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Note

Frequent Unanticipated Alleles of *lethal giant larvae* in Drosophila Second Chromosome Stocks

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ABSTRACT

Forty years ago, a high frequency of *lethal giant larvae* (*lgl*) alleles in wild populations of *Drosophila melanogaster* was reported. This locus has been intensively studied for its roles in epithelial polarity, asymmetric neural divisions, and restriction of tissue proliferation. Here, we identify a high frequency of *lgl* alleles in the Bloomington second chromosome deficiency kit and the University of California at Los Angeles Bruinfly FRT40A-lethal P collection. These unrecognized aberrations confound the use of these workhorse collections for phenotypic screening or genetic mapping. In addition, we determined that independent alleles of *insensitive*, reported to affect asymmetric cell divisions during sensory organ development, carry *lgl* deletions that are responsible for the observed phenotypes. Taken together, these results encourage the routine testing of second chromosome stocks for second-site alleles of *lgl*.

STUDIES of *lethal giant larvae* [*l(2)gl* or *lgl*] provided founding genetic evidence for the existence of tumor suppressor genes. Originally isolated by Bridges in 1933 (Bridges and Brehme 1944), *lgl* mutant cells were later characterized to have potent malignant properties (Gateff and Schneiderman 1967). In the past decade, Lgl has received considerable attention for its varied functions in apico-basal polarity and epithelial maintenance (Manfruelli *et al.* 1996; Bilder *et al.* 2000; Bilder 2004), in asymmetric division of embryonic neuroblasts (Ohshiro *et al.* 2000; Peng *et al.* 2000), and during multiple asymmetric divisions in adult mechanosensory organ development (Justice *et al.* 2003; Langevin *et al.* 2005).

The frequency of mutant *lgl* alleles in wild populations is extraordinary: ~3–6% of lethal second chromosomes extracted from wild flies collected in the former Soviet Union (Golubovsky 1978) and in California (Green and Shepherd 1979) failed to complement *lgl*. This high frequency is due to the fact that *lgl* is the second protein-coding gene downstream of the subtelomeric region of chromosome 2L, and many spontaneous *lgl* alleles actually represent terminal deletions of 2L (Mechler *et al.* 1985).

The high frequency of *lgl* alleles and terminal deletions of chromosome 2L are not well recognized among Drosophila researchers. We were led to appreciate this during three independent studies using popular public stock collections and previously published mutants, all of which revealed confounding second-site deletions uncovering *lgl*. Therefore, the possibility of 2L tip aberrations should be regularly considered when analyzing second chromosome stocks of *Drosophila melanogaster*.

The Bloomington deficiency kit contains frequent alleles of *lgl*: The Bloomington deficiency kit is a tiling set of deletions that uncovers ~95% of *D. melanogaster* euchromatin (http://flystocks.bio.indiana.edu/Browse/df-dp/dfkit.htm) and is commonly used to map lethal mutants. In addition, it is actively screened for zygotic phenotypes in homozygous deficiency embryos or for the ability of deficiencies to dominantly modify phenotypes of interest (*e.g.*, Guo *et al.* 2003; Mason *et al.* 2004; Anderson *et al.* 2005; Norgate *et al.* 2007).

While screening the second chromosome deficiency kit for loci required for embryonic neuroblast asymmetry, we found that *Df(2R)en-A* and *Df(2R)CB21* homozygotes failed to localize Miranda asymmetrically in dividing neuroblasts (supporting information, File S1, and data not shown). However, we eventually determined that these stocks,

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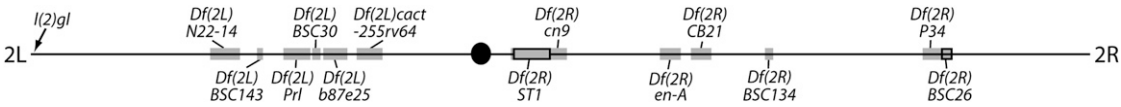

Figure 1.—The Bloomington second chromosome deficiency kit contains a high frequency of *lgl* alleles. The mapped deficiencies that fail to complement the null allele *lgl[4]* are shown; these include deficiencies on both the left and right arms of chromosome 2. *Df(2R)ST1-Df(2R)cn9* and *Df(2R)P34-Df(2R)BSC26* are pairs of deficiency stocks that delete partially overlapping sequence.

which contain cytologically visible deletions in the right arm of chromosome 2, were also deleted for *lgl* (Figure 1), whose loss underlies their neuroblast defects.

