Drosophila *small ovary* encodes a zinc-finger repressor required for ovarian differentiation

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Abstract

Repression is essential for coordinated cell type-specific gene regulation and for controlling the expression of transposons. In the *Drosophila* ovary, stem cells regeneration and differentiation require well-controlled expression, with de-repression leading to tissue degeneration and ovarian tumors. Additionally, the ovary is acutely sensitive to deleterious consequences of transposon de-repression. The *small ovary* (*sov*) locus was identified in a female sterile screen shows dramatic effects ovarian morphogenesis. We mapped the locus to the uncharacterized gene *CG14438* and reveal it encodes a zinc-finger protein that colocalizes with the essential Heterochromatin Protein 1 (HP1a). The distinct functions of Sov we report, including repression of inappropriate signaling, transposon silencing, and effects on position-effect variegation in the eye, suggest a central role in heterochromatin stabilization.

Introduction

Multicellular organisms rely on stem cells. A combination of extrinsic and intrinsic signals function to maintain the balance between self-renewal of stem cells and the differentiation of progenitors needed for tissue homeostasis and development. The *Drosophila* adult ovary is a well-studied model for development (Fuller and Spradling 2007). It is organized into ~16 ovarioles, which are assembly lines of progressively maturing egg chambers. At the anterior tip of each ovariole is a structure called the germarium, harboring 2-3 germline stem cells (GSCs), which sustain egg development throughout the life of the animal. Surrounding the GSCs and their cystoblast daughters is a stem cell niche, a collection of nonproliferating somatic cells composed of terminal filament and cap cells. Like the GSCs, somatic stem cells divide to produce new stem cells and escort cells, which encase the germline. Each cystoblast is fated to differentiate and will undergo four rounds of cell division with incomplete cytokinesis to

49 produce a germline cyst interconnected by the fusome, a branched cytoskeletal structure.
50 Germline cysts are surrounded by two escort cells. Toward the posterior of the germarium, a
51 monolayer of somatic follicle cells replaces the escort cells to form an egg chamber.

52 Egg chamber development requires the careful coordination of distinct germline and
53 somatic cell populations through signaling within a complex microenvironment. For example,
54 extrinsic signals and transducers including Jak/Stat and the BMP homolog, Decapentapalegic
55 (Dpp), are required to maintain the proliferative potential of GSCs (Bausek 2013; Gilboa 2015;
56 S. Chen, Wang, and Xie 2011). Oriented divisions displace the primary cystoblast cell away
57 from the stem cell preserving signals and permit the expression of differentiation factors, such
58 as *bag-of-marbles* (*bam*). The importance of bam is revealed in bam loss-of-function mutants,
59 resulting in tumorous germaria filled with undifferentiated germline cells containing many
60 spectrosomes, the dot-fusome structure characteristic of GSCs and primary cystoblasts, as a
61 result of complete cytokinesis (Lin and Spradling 1995; D. McKearin and Ohlstein 1995).
62 Controlling this signaling requires repression in addition to activation.

63 Dynamic changes in chromatin landscapes within the germline and somatic cells are
64 critical for oogenesis (Barton et al. 2016; X. Li et al. 2017; Börner et al. 2016; Peng et al. 2016;
65 Soshnev et al. 2013; McConnell, Dixon, and Calvi 2012). Nucleosomes generally repress
66 transcription by competing for DNA binding with transcription factors (Lorch and Kornberg
67 2017; Kouzarides 2007; Jenuwein and Allis 2001). Further levels of repression depend largely
68 on histone modifications. For example, the activities of both the H3K9 methyltransferase
69 SETDB1, encoded by *eggless* (*egg*) (X. Wang et al. 2011; Clough et al. 2007; Clough,
70 Tedeschi, and Hazelrigg 2014), and the H3K4 demethylase encoded by *lysine-specific*
71 *demethylase 1* (Di Stefano et al. 2007; Rudolph et al. 2007; Eliazer, Shalaby, and Buszczak

72 2011; Eliazer et al. 2014) are required in the somatic cells of the germarium for GSC
73 maintenance, normal patterns of differentiation, and germline development. Genome-wide
74 profiling hints at a progression from open chromatin in germline stem cells to a more closed
75 state during egg chamber differentiation (T. Chen and Dent 2014). Disrupting this progressive
76 repression in the ovary could result in stem cell over-proliferation and defective development.
77 There are other roles for regulated chromatin states in oogenesis. The germline has
78 been repeatedly hijacked to promote the vertical transmission of transposons resulting in a
79 loss of fitness in the *Drosophila* host (Charlesworth and Langley 1989; Pelisson et al. 2002).
80 This increased mobilization of transposons in gonads is countered by host responses, such as
81 induced heterochromatin formation, to repress transposon activity. The proximity of condensed
82 heterochromatin can dampen the activity of genes and transgenes (Elgin and Reuter 2013).
83 The conserved Heterochromatin-Protein 1a (HP1a), encoded by *Su(var)205*, is critical for
84 heterochromatin formation (James and Elgin 1986; Eissenberg et al. 1990; Clark and Elgin
85 1992).
86 Originally described over 40 years ago (J. D. Mohler 1977), the *small ovary* (*sov*) locus
87 mutants have range of phenotypes including disorganized ovariole structure, egg chamber
88 fusions, and undifferentiated tumors, culminating in complete ovarian degeneration (Wayne et
89 al. 1995). In the present work, we demonstrate *sov* encodes an unusually long 21 C_2H_2 Zinc
90 finger (ZnFs) nuclear protein that colocalizes with HP1. Previous work showed this ZnF protein
91 in complex with HP1a (Alekseyenko et al. 2014). RNA-seq analysis shows that *sov* activity is
92 required to repress the expression of a large number of genes in the ovary as well as
93 transposons. Additionally, *sov* mutations are strong dominant suppressors of Position Effect
94 Varigation (PEV) in the eye, a key characteristic of HP1 (Elgin and Reuter 2013), which is

95 consistent with Sov and HP1a colocalization in the nucleus. These data indicate that Sov is a
96 potent repressor of gene expression.
97
98 **Results**
99 *small ovary is CG14438*
100 To localize *sov*, we used complementation of female-sterile and lethal *sov* mutations with
101 preexisting and custom-generated deficiencies, duplications, and transposon insertions to
102 refine previous mapping (J. D. Mohler 1977; D. Mohler and Carroll 1984). Our results (Fig
103 1A,B) suggested that either the protein-coding *CG14438* gene or the intronic, noncoding
104 *CR43496* gene was *sov*. The *sov* locus mapped to the non-complementing deletion *Df(1)sov*.
105 Rescue of female sterility and/or lethality of sov^{2}, sov^{ML150}, sov^{EA42} and *Df(1)sov* with
106 $Dp(1;3)sn^{13a1}$ (molecularly undefined, not shown), *Dp(1;3)DC486*, and *PBac{GFP-sov}*
107 confirmed our mapping. We could not replicate separability of sov^{EA42} sterility and lethality with
108 $Dp(1;3)sn^{13a1}$ (Wayne et al. 1995). Female sterile and lethal alleles of *sov* map identically.
109 We performed genome sequencing to determine if the lesions in *sov* alleles occur in
110 *CG14439* or *CR43496* (Table S1). While *CR43496* contained three polymorphisms in *sov*
111 alleles relative to the reference, we identified the same polymorphisms in all mutant *sov* alleles
112 and in sov^{+} alleles in w^{1118} and *Oregon-R* lines. Thus, *CR43496* is highly unlikely to be *sov.* In
113 contrast, we found disruptive mutations in *CG14438* (Fig 1C), which encodes an unusually
114 long 3,313 residue protein with 21 C_2H_2 ZnFs, nuclear localization motifs, and coiled-coil
115 regions (Fig 1C). We identified a nonsense mutation (G to A at position 6,764,462) in the
116 *CG14438* open reading frame of sov^{EA42} which is predicted to truncate the *sov* protein before
117 the ZnF domains. We found a frameshift insertion (T at position 6,769,742) located towards the

