGCTTTAGTATTAAAATTTTACATTAGATGT	300 bp Deletion, ~3,200 bp Insert
Ins3	GTGAGTCCCGAGTCCAGCTAAAAGAACCCCTTCCCCATCAACTCCTCTCAATCAC CCCGATTTGTCTTCATCAGTGTTTTGTTTGATGGCTTCATTGGCCAGGATACGTGG TGTGTTTTCCTGACTGGGTGGGGGTAAAGGGGAATAATCAATCAGCGTCTGGTTA GCCGGGAGAGTTTCCATTGATAAATACTGGGCACGCAATAGAGAGTCGGTGGTG AAGGTCTGGGAAAATCCACTTCCTTCACTAATATTCTGTCATTTGTAATTGTCTGC AATTAATAAGTTCTGGAATTGATTAACTTAATTGAGGTGGGAAAGGGATGGGGC GCAGAAACAATTAGACTGTGGGGAGAAATCTTTCTTGGCCGTATAAAAAGGTAA AATGCCGCAAGAAATGAAAAGAGATGAGCACAAAAAAAAATCAGCGTACAAAAA GAAAACAACAACCCGTTAGCAGGACTCATATACAATATTTCAGATACCCTTTTTT AAGATGTCACTAAAATTGTATTTAATGTAATATCCTTTATGCGGTATCTG	1 bp Deletion, ~3,200 bp Insert
Ins4	GCCTCTTCGCACACAACCTCTCCGCAGCAGCAACAACAGCAGCAGCTCCAACAA TCACAGCGAGGAGTTGGTTCCAATGTTAGCGCTCCCAGCAGCTCACTCACTAGCA CGGGCACAGATCAGCAGGCGGTGCACCCATCCAGTTGCCATCAACAACAGCAGC AGCAGCAGCAACAACACCATCACGCCCACCCGCATCCGCACTCGCATCATCACC ACCATCATCACCATCACCATGAGACGGCGCATCAGCACAGCAGCGAGGTATGGA CGCCCGCCTCCTCAAAACAATCAGAGCGAAATAAATCTAGAAAATAATCGAATC ACTTAATGGTCTTTTAATTCAACATCCTTGATCCAACGCTATTCATGTAACAATTT TTAGGTACCTTTACAACACGTATATTTGAATAGACCGGCGTATTACAAACAATTA AGATCTGTTGCCCATTTTGCCCAAGAGTATGCCACAAATATGGCATGTGCAAAGT AGCCCCAAAAATATGCCACGAAAGTCTGGTAAC	2 bp Deletion, ~3,200 bp Insert

Table S6. Oligopaints Probe Libraries, Related to STAR Methods

Probe Set	Oligo Paints Library	Coordinates	Conjugated Fluorophore
ss RNA	ss-Full-RNA	3R: 16,370,515-16,435,663	Cy3
ss DNA	ss-Ext-Univ	3R: 16,370,516-16,435,663	Cy3
ss Upstream DNA	50-kb extension (left)	3R: 16,320,533-16,370,533	Alexa 488
ss Downstream DNA	50-kb extension (right)	3R: 16,435,681-16,485,681	Cy5