These findings prompted us to systematically test the second chromosome deficiency kit for failure to complement *lgl[4]*, a characterized null allele. Thirteen of 98 stocks were indeed mutant for *lgl*, comprising deficiencies of diverse regions of both the left and right arms of the second chromosome (Figure 1 and Table S1). Because the affected stocks were collectively generated in several laboratories using different strategies, we infer that many additional deficiencies not included in the standard kit are contaminated with *lgl* mutations and/or 2L terminal deletions.

The University of California at Los Angeles Bruinfly FRT40A-lethal P collection contains frequent alleles of *lgl*: Collections of lethal stocks bearing randomly inserted transposable elements have been valuable tools for genetic screening (St Johnston 2002; Bellen *et al.* 2004). Recently, the University of California at Los Angeles Undergraduate Research Consortium in Functional Genetics recombined large collections of lethal *P* elements onto FRT chromosomes (Chen *et al.* 2005; Call *et al.* 2007), thus enabling their use in clonal screens.

We investigated whether these stocks might uncover novel maternally provided genes involved in cuticle patterning. In screening 2L Bruinfly stocks, we identified several that produced cuticular defects in germline clones resembling cobblestones (Figure 2). This phenotype is characteristic of loss-of-function mutations in the neoplastic tumor suppressor genes *scribble*, *discs large*, and *lgl* (Bilder *et al.* 2000; Tanentzapf and Tepass 2003). Suspicious of the high rate of *lgl* phenocopy, we tested whether these stocks harbored alleles of *lgl* and indeed found several lines that failed to complement *lgl[4]* (Figure 2 and Table S2).

The Bruinfly consortium performed excision experiments to ask whether lethality mapped to the *P* elements (Chen *et al.* 2005; Call *et al.* 2007), and as might be expected, many stocks were not revertible. Curiously, some stocks that generated *lgl*-like embryos were restored to viability upon P excision and complemented *lgl[4]* in our initial tests. A potential explanation is that such stocks are composed of mixed populations of second chromosomes. To test this, we repeated *lgl* complementation tests as cohorts of single-fly crosses from each of 24 randomly selected Bruinfly 2L stocks. Five stocks contained *lgl* alleles, but two of these were indeed mixed with respect to 2L tip status (Table S2). Therefore, conventional complementation/excision tests using multiple fly crosses can falsely attribute phenotypes to the transposon insertion and/or report incorrectly on the integrity of the second chromosome.

Previously described alleles of *insensitive* are deficient for *lgl*: Drosophila external mechanosensory organs are multicellular structures that develop via asymmetric divisions of individual sensory organ precursors (SOPs) (Lai 2004). The Notch-signaling pathway generates asymmetry at each division, yielding four distinct cells in the mature organ. The shaft and socket cells are visible from the exterior and have large nuclei, while the sheath and neuron are strictly internal and have smaller nuclei; a fifth glial cell undergoes apoptosis and is absent from normal organs. The complete loss of Notch activity yields sensory lineages that exclusively generate neurons, while the activation of Notch signaling in all cells of the sensory lineage yields organs composed of multiple sockets.