118 end of *CG14438* in *sov*^{2} that encodes 30 novel residues followed by a stop codon within the C-
119 terminal ZnF, as well as removing the terminal predicted NLS motif (Fig 1C). We found a
120 missense mutation (C to G at position 6,763,888) in *sov*^{ML150} that results in a glutamine to
121 glutamate substitution within a predicted coiled-coil domain. While this is a conservative
122 substitution, and while glutamate residues are common in coiled-coil domains, glutamine to
123 glutamic acid substitutions are especially disruptive in the coiled-coil region of the sigma
124 transcription factor (Hsieh, Tintut, and Gralla 1994). We conclude that *CG14438* is *sov*.
125
126 *sov expression*
127 To determine where *sov* is expressed and if it encodes multiple isoforms, we analyzed
128 expression in adult tissues by RNA-Seq. We noted that *sov* is broadly expressed as a single
129 mRNA isoform in adult tissues, with highest expression in ovaries (Fig 1D). The modENCODE
130 (Graveley et al. 2011; J. B. Brown et al. 2014) and FlyAtlas (Robinson et al. 2013; Leader et al.
131 2018) reference sets show a similar enrichment in the ovary, as well as in early embryos. The
132 enrichment of *sov* in the ovary is consistent with its role in oogenesis.
133 To determine if and where Sov was expressed in the ovary, we examined the
134 distribution of GFP-Sov in developing egg chambers (Fig 2A). We observed GFP-Sov signal in
135 all cell types. By pairing localization analysis of Sov with antibodies recognizing Vasa (Vas) to
136 label the germline (Fig 2B), we observed particularly striking nuclear localization of Sov
137 surrounded by perinuclear Vas in the germline cells within region 1 of the germarium, with
138 highest levels evident within the GSCs (dashed circles). We also used Traffic jam (Tj) to label
139 the soma (Fig 2C). We observed Sov nuclear staining in the Tj positive somatic and follicle

140 stem cells (dashed ovals) (Fig 2C). These data indicate that Sov is expressed in the germline
141 and soma in the ovary.
142
143 *Sov function*
144 To examine *sov* function in the germarium, we compared a strong allelic combination,
145 sov^{2}/sov^{EA42} to cell-type specific knockdown of *sov* using a UAS short hairpin *sov* RNAi
146 construct (P{TRiP.HMC04875}; hereafter, sov^{RNAi}). The germaria of heterozygous *sov* females
147 are wild type (Fig 3A), but the *sov* mutants often show an ovarian tumor phenotype, with
148 greater than the expected number of germ cells with dot spectrosomes (Fig 3B), indicating that
149 germ cells undergo complete cytokinesis. Ovarian tumors were observed in ~40% of *sov*
150 mutants (N=12/31 sov^{EA42}/sov^{2} egg chambers versus N=0/47 controls). These findings are
151 consistent with GSC hyperproliferation and/or failed differentiation. We also observed tumors
152 and nurse cell nuclei residing within common follicles (Fig 3B) in about one-third of *sov*
153 mutants (N=11/31 sov^{EA42}/sov^{2} versus N=0/24 in controls). This phenotype occurs when follicle
154 cells either fail to separate egg chambers, or where those chambers fuse (Goode, Wright, and
155 Mahowald 1992). In older *sov* ovaries, we observed extensive cell death (Fig S1). Additionally,
156 we found that rare sov^{EA42}/Y males that escaped lethality had no germ cells (Fig S2). Thus, *sov*
157 functions widely.

158 To examine cell-type specific functions of *sov*, we used *tj-GAL4* to express sov^{RNAi} in
159 somatic escort and follicle cells and *nanos-GAL4* (*nos-GAL4*) to express sov^{RNAi} (or
160 $mCherry^{RNAi}$ controls) in the germline. Relative to the controls, the $tj>sov^{RNAi}$ germaria (Fig
161 3C,D) were abnormal, showing ovarian tumor phenotypes similar to those seen in *sov*
162 mutants. Germariums were often filled with germline cells with dot spectrosomes and the

163 follicle cells encroached anteriorly. Similar results were also observed using other somatic
164 drivers (*c587-GAL4* and *da-GAL4*). Interestingly, *nos>sov*^{RNAi} did not impaired germaria
165 morphology (Fig 3E,F) and egg chambers representing all 14 morphological stages of
166 oogenesis appeared phenotypically normal. However, eggs produced from *nos>sov*^{RNAi}
167 mothers arrested during early embryogenesis, indicating that maternal *sov* is required for
168 embryonic development. While the expression patterns and germline RNAi suggest that *sov* is
169 deployed in both the soma and germline, Wayne et al. (1995) reported *sov* to be somatic line
170 dependent. We conducted a germline clonal analysis and found instead that *sov*$^{+}$ is required in
171 the germline (Fig S3). Additionally, *sov*^{RNAi} resulted in embryonic germline defects in a high-
172 throughput study (Jankovics et al. 2014). Taken together, these data indicate that there are
173 both somatic and germline requirements for *sov* in oogenesis.

174 To explore the differentiation of the germline further, we examined the distribution of
175 Bam, which is expressed in germarium region 1 germ cells (D. M. McKearin and Spradling
176 1990; D. McKearin and Ohlstein 1995; Ohlstein and McKearin 1997). In the absence of Bam,
177 germ cells hyper-proliferate, resulting in tumors composed of 2-cell cysts. Given the
178 prevalence of undifferentiated tumorigenic germ cells upon somatic *sov*^{RNAi} knockdown, we
179 asked if *sov* has a non-autonomous role in promoting Bam expression in germ cells. Indeed,
180 somatic knockdown of *sov* results in significantly less Bam expressed in germ cells relative to
181 controls (Fig 3G,H; Fig S4). Both *tj-GAL4>sov*^{RNAi} and *c587-GAL4>sov*^{RNAi} resulted in strong
182 repression of Bam expression. Germline depletion of *sov* did not alter Bam localization (Fig
183 S4). The reduction in Bam expression in the germline is consistent with a non-autonomous role
184 of Sov function in the soma for differentiation signals directed to the germline.

185 To examine the defective follicle encapsulation of the germline cysts following *sov*
186 depletion (resulting in fused follicles), we used the oocyte-specific expression of Oo18 RNA
187 binding protein (Orb) (Lantz et al. 1994) to count the number of oocytes per cyst. Orb specifies
188 the future single oocyte at the posterior of the 16-germ cell egg chamber in control egg
189 chambers (Fig 3I, Fig S2D). When we examined ovaries where *sov* expression had been
190 knocked down in somatic cells, we found examples of both egg chambers with either too many
191 oocytes (Fig 3J) or no oocytes (Fig 3K, Fig S4). In a wild type 16-cell germline cyst, one of the
192 two cells that has four ring canals becomes the oocyte, and this feature can be used to
193 determine if extra germline divisions had occurred in cysts, or if multiple cysts were enveloped
194 by the follicle cells. We saw that the egg chambers with multiple Orb+ cells always had more
195 than 16 germ cells, and in the example shown, all three Orb+ cells had four ring canals,
196 indicating that egg chamber fusion had occurred (Fig 3J). Taken together, we conclude that
197 Sov functions in the soma to ensure the proper differentiation of the germline.
198
199 *Sov represses gene expression in the ovary*
200 To provide mechanistic insights into the function of Sov, we performed transcriptome profiling
201 using triplicated Poly-A$^+$ RNA-seq analyses of ovaries from *sov*, *sov*^{RNAi}, and control females.
202 The gene expression profiles of ovaries from sterile females were markedly different from
203 controls, primarily due to de-repression in the mutants (Fig 4A; Table S2). Among genes
204 showing differences in expression (FDR *p*adj < 0.05), we found 1,752 genes with >4-fold
205 increased expression in mutants, while there were only 172 genes with >4-fold decreased
206 expression (Table S3). To explore where these de-repressed genes are normally expressed,
207 we examined their expression in other female tissues and in testes in a set of quadruplicated

208 RNA-seq experiments (a lab resource for this and other studies; Fig 4B). Many of the genes
209 suppressed by Sov in ovaries were highly expressed in other female tissues, or in the testis,
210 indicating that wildtype Sov prevents ectopic gene expression.

211 To determine what types of genes are de-repressed in *sov* mutants, we performed
212 Gene Ontology (GO)(Gene Ontology Consortium 2015) term analysis (Fig S5; Table S4). The
213 genes with lower expression in *sov* mutants had only a few significant GO terms that were
214 oogenic in nature (as expected given the general lack of mature eggs in *sov* mutants). For
215 example, there was poor expression of the chorion genes that are required to build the
216 eggshell (Orr-Weaver 1991), as well as genes required for follicle cell development. In
217 contrast, the de-repressed genes in *sov* mutants showed that there was a significant
218 enrichment of genes with cell signaling GO terms, including neuronal communication. We
219 found that many genes repressed by Sov in the ovary are indeed expressed in the head. For
220 example, the *heartless* (*htl*) locus, which encodes a FGFR tyrosine kinase receptor important
221 for neuron/glia communication (Stork et al. 2014), was de-repressed in *sov* ovaries and highly
222 expressed in the head (Fig 4C). This intriguing result suggests that *sov* normally functions to
223 repress a host of signaling pathways. This de-repressed signaling in *sov* mutants is likely
224 catastrophic for communication between various somatic cells and the germline during egg
225 chamber development. This could well explain the variety of mutant phenotypes observed in
226 *sov* mutants.