Insensitive (*insv*) was originally isolated from expression profiling as a SOP-specific transcript (Reeves and Posakony 2005). Two lethal (and presumably null) deletion alleles were described, *insv[23B]* and *insv[23L]*,

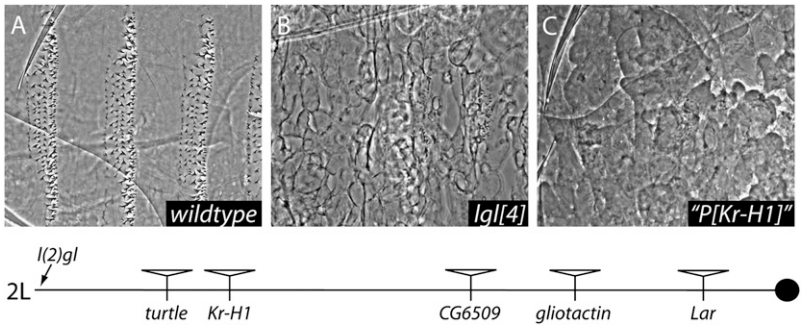

Figure 2.—The Bruinfly FRT40A collection contains a high frequency of *lgl* alleles. (Top) Cuticle preparations from wild-type (A), *lgl[4]* germline clones (B), and *Kruppel-Homolog 1* (*Kr-H1*) (Bruinfly 13097) germline clones (C). Loss of maternal and zygotic *lgl* disrupts cuticle development, and the mimic of this phenotype in the *Kr-H1* line is due to loss of *lgl*. (Bottom) Five of 24 randomly selected FRT40A-lethal P stocks, containing transposons distributed over the entirety of chromosome 2L, harbored *lgl* alleles.

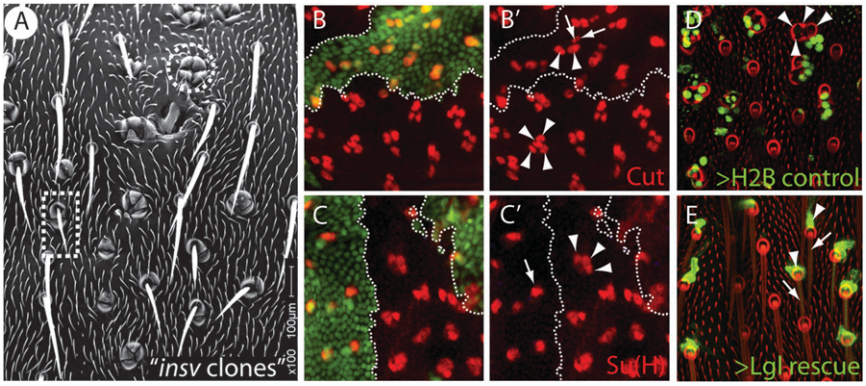

Figure 3.—The loss of *lgl* from *insv* mutant chromosomes is responsible for their reported multiple socket phenotypes. (A) Scanning electron micrograph of *insv[23B]* notum clones, which differentiate sensory organs with extra socket cells. The dotted circle highlights a four-socket organ, while the dotted square indicates an organ with two sockets and a malformed shaft. (B, B′) Cut staining in a 24-hr after puparium formation (APF) notum bearing *insv[23B]* clones, marked by the absence of nuclear GFP. Wild-type organs contain two large (arrowheads) and two small (arrows) Cut^+ nuclei, while *insv* mutant organs contain four large Cut^+ nuclei. (C, C′) Suppressor of Hairless [Su(H)] staining in a 24-hr APF notum bearing *insv[23B]* clones marked by the absence of nuclear GFP. Wild-type organs contain a single $Su(H)^+$ cell (arrow), while *insv* mutant organs contain three to four $Su(H)^+$ cells (arrowheads). (D) PCR tests demonstrated that the available *insv* stocks are actually doubly mutant for *lgl* (data not shown). Expression of *UAS-H2B-RFP* in *lgl-insv* double mutant MARCM clones does not affect the multiple socket phenotype (arrowheads). H2B-RFP is green, and cuticular structures including socket and shaft cells are red. (E) Expression of *UAS-lgl-GFP* (green) fully rescues the bristle phenotypes of *lgl-insv* double mutant clones with single sockets (arrowheads) and single shafts (arrows).

and both of these yielded multiple socket organs in adult thorax clones (Figure 3A). The similarity between *insv* clones and clones of Notch-inhibitory factors such as *numb* or *lgl* suggested that Insv is a novel factor that represses Notch activity in the sensory organ lineage (Justice *et al.* 2003; Langevin *et al.* 2005; Reeves and Posakony 2005).

We observed that *insv* mutant sensory organs differentiated four cells with large nuclei (as marked by Cut), suggesting the conversion of inner cell fates into outer cell fates (Figure 3B). Indeed, *insv* mutant organs failed to differentiate sheath cells (marked by Prospero) or neurons (marked by Elav) and instead contained three to four $Su(H)^+$ socket cells (Figure 3C and data not shown). Such cell fate transformations were highly reminiscent of *lgl* clones. Consistent with this, *insv* clones were deficient for Lgl protein. However, we were surprised to observe that Lgl was absent both in epithelial cells and in SOPs of the *insv* clones (data not shown), as *insv* is specifically expressed by SOPs (Reeves and Posakony 2005).

Because both *insv* and *lgl* are located on chromosome 2L, we considered whether *insv* alleles were deficient for *lgl*. We observed that *insv* stocks generated overgrown larvae with tumorous discs and demonstrated their loss of *lgl* using complementation and PCR tests (data not shown). Thus, the available chromosomes are doubly mutant for *lgl* and *insv*. We next used the MARCM system and *Ubx-Flp* to induce *lgl* and *insv* thorax clones for rescue assays. When *UAS-Histone2B-RFP* was activated in such clones, their sensory organs differentiated with multiple sockets (Figure 3D and File S1). However, expression of *UAS-lgl-GFP* in these clones completely restored normal external fates with single sockets and shafts in the mutant organs (Figure 3E). Therefore, the deletion of *lgl* accounts for the sensory organ defects produced by available *insv* chromosomes.

Conclusion: We found that commonly used tool-kit stock collections and published second chromosome mutants harbor second-site terminal deletions of chromosome 2L, thereby removing *lgl*. As this was previously noted to affect a set of deficiency and lethal P stocks (Brumby *et al.* 2004; Mason *et al.* 2004; Menut *et al.* 2007) that are substantially nonoverlapping with the ones that we characterized, the 2L problem is undoubtedly broader than currently recognized.

Unanticipated *lgl* alleles confounded interpretation of the previously described *insv* mutant (Reeves and Posakony 2005). Similarly, the neoplastic tumors induced by *wingless* mutant cells blocked for apoptosis (Perez-Garijo *et al.* 2005) also appear to be due to the loss of *lgl* from the *wingless* mutant chromosome studied (G. Morata, personal communication). We even found that the *numb[1]* stock (available at the Bloomington Stock Center), which disrupts the prototypical asymmetric cell fate determinant in Drosophila (Uemura *et al.* 1989; Rhyu *et al.* 1994), is deficient for *lgl*.

The observation that *lgl* mutations exhibit dose-sensitive interactions with a variety of loci (Bilder *et al.* 2000; Tanentzapf and Tepass 2003; Zarnescu *et al.* 2005) suggests that unanticipated heterozygosity for *lgl* can confound dominant modifier screens. Notably, *lgl* was accidentally discovered to modify a *crumbs* gain-of-function phenotype via a *kekkon* P insertion chromosome that fortuitously carried an allele of *lgl* (Tanentzapf and Tepass 2003). Presumably, at least some other genetic interactions involving second-site *lgl* alleles have not been appropriately tracked down.

There are further consequences stemming from the fact that most spontaneous *lgl* alleles represent terminal deletions. Subtelomeric heterochromatic regions in Drosophila generate large numbers of Piwi-interacting RNAs (piRNAs), which mediate post-transcriptional control of transposons (Brennecke *et al.* 2007) and epigenetic

transcriptional activation (Yin and Lin 2007). In addition to Lgl-regulated cuticle patterning, imaginal disc growth, embryonic neuroblast asymmetry, and adult sensory organ development, then, 2L terminal deletions may also affect piRNA-controlled processes, potentially including telomeric silencing (Mason *et al.* 2004). In conclusion, these observations suggest that Drosophila geneticists should routinely test second chromosome stocks to verify the absence of unanticipated *lgl* alleles.