227 The general repressive role of *sov* was also revealed by a dramatic and coherent
228 elevation of transposon expression in the mutants (Fig 4D; Table S3). 138 transposable
229 element classes were detected in our gene expression profiling of *sov* mutants and controls.
230 Of these, 91 had increased expression (FDR *p*adj < 0.05) >4-fold in *sov* mutants, while none

231 had >4-fold decreased expression. The DNA, LINE, and LTR families of transposable
232 elements were all de-repressed in *sov* mutants. Interestingly, the somatic knockdown of *sov*
233 resulted in the most dramatic de-repression of the *gypsy* and *copia* classes of transposons,
234 which are *Drosophila* retroviruses that develop in somatic cells and are exported to the
235 developing germline (Yoshioka et al. 1990). Thus, wild type *sov* may be important to protect
236 the germline from infection. Equally interesting, germline knockdown resulted in greatest de-
237 repression of the *HeT-A*, *TAHRE*, and *TART* transposons that compose the telomere (Mason,
238 Frydrychova, and Biessmann 2008). Poorly formed telomeres result in loss of material from
239 chromosome ends and telomere fusion, which results in poor anaphase separation and
240 aneuploidy (Cenci, Ciapponi, and Gatti 2005). This finding suggests that the general role of
241 *sov* in silencing transposon expression includes control of both transposon functions
242 necessary for normal cellular metabolism, exemplified by the telomere transposons, as well as
243 detrimental activities of retrovirus-like elements. In addition to widespread dis-regulation of
244 signaling and development pathways in *sov* mutants, transposon dysgenesis may contribute to
245 the severe defects in *sov* ovarian development.
246
247 *Sov is a Suppressor of PEV and co-localizes with HP1*
248 The repressive function of Sov is reminiscent of HP1a function as general repressor of gene
249 expression. HP1a was first characterized in *Drosophila* as a suppressor of PEV, a process that
250 reduces gene expression due to spreading of heterochromatin into a gene region (Clark and
251 Elgin 1992). To test the hypothesis that *sov* negatively regulates gene expression by
252 promoting heterochromatin formation, we examined the role of *sov* in PEV in the eye, where
253 patches of white^{+} (red pigmented) and white^{-} (not red pigmented) eye facets are easily

254 observed (Fig 5A). If, like HP1a, Sov represses gene expression by promoting
255 heterochromatin formation (Eissenberg et al. 1990), then it should suppress PEV, which would
256 be seen as increased eye pigmentation. We obtained five different variegating w^+ transgene
257 insertions associated with either the heterochromatic pericentric region of chromosome arm 2L
258 or spread along the length of the heterochromatin-rich chromosome 4. In control animals,
259 these insertions show a characteristic eye variegation pattern (Fig 5B). Consistent with the
260 allelic strengths seen in previous experiments, the weak sov^2 allele did not suppress PEV (Fig
261 5C) but the stronger sov^{ML150}, sov^{EA42}, and *Df(1)sov* mutations dominantly suppressed PEV
262 (Fig 5D–F). These data demonstrate a role for Sov in heterochromatin formation.
263 The strong repressive function of Sov is reminiscent of HP1. If Sov is in complex with
264 HP1a (Alekseyenko et al. 2014), then Sov and HP1 should colocalize in cells. To see if this
265 was true, we followed HP1a-RFP and GFP-Sov localization in 1–2 hr live embryos where both
266 are abundantly expressed (Rudolph et al. 2007). During *Drosophila* cleavage, nuclei undergo
267 rapid synchronous nuclear divisions prior to cellularization at cleavage division/nuclear cycle
268 (NC) 14 (Foe and Alberts 1983). HP1a and Sov colocalized in all NC 10–12 embryos we
269 examined (Fig 6A; N=7 embryos). During prophase, both HP1a and Sov were enriched in
270 regions of condensed DNA (Fig 6A, 4:30). Whereas low levels of HP1a decorated DNA
271 throughout division, Sov was depleted during mitosis (Fig 6A, 10:00 and 11:10). Upon re-entry
272 into interphase, Sov localization to nuclei resumed and was coincident with HP1a. Formation
273 of heterochromatin is contemporaneous with or slightly proceeds HP1a apical subnuclear
274 localization in NC 14 (Rudolph et al. 2007; Yuan and O'Farrell 2016). At this stage, we
275 observed a strong colocalization of HP1a and Sov. Measuring the distribution of HP1a and Sov
276 (Fig 6B,B') confirmed high levels of colocalization within HP1a subnuclear domains (Fig 6C,

277 shaded region). These data support the idea that HP1a and Sov are deposited maternally
278 where they assemble into a complex.
279
280 **Discussion**
281 Sov is a novel heterochromatin-associated protein
282 Genomes contain large blocks of DNA of potentially mobile transposons and immobile mutated
283 derivatives (Vermaak and Malik 2009). The cell keeps these transposons from wreaking havoc
284 on the genome by actively suppressing their expression through condensation into
285 heterochromatin. However, some tightly regulated expression from heterochromatin is required
286 for normal cellular function. For example, the telomeres of *Drosophila* are maintained by
287 transposition of mobile elements from heterochromatic sites (Mason, Frydrychova, and
288 Biessmann 2008), and histone and rRNA genes are located within heterochromatic regions
289 (Yasuhara and Wakimoto 2006). Weakening of heterochromatin by suppressors of variegation
290 results in de-repression of gene expression at the edges of heterochromatin blocks,
291 suggesting that the boundaries between repressed and active chromatin can expand and
292 contract (Weiler and Wakimoto 1995; Reuter and Spierer 1992). HP1a, encoded by
293 *Su(var)*205*,* is a central component of heterochromatin (Ebert et al. 2006) that shows the same
294 strong suppression of variegation that we observed in *sov/*+ flies. Similarly, HP1a is also
295 required to repress the expression of transposons (Vermaak and Malik 2009).
296 Repression is often stable and emerging themes suggest a robust set of activities,
297 rather than a single component, maintain a chromatin state. For example, long-term repression
298 is stabilized with complexes, such as the repressive Polycomb Group (PcG) which provides an
299 epigenetic memory function (Kassis, Kennison, and Tamkun 2017). In order to create a stable

300 epigenetic state, the PcG complexes have subunits that modify histones and subunits that bind
301 these modifications. In addition to being tethered to the chromatin via histones, the PcG
302 complexes also include DNA-binding proteins, such as the YY1-like ZnF protein Pleiohomeotic
303 (Pho) that further reinforce localization. The DNA anchor proteins in PcG complexes are at
304 least partially redundant with the histone-binding components (J. L. Brown et al. 2003),
305 suggesting that localization is robust due to multiple independent localization mechanisms.
306 In mammals, HP1a also has both histone and DNA anchoring. DNA anchors are
307 provided by a family of KRAB ZnF proteins that have undergone a massive radiation during
308 evolution (Yang, Wang, and Macfarlan 2017; Ecco, Imbeault, and Trono 2017). KRAB proteins
309 are essential for repression of transposons and more generally for a properly regulated
310 genome. The KRAB family members use a KRAB-Associated Protein (KAP1) adapter protein
311 to associate with HP1a and the SETDB1 methylase that modifies histones to enable HP1a
312 binding (Fig 7). Although KRAB proteins have not been identified in *Drosophila*, the *Drosophila*
313 *bonus* (*bon*) gene encodes a KAP1 homolog (Beckstead et al. 2005). *Drosophila* also has a
314 SETDB1 encoded by *egg*, and loss of *egg* results in ovarian phenotypes reminiscent of those
315 observed in *sov* loss-of-function females (Clough et al. 2007; Clough, Tedeschi, and Hazelrigg
316 2014; X. Wang et al. 2011). Like *sov* and HP1a, *bon* is a modifier of variegation, raising the
317 possibility that they collectively coordinate gene regulation. In support of this hypothesis, Bon,
318 Sov, and Egg were all identified in the same biochemical complex as HP1a (Alekseyenko et al.
319 2014). It is tempting to speculate that the single very long and ZnF-rich Sov protein plays the
320 same direct tethering role as the large family of KRAB ZnF proteins in mammals (Fig 7). If Sov
321 uses subsets of fingers to bind DNA, it could localize to many different sequences in a
322 combinatorial fashion. Additionally, the complex containing Sov and HP1a also contains RNA

323 (Alekseyenko et al. 2014) and could act as a tether via a series of RNA intermediates as
324 occurs in sex chromosome inactivation, for example (J. T. Lee 2009). As in the case of Pho in
325 the PcG complexes, Sov might be a robustness factor rather than an absolute requirement, as
326 we did not observe gross delocalization of HP1a following *sov*^{RNAi} (not shown). Further work
327 will be required to fully understand the relationship between Sov and HP1a.
328
329 Repression by Sov promotes germline differentiation
330 The fact that there have been many female sterile alleles of *sov* isolated suggests that
331 the ovary is particularly sensitive to reduced *sov* activity. The female sterility phenotype is
332 complex and somewhat variable, but partial *sov* loss of function is characterized by somatic-
333 dependent differentiation defects. Phenotypes include defective follicle cell encasement of egg
334 chambers, resulting in follicles with multiple oocytes, and accumulation of germline tumor cells
335 that undergo complete cytokinesis instead of forming mature and differentiated 16-cell egg
336 chambers. Stronger alleles also show a germline-dependent block in development and
337 lethality. Diverse functions sometimes result from isoform and/or localization diversity. While
338 *sov* functions seem diverse, the locus expresses a single major isoform and Sov seems to be
339 consistently located in the nucleus. It is more likely that one mechanism account for all *sov*
340 function. We suggest that Sov in regulates the chromatin landscape that helps repress stem
341 cell identity and promote differentiation. This same mechanism represses ectopic signaling and
342 tissue-inappropriate responses in the ovary, controls heterochromatin formation in the eye and
343 represses transposons.
344 We observed dramatic de-repression of transposons in *sov* mutants. In addition to our
345 observations, Czech et al (2013) found evidence for a role of *sov* in transposon repression in a

346 genome-wide screen. Transposon repression raises the possibility that Sov interacts with the
347 Piwi pathway (Brennecke et al. 2007; Yin and Lin 2007; Teixeira et al. 2017) which directly
348 interacts with HP1a to facilitate heterochromatin formation and promote transposon silencing
349 (Brower-Toland et al. 2007; S. H. Wang and Elgin 2011). However, genes involved more
350 strictly with transposable element regulation, such as Piwi, do not have strong dosage effects
351 on PEV (Gu and Elgin 2013), while *sov* and *HP1a*/*Su(var)205* do. This distinction suggests
352 that Sov has a more general effect on heterochromatin, rather than specificity for transposon
353 repression. Nevertheless, interplay between Piwi, Sov, and HP1a remains possible.
354

355 Conclusions

356 Our data show that *sov* encodes a repressor of gene expression and transposons in the
357 ovary. In the absence of *sov*, genes that are normally expressed at very low levels in ovary,
358 are activated. These same genes show dynamic patterns of expression in other adult tissues,
359 suggesting that they are de-repressed in the absence of Sov. The failure to restrict gene
360 expression results in a range of phenotypes including ovarian tumors, defective oogenesis,
361 and tissue degeneration. These data support the idea that Sov encodes a novel and essential
362 protein that is generally repressive, possibly via interactions with HP1a.
363

364 **Materials and Methods**

365 We have adopted the FlyBase-recommended resources table which includes all genetic,
366 biological, cell biology, genomics, manufactured reagents and algorithmic resources used in
367 this study (Table S5).
368

369 Flies and genetics

370 The *small ovaries* (*sov*) locus was defined by three non-complementing female sterile
371 mutations including sov^2, which mapped to ~19 cM on the X chromosome in the 5D5-6;6E4-5
372 interval (D. Mohler and Carroll 1984; J. D. Mohler 1977), refined to 6BD (Wayne et al. 1995).
373 Cook *et al.* (2012) placed *sov* in the 4-gene *CG14438–shf* interval (Fig. 1; Table S5). *sov*
374 mutations complemented shf^2, but not $P\{SUPor\text{-}P\}sov^{KG00226}$ and $P\{GawB\}sov^{NP6070}$,
375 suggesting that *CG14438* or *CR43496* were *sov*, which we confirmed by generating *Df(1)sov*
376 (X:6756569..6756668; 6770708, 6C12;6C13) as a FLP-induced recombination event between
377 $P\{XP\}sov^{d07849}$ and *PBac{RB}e03842* (Parks et al. 2004; Cook et al. 2012) to remove only
378 *CG14438* and *CR43496.*

379 We used the dominant female sterile technique for germline clones (Chou and Perrimon
380 1996). Test chromosomes were free of linked lethal mutations by male viability (sov^2) or rescue
381 by *Dp(1;3)DC486* (sov^{EA42} and *Df(1)sov*), were recombined with $P\{ry^{+t7.2}=neoFRT\}19A$, and
382 verified by complementation tests and PCR. We confirmed $P\{ry^{+t7.2}=neoFRT\}19A$ functionality
383 by crossing to $P\{w^{+mC}=GMR\text{-}hid\}SS1$, y^1 w^* $P\{ry^{+t7.2}=neoFRT\}19A$; $P\{w^{+m*}=GAL4\text{-}ey.H\}SS5$,
384 $P\{w^{+mC}=UAS\text{-}FLP.D\}JD2$ and scoring for large eye size. We crossed females with FRT
385 chromosomes to $P\{w^{+mC}=ovoD1\text{-}18\}P4.1$, $P\{ry^{+t7.2}=hsFLP\}12$, y^1 w^{1118} sn^3
386 $P\{ry^{+t7.2}=neoFRT\}19A/Y$ males for 24 hours of egg laying at 25°C. We heat-shocked for 1hr at
387 37°C on days 2 and 3. We dissected females (5d post-eclosion) to score for ovo^{D1} or wildtype
388 morphology.

389 We generated *PBac{GFP-sov}* from P[acman] BAC clone CH322-191E24 (X:6753282--
390 6773405) (Venken et al. 2009) grown in the SW102 strain (Warming et al. 2005). In step one,
391 we integrated the positive/negative marker CP6-RpsL/Kan (CP6 promoter with a bi-cistronic

392 cassette encoding the RpsL followed by the Kan) between the first two codons of *sov*, and
393 selected (15 µg/ml Kanamycin). We integrated at the galK operon in DH10B bacteria using
394 mini-lambda-mediated recombineering (Court et al. 2003). We amplified DH10B::CP6-
395 RpsL/Kan DNA using primers N-CG14438-CP6-RN-F and -R. Correct events were identified
396 by PCR, as well as resistance (15 µg/ml Kanamycin) and sensitivity (250 µg/ml Kan). In step
397 two, we replaced the selection markers with a multi-tag sequence (Venken et al. 2011),
398 tailored for N-terminal tagging (N-tag) and counter-selected (250 µg/ml Kanamycin). The N-tag
399 (3xFlag tag, TEV protease site, StrepII tag, superfolder GFP, FlAsH tetracysteine tag, and
400 flexible 4xGlyGlySer (GGS) linker) was *Drosophila* codon optimized in a R6Kγ plasmid. We
401 transformed plasmid into EPI300 for copy number amplification. We confirmed correct events
402 by PCR and Sanger DNA sequencing. Tagged P[acman] BAC clone DNA was injected into y^1
403 *M{vas-int.Dm}ZH-2A w*; PBac{y+-attP-3B}*^{VK00033} embryos, resulting in w^{1118}*; PBac{y[+mDint2]*
404 w^{+mC}*=FTSF.GFP-sov}*^{VK00033}.
405
406 Microscopy
407 We fixed ovaries in 4 or 5% EM-grade paraformaldehyde in PBS containing 0.1 or 0.3%
408 Triton X-100 (PBTX) for 10-15min, washed 3x 15 min in PBTX, and blocked >30min in 2%
409 normal goat serum, 0.5-1% bovine serum albumin (BSA) in PBS with 0.1% Tween-20 or 0.1%
410 Triton X-100. Antibodies and DAPI were diluted into blocking buffer. We incubated in primary
411 antibodies overnight at 4 ºC and secondaries 2–3hr at room temperature. Embryos (1–2 hr)
412 were prepared for live imaging in halocarbon oil (Lerit et al. 2015). We imaged ovaries and
413 embryos using a Nikon Ti-E system or Zeiss LSM 780 microscope and eyes with a Nikon SMZ.
414

415 DNA-Seq

416 Genomic DNA was extracted from 30 whole flies per genotype (Huang, Rehm, and
417 Rubin 2009)(Sambrook and Russell 2006) to prepare DNA-seq libraries (Nextera DNA Library
418 Preparation Kit). We used 50 bp, single-end sequencing (Illumina HiSeq 2500, CASAVA base
419 calling). Sequence data are available at the SRA (SRP14438). We mapped DNAseq reads to
420 FlyBase r6.16 genome with Hisat2 (-k 1 --no-spliced-alignment)(Kim, Langmead, and Salzberg
421 2015). We used mpileup and bcftools commands from SAMtools within the genomic region
422 X:6756000–6771000 (H. Li et al. 2009; H. Li 2011) for variant calling and snpEFF to determine
423 the nature of variants in *sov* mutants (Cingolani et al. 2012).