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FILE S1

Supplemental Experimental Procedures

Bloomington deficiency screening

We stained collections of Bloomington embryos with rabbit anti-Miranda (gift of Fumio Matsuzaki) and identified stocks with Mendelian segregation of delocalized Miranda in neuroblasts. We subsequently tested the entire current 2nd chromosome collection (DK2) for failure to complement *lgl[4]*.

Bruinfly FRT-P lethal screening

We induced germline clones in *hs-FLP/+; P lethal FRT40A/ovo[D], FRT40A* females by heat-shocking for 1 hr at 38°C during the first instar. We mated these to sibling males and prepared cuticles from 16-24 hr embryos, which were scored for the presence of epithelial patterning defects. We subsequently tested 4-10 individual flies from 24 randomly selected Bruinfly stocks for failure to complement *lgl[4]*.

Analysis of insensitive

We analyzed thorax clones in *Ubx-FLP/+; insv [23B] (*or *insv[23L]), FRT40A/ ubi-GFP, FRT40A* animals. We dissected 24 hr APF pupae and stained these with rabbit anti-GFP (Molecular Probes), rat anti-Elav Developmental Studies Hybridoma Bank, DSHB), mouse anti-Prospero (DSHB), rat anti-Suppressor of Hairless (gift of Francois Schweisguth), rabbit anti-Numb (gift of Yuh Nung Jan), rabbit anti-Lgl (gift of Scott Goode), followed by Alexa-coupled secondary antibodies (Molecular Probes). Following the determination that *insv* chromosomes harbored *lgl*, we performed rescue tests in *Ubx-FLP/+; tub-Gal80, FRT40A/lgl*, *insv [23B], FRT40A; neur-Gal4, UAS-lgl-GFP (or UAS-Histone2B-RFP*) animals.

TABLE S1

Complementation tests of the DK2 Bloomington Deficiency Kit with lgl[4]

stock name		Df name	single fly cross to lgl[4]	complements lgl[4]?
DK2-1	3638	Df(2L)net-PMF	1	yes
			2	yes
DK2-2	6283	Df(2L)BSC4	1	yes
			2	yes
DK2-3	8672	Df(2L)BSC106	1	yes
			2	yes
DK2-4	6608	Df(2L)BSC16	1	yes
			2	yes
DK2-5	3084	Df(2L)ast2	1	yes
			2	yes
DK2-7	7144	Df(2L)BSC37	1	yes
			2	yes
DK2-8	6648	Df(2L)dpp[d14]	1	yes
			2	yes
DK2-9	90	Df(2L)C144	1	yes
			2	yes
DK2-10	1567	Df(2L)JS17	1	yes
			2	yes
DK2-11	6875	Df(2L)BSC28	1	yes
			2	yes
DK2-12	6965	Df(2L)BSC31	1	yes
			2	yes
DK2-13	6507	Df(2L)drm-P2	1	yes
			2	yes
DK2-14	5330	Df(2L)ed1	1	yes
			2	yes
DK2-15	693	Df(2L)sc19-8	1	yes
			2	yes
DK2-16	9270	Df(2L)ED250	1	yes
			2	yes
DK2-17	8835	Df(2L)BSC110	1	yes
			2	yes
DK2-18	8674	Df(2L)BSC109	1	yes
			2	yes
DK2-19	7497	Df(2L)Exel6011	1	yes
			2	yes
DK2-20	781	Df(2L)cl-h3	1	yes
			2	yes
DK2-21	490	Df(2L)E110	1	yes
			2	yes
DK2-22	6299	Df(2L)BSC5	1	yes
			2	yes
DK2-23	6338	Df(2L)BSC6	1	yes
			2	yes
DK2-24	6374	Df(2L)BSC7	1	yes
			2	yes
DK2-25	2414	Df(2L)spd[j2]	1	yes
			2	yes
DK2-26	5420	Df(2L)Dwee1-W05	1	yes
			2	yes
DK2-27	4956	Df(2L)XE-3801	1	yes
			2	yes
DK2-28	7147	Df(2L)BSC41	1	yes