424

425 RNA-Seq

426 Stranded PolyA+ RNA-seq libraries from *sov* and control ovaries (Table S5) were
427 created (H. Lee, Cho, et al. 2016) and are available at GEO (GSE113977). We extracted total
428 RNA (Qiagen RNeasy Mini Kit) in biological triplicate from 15 ovaries (4-5d post-eclosion) and
429 used 200 ng with 10 pg of ERCC spike-in control RNAs (pools 78A or 78B) for libraries (Jiang
430 et al. 2011; Zook et al. 2012; Pine et al. 2016; H. Lee, Pine, et al. 2016). We used 50 bp,
431 single-end sequencing as above. Tissue expression analysis are from GEO accession
432 GSE99574, a resource for comparing gene expression patterns.

433 We mapped RNA-seq reads to FlyBase r6.21 with Hisat2 (-k 1 --rna-strandness R --
434 dta)(Kim, Langmead, and Salzberg 2015). We determined read counts for each attribute of the
435 FlyBase r6.21 GTF file (with ERCC and transposable element sequences), with HTSeq-count
436 (Anders, Pyl, and Huber 2015). Transposon sequences were from the UCSC Genome
437 Browser RepeatMasker track (Casper et al. 2018; Smit, Hubley, and Green 2013-2015).

438 We conducted differential expression analysis with DESeq2 (pAdjustMethod = "fdr")
439 (Love, Huber, and Anders 2014). We removed genes with read counts $\leq$1 and read counts for
440 transposable elements with >1 location were summed for the DESeq2 analysis. *Df(1)sov/sov*^{2}
441 replicate 3 and *sov*^{2}*/w*^{1118} replicate 1 were failed. For *sov* mutant *vs.* control DESeq2 analysis,
442 all *sov* mutants were compared to all wild type controls. For tissue types, we compared each
443 sexed tissue to sexed whole organism. We used reads per kb per million reads (RPKM) for
444 gene-level expression.
445 For heatmaps, we calculated Euclidean distance, performed hierarchical cluster
446 analysis (agglomeration method = Ward), and mean-subtracted scaled across genotypes.
447 Mean sample RPKM correlation values (Table S1) were calculated by cor.test() function with
448 Pearson correlation coefficient in R (R Core Team 2017). We represented de-repressed genes
449 as mean-subtracted scaled values across tissues in the heatmap (Table S6).
450 For read density tracks, replicate raw read files were combined. Bedgraph files were
451 created with bedtools genomecov (Quinlan and Hall 2010) visualized on the UCSC genome
452 browser (Kent et al. 2002). Tracks were scaled by the number of reads divided by total reads
453 per million.
454 We used ClueGO (Bindea et al. 2009), with Cytoscape (Shannon et al. 2003) for one-
455 sided enrichment analysis (see Table S4).
456 Images were assembled using ImageJ and Photoshop. We used ROI tool in ImageJ,
457 plotted/analyzed image data (Microsoft Excel and GraphPad Prism), and calculated
458 significance by D'Agnostino and Pearson normality tests, followed Student's two-tailed t-test or
459 Mann-Whitney tests.
460

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471

472 **Author contributions**

473 LB, KC, BO, and DAL designed the project and analyzed data. LB, KC, CW, and KJTV
474 performed genetics. LB and HY performed genomics. LB, EAC, CW and DAL performed
475 imaging. LB, BO, and DAL wrote the manuscript. All authors reviewed data and provided
476 feedback on the manuscript.

477

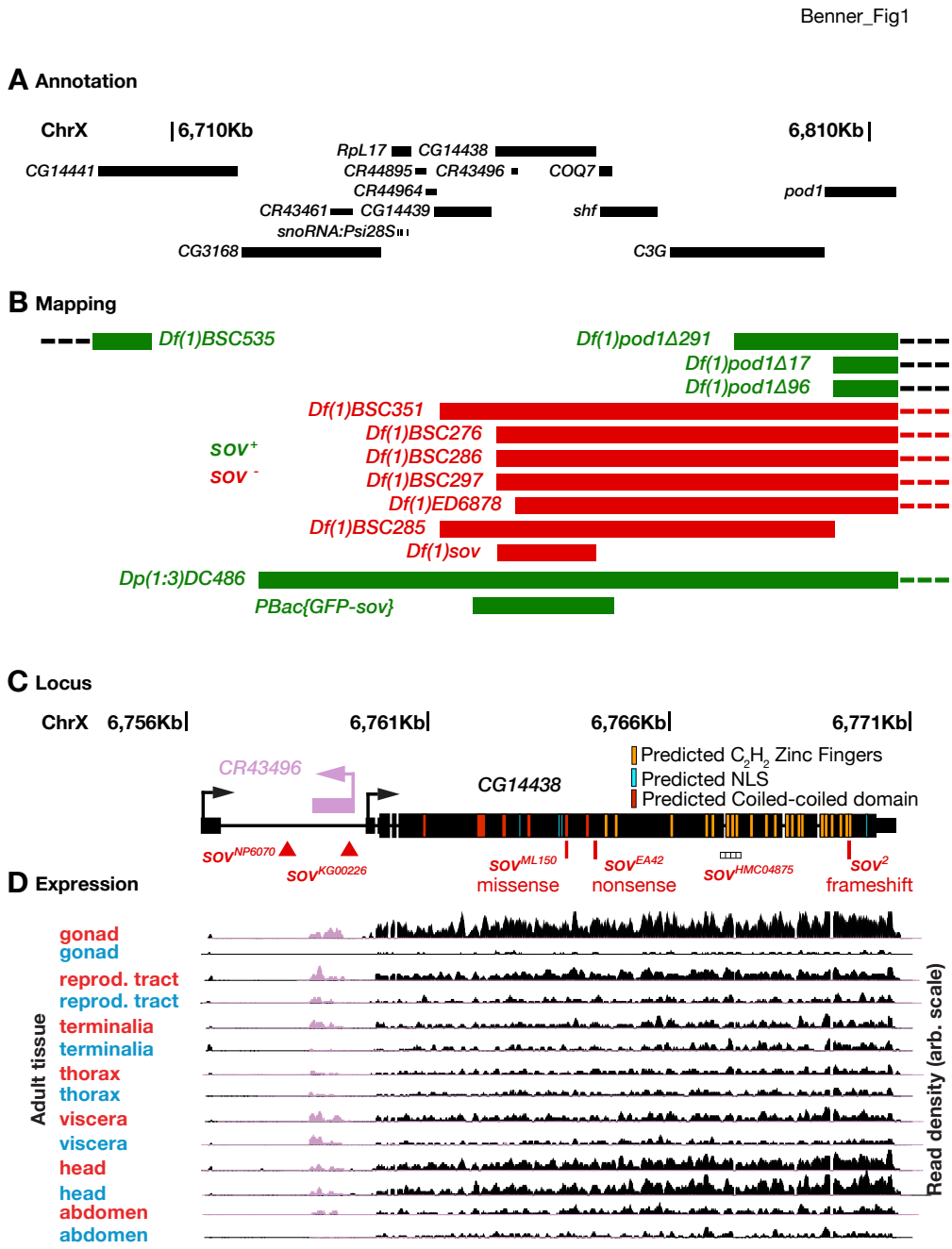

Figure 1. *sov* is *CG14438*. (A) Genes of the genomic interval X:6710000–6810000 (Gramates et al. 2017). (B) Deficiency (*Df*) and Duplication (*Dp*) mapping. (C) Schematic of the *CG14438* (black) and CR43496 (purple) genes. Transcription start (bent arrows), introns (thin lines) non-coding regions (medium lines) and coding regions (thick lines) are shown. Transposon insertions (triangles), point mutations (red lines), the region targeted by the shRNAi transgene (base-paired), and Sov protein features are shown. Non-complementing (*sov⁻*, red) and complementing (*sov*⁺, green) alleles and rearrangements are shown. (D) RNA expression tracks by tissue type from female (red) or male (blue) adults.

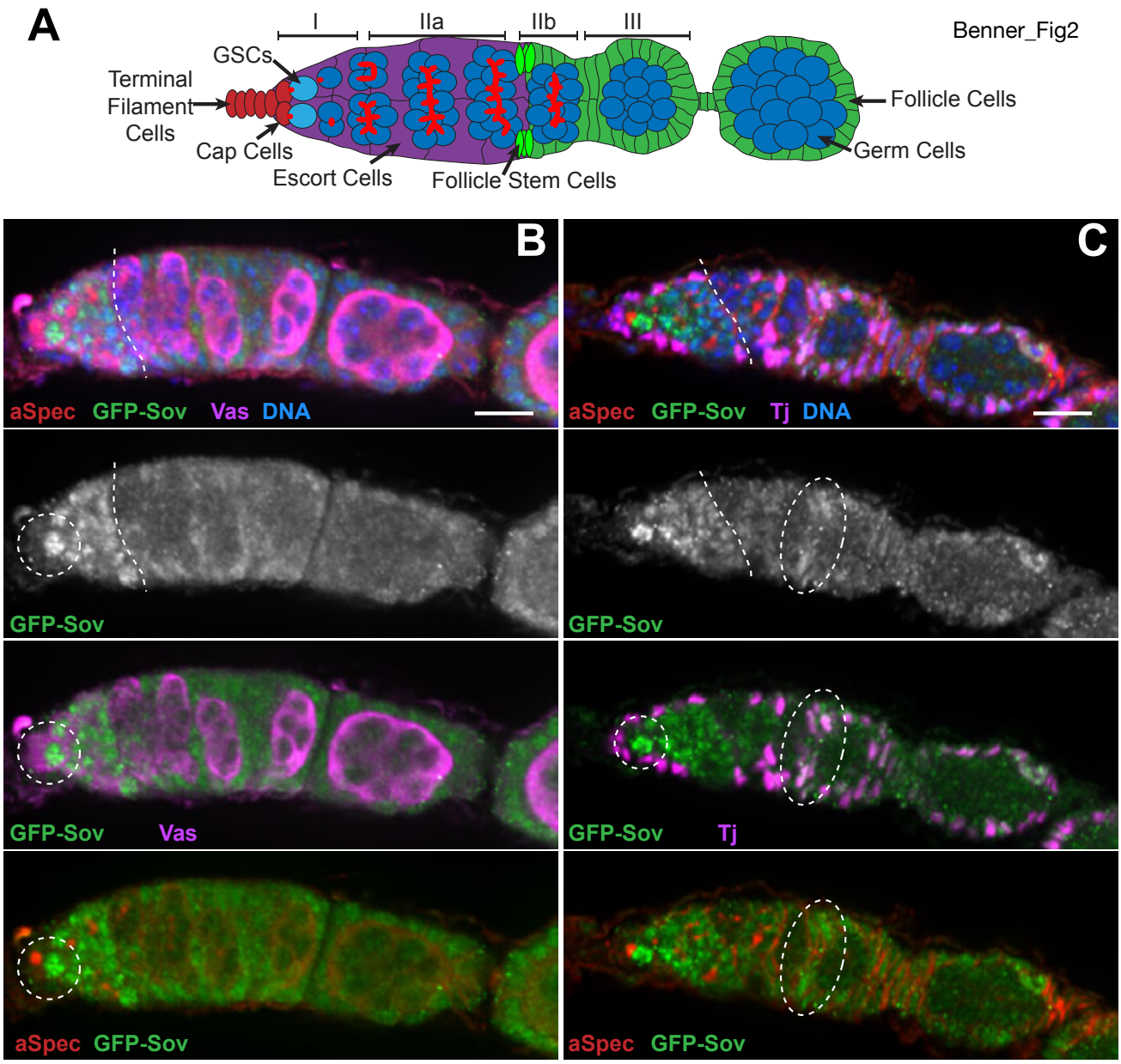

Figure 2. Sov germarium expression. (A) Cartoon showing germarium regions I-III and young egg chamber with cell types. (B,C) 1d post-eclosion, visualized for GFP-Sov (green), anti-αSpectrin (red). Images show single optical sections. (B) Sov contrasted with anti-Vas germline (magenta), or (C) -Tj somatic staining (magenta). GFP-Sov expression regions of interest (see text) are shown (dashed lines). Bars: 10μm.

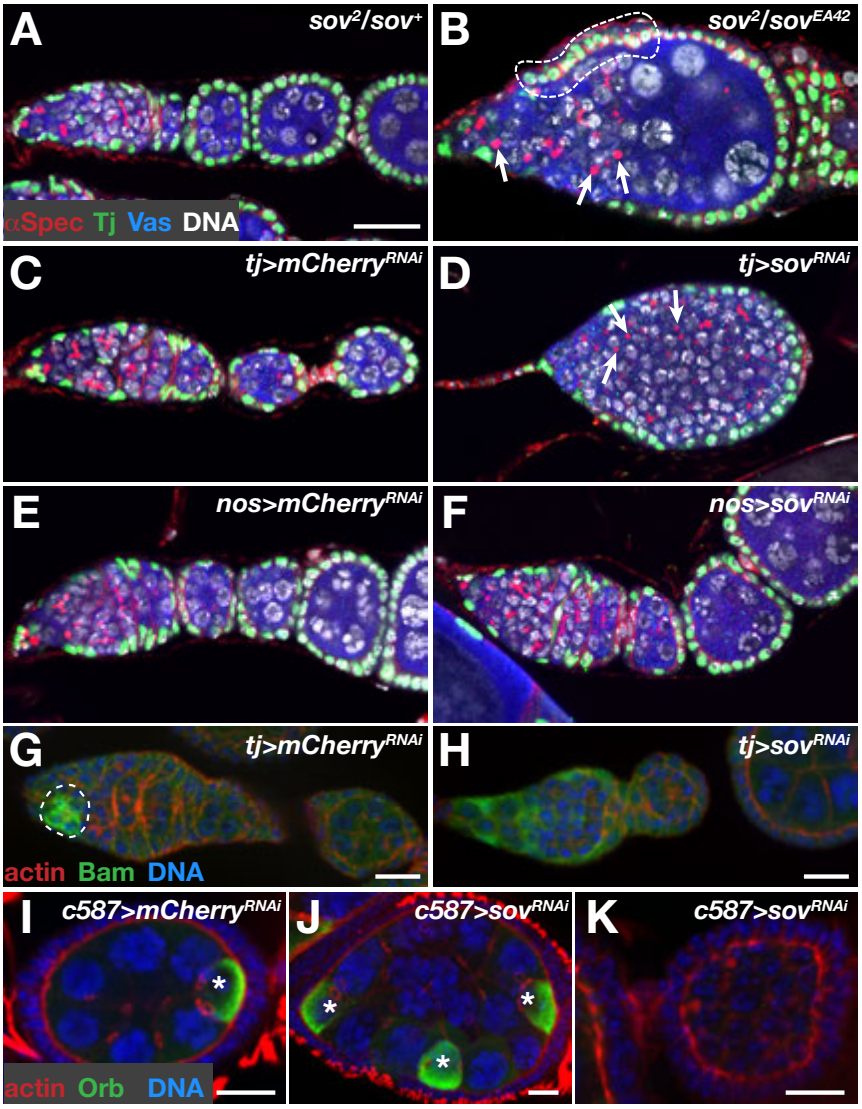

Figure 3. *sov* mutant defects. (A-K) images of indicated genotype (white). (A-F) Germaria (4-5d post-eclosion) of stained with anti-Vas (blue), -Tj (green), -αSpectrin (red), and DAPI (white). Dot spectrosomes (arrows) and displaced follicle cells (dashed lines) are shown. Images are single optical sections. (G, H) Germaria (1d post-eclosion) stained with anti-Bam (green), -actin (red), and DAPI (blue). Bam region of interest is shown (dashed lines). (I–K) Germaria (1d post-eclosion) anti-Orb (green), -actin (red), and DAPI (blue). Orb+ cells shown (*). Single optical sections (A-H) and maximum intensity projections (I-K). Bars: (A–F) 20μm, (G–K) 10μm.