			2	yes
DK2-29	9502	Df(2L)BSC142	1	yes
			2	yes
DK2-30	140	Df(2L)Trf-C6R31	1	yes
			2	yes
DK2-31	179	Df(2L)TE29Aa-11	1	yes
			2	yes
DK2-32	8836	Df(2L)BSC111	1	yes
			2	yes
DK2-33	9298	Df(2L)ED611	1	yes
			2	yes
DK2-34	2892	Df(2L)N22-14	1	no
			2	no
DK2-35	6478	Df(2L)BSC17	1	yes
			2	yes
DK2-36	1045	Df(2L)Mdh	1	yes
			2	yes
DK2-37	8469	Df(2L)BSC50	1	yes
			2	yes
DK2-38	3366	Df(2L)J2	1	yes
			2	yes
DK2-39	9503	Df(2L)BSC143	1	no
			2	no
DK2-40	7142	Df(2L)BSC32	1	yes
			2	yes
DK2-41	9505	Df(2L)BSC145	1	yes
			2	yes
DK2-42	7143	Df(2L)BSC36	1	yes
			2	yes
DK2-43	5869	Df(2L)FCK-20	1	yes
			2	yes
DK2-44	3079	Df(2L)Prl	1	no
			2	no
DK2-45	6999	Df(2L)BSC30	1	no
			2	no
DK2-47	3138	Df(2L)b87e25	1	no
			2	no
DK2-48	9506	Df(2L)BSC147	1	yes
			2	yes
DK2-49	3588	Df(2L)TE35BC-24	1	yes
			2	yes
DK2-50	1491	Df(2L)r10	1	yes
			2	yes
DK2-51	2583	Df(2L)cact-255rv64	1	no
			2	no
DK2-52	420	Df(2L)TW137	1	yes
			2	yes
DK2-53	567	Df(2L)pr-A16	1	yes
			2	yes
DK2-54	167	Df(2L)TW161	1	yes
			2	yes
DK2-55	7531	Df(2L)Exel6049	1	yes
			2	yes
DK2-56	9510	Df(2L)BSC151	1	yes
			2	yes
DK2-57	4959	Df(2L)C'	1	yes
			2	yes
DK2-58	749	In(2R)bw[VDe2L]Cy[R]	1	yes

			2	yes
DK2-59	739	Df(2R)M41A4	1	yes
			2	yes
DK2-60	1007	Df(2R)nap9	1	yes
			2	yes
DK2-61	1888	Df(2R)ST1	1	no
			2	no
DK2-62	3368	Df(2R)cn9	1	no
			2	no
DK2-63	198	Df(2R)H3C1	1	yes
			2	yes
DK2-65	3591	Df(2R)Np5	1	yes
			2	yes
DK2-66	4966	Df(2R)w45-30n	1	yes
			2	yes
DK2-67	6917	Df(2R)BSC29	1	yes
			2	yes
DK2-69	1743	Df(2R)B5	1	yes
			2	yes
DK2-70	1702	Df(2R)X1	1	yes
			2	yes
DK2-71	190	Df(2R)en-A	1	no
			2	no
DK2-72	1145	Df(2R)en30	1	yes
			2	yes
DK2-73	7145	Df(2R)BSC39	1	yes
			2	yes
DK2-74	4960	Df(2R)CB21	1	no
			2	no
DK2-75	7146	Df(2R)BSC40	1	yes
			2	yes
DK2-76	5879	Df(2R)BSC3	1	yes
			2	yes
DK2-77	754	Df(2R)vg-C	1	yes
			2	yes
DK2-80	7875	Df(2R)Exel7130	1	yes
			2	yes
DK2-81	9496	Df(2R)BSC134	1	no
			2	no
DK2-82	7876	Df(2R)Exel7131	1	yes
			2	yes
DK2-85	3520	Df(2R)Jp8	1	yes
			2	yes
DK2-86	7445	Df(2R)BSC49	1	yes
			2	yes
DK2-87	7414	Df(2R)BSC44	1	yes
			2	yes
DK2-88	9596	Df(2R)BSC161	1	yes
			2	yes
DK2-90	5574	Df(2R)k10408	1	yes
			2	yes
DK2-91	7441	Df(2R)BSC45	1	yes
			2	yes
DK2-92	6779	Df(2R)14H10Y-53	1	yes
			2	yes
DK2-93	6780	Df(2R)14H10W-35	1	yes
			2	yes
DK2-94	1547	Df(2R)PC4	1	yes