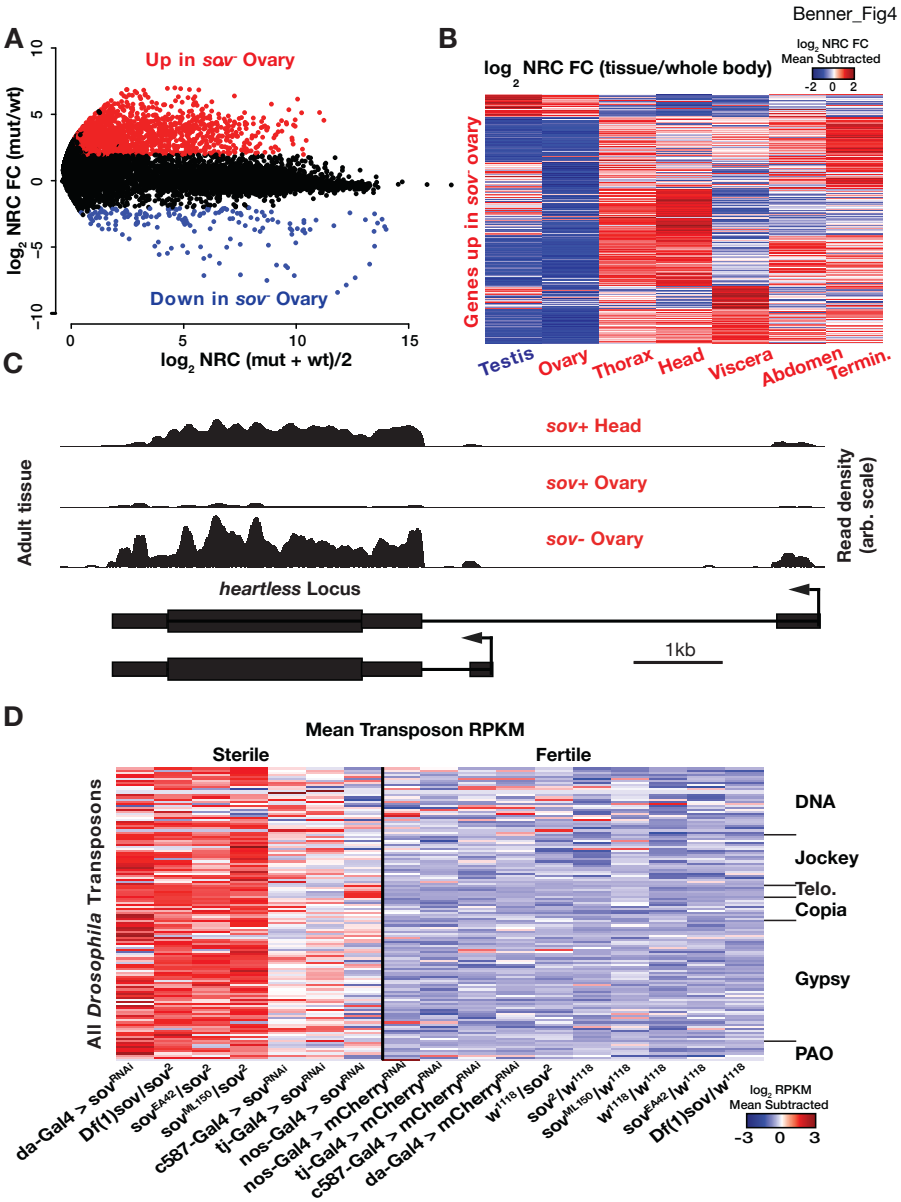

Figure 4. *sov* mutant transcriptome. (A) Relative expression in *sov* mutant vs *sov*+ ovaries plotted against the mean expression in both sample types. Units are Log_2 normalized read counts (NRC). Data points are genes, those with >4-fold change (log_2 2, red) or <–4 (log_2 -2, blue) and FDR *p*adj value <0.05 are highlighted. (B) Tissue-biased expression in wildtype for genes de-repressed in *sov* mutant ovaries. Heatmap from mean-subtracted ratios scaled across tissues (red=higher; blue=lower). (C) The *heartless* locus (see Fig1 for format) showing RNA-seq normalized read densities of *sov*+ from head and *sov*⁺ or *sov*⁻ ovarian tissues. (D) Transposable element expression in *sov* mutants and control alleles (as indicated). Heatmap from mean-subtracted reads (in RPKM. red=higher; blue=lower) scaled for each transposable element (rows) across genotypes (columns). Some element subtypes are shown for DNA, non-LTR (Jockey), telomeric repeat (Telo), and LTR (Gypsy, Copia, and PAO) are indicated.

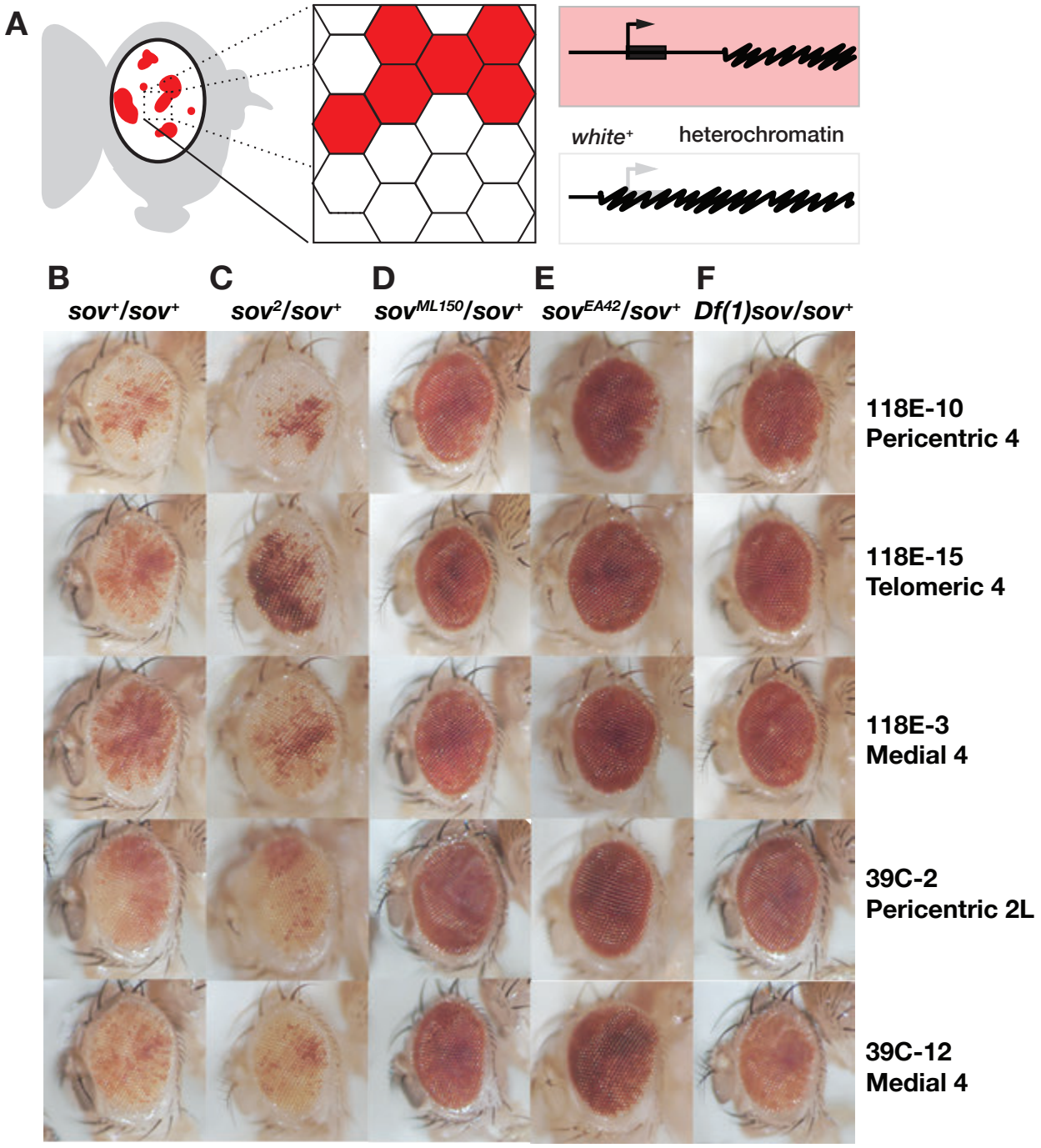

Figure 5. Sov is a dominant suppressor of position effect variegation. (A) Cartoon of position effect variegation (PEV) in the eye. Expression of the *white* gene (bent arrow, thick bar, red) near heterochromatin (squiggled) region can be silenced (white) by spreading. (B–F) Eyes with variegated expression of *P{hsp26-pt-T}* transgene inserted into different chromosomal positions (rows) in different *sov* backgrounds (columns).