			2	yes
DK2-95	757	Df(2R)P34	1	no
			2	no
DK2-96	6866	Df(2R)BSC26	1	no
			2	no
DK2-97	6647	Df(2R)BSC22	1	yes
			2	yes
DK2-98	3467	Df(2R)AA21	1	yes
			2	yes
DK2-99	7896	Df(2R)Exel7162	1	yes
			2	yes
DK2-100	6609	Df(2R)BSC19	1	yes
			2	yes
DK2-101	5246	Df(2R)Egfr5	1	yes
			2	yes
DK2-103	3909	Df(2R)59AD	1	yes
			2	yes
DK2-104	7273	Df(2R)vir130	1	yes
			2	yes
DK2-106	9691	Df(2R)BSC155	1	yes
			2	yes
DK2-107	2604	Df(2R)Px2	1	yes
			2	yes
DK2-108	9069	Df(2R)ED4065	1	yes
			2	yes
DK2-110	4961	Df(2R)Kr10	1	yes
			2	yes

Two male flies were individually tested from each stock. For stocks that failed to complement lgl, an additional single fly cross was performed as confirmation.

TABLE S2

Complementation tests of 24 randomly selected FRT40A Bruinfly lines with lgl[4]

Kyoto ID	Bruinflyl ID	insertion site	annotated gene disrupted	single fly crosses that fail to complement lgl[4]
111624	14733	023E01_	CG3347	0 of 8
111512	13053	35D04	gliotactin	10 of 10
111516	13097	026B05	Kruppel homolog 1	10 of 10
111066	20404	035F01	Cropped	0 of 4
111067	10363	029E04-029E06	raw	0 of 4
111079	10386	033A01-033A02	crooked legs	0 of 4
111083	10391	038B03-038B05	nebbish	0 of 4
111097	10435	031D01	no mitochondrial derivative	0 of 4
111106	10451	024E01_	turtle	5 of 10
111108	10453	030A02-030A06	taiman	0 of 4
111111	10457	023B06	overgrown hematopoietic organs	0 of 4
111122	10473	025C01	viking	0 of 10
111275	10959	022C01	CG31672	0 of 4
111278	10965	023F03	Pdsw	0 of 4
111334	11115	036A11	cytochrome C proximal	0 of 4
111356	11166	021C02	ebi	0 of 4
111366	11212	024C05	lethal (2) k16918	0 of 4
111369	11218	034A04	Target of rapamycin	0 of 4
111429	12169	021E02_	dribble	0 of 4
111431	12173	035B08	moladietz	0 of 4
111462	12309	033A01-033A02	unknown	0 of 4
111463	12310	023D01-023D02,029A03-029A05	unknown	0 of 4
111558	13692	032F02	CG6509	7 of 10
111564	13853	038A01	Lar leukocyte antigen related	8 of 8

Initially, 4-6 male flies were individually tested from each stock. For stocks that exhibited some failure to complement lgl, additional single fly crosses were performed to assay a total of 10 chromosomes from each stock. The gliotactin, Kr-H1, and Lar insertions were 100% lgl, whereas the turtle and CG6509 insertions were 50-70% lgl.