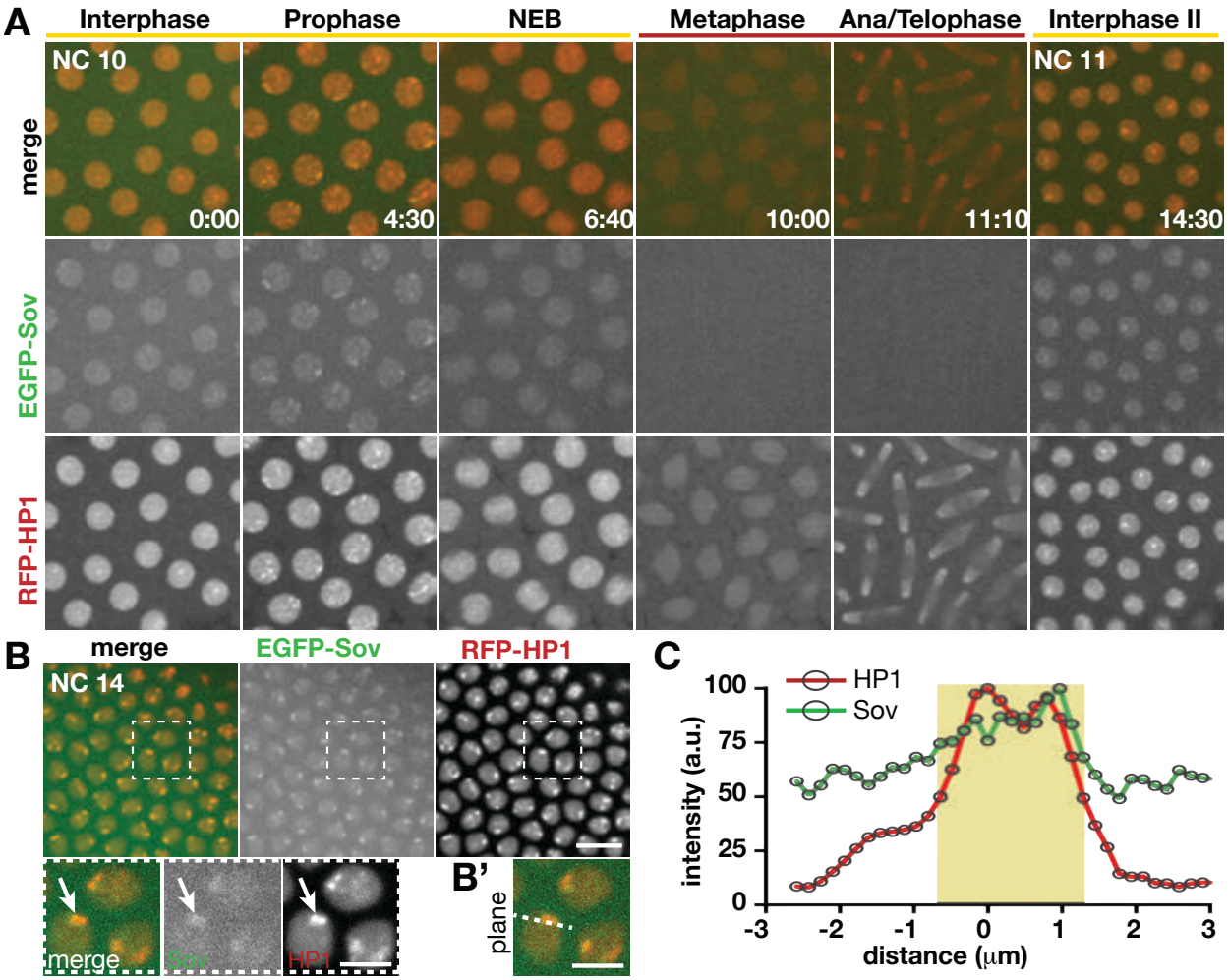

Figure 6. Sov colocalizes with HP1a. Stills from live imaging of embryos expressing GFP-Sov and RFP-HP1a. (A) Localization of GFP-Sov and RFP-HP1a (rows) in stills from live imaging of an embryo expressing progressing from NC 10 (left) to NC 11 (right) with stages (columns, nuclear envelope breakdown=NEB) and time (min:s in merge row) shown. (B) expressing GFP-Sov and RFP-HP1a (columns) at NC 14. Boxed regions are magnified in insets below. An HP1a subnuclear domain is shown (arrows). (B') Single optical section containing peak HP1a fluorescence of the same region shown in (B). Dashed line indicates region used for linescan analysis. (A,B) Maximum projections through 1.5 μm volume at 1F/30s. Bars: 10 μm; insets, 5 μm. (C) Histogram of HP1a and Sov fluorescence intensity measured in (B'). Fluorescence levels (arbitrary units) normalized to the peak fluorescence intensity for each channel and the distance (μm) to peak HP1a signal. Line scans were scaled –3 to 3μm, where 0.0 is peak HP1a fluorescence. Half maximum HP1a fluorescence is shown (yellow shade).

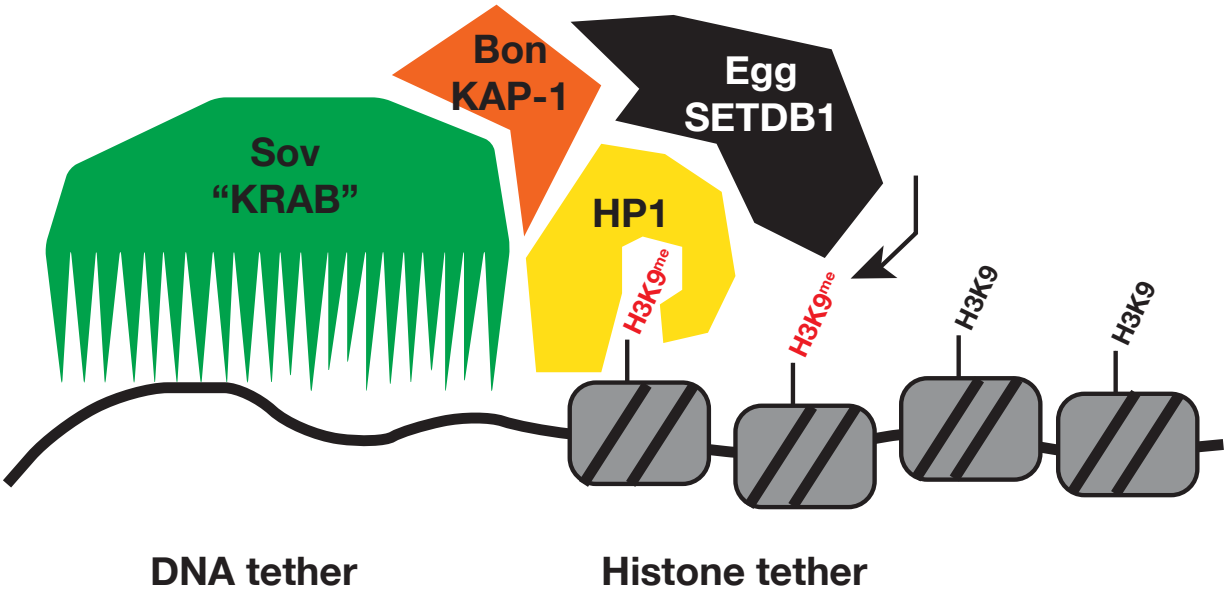

Figure 7. Working model of *sov* function. Sov protein function and attributes are analogous to the mammalian KRAB proteins (green). Like KRAB proteins, Sov is in complex with HP1a (yellow), Bon (mammalian KAP-1, orange) and Egg (mammalian SETDB1, black) providing a DNA tether that functions along with the H3K9me tether written by Egg (arrow, red lettering) and bound by HP1, to stabilize a repressed state.

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