

G.3.2 is a novel allele of the gene *connector enhancer of ksr* (*cnk*) in *Drosophila melanogaster*

Hanadi Chammout¹, Delia L. Adkins², Aleece K. Al-Olimat², Zeinab Alsaad¹, Beatrice M. Altopp³, Tuqa Amer³, Feyi O. Apampa³, Gwendolyn R. Avery², Isaac I. Bazzi¹, Emilia D. Beck², Elise L. Beier³, B. Shafer Belisle³, Lane Benton², Madison M. Bolyard², Olivia E. Brain², Eldon T. Buckner², Shria Roy Chowdhury¹, Jennifer R. Cifranic², Liam Cleary³, Tyler R. Clum², Autumn M. Cruz², Meghan V. DeGray³, Isabel L. Echeverry³, Haya El dana¹, Sarah K. Elkadri¹, Paige L. Estep², Luke R. Falke², Hannah J. Foor², Anika S. Gullapalli¹, Sandro S. Hakim¹, Hussein B. Hazime¹, Lauren E. Heininger², Emma G. Hoeft², Lauren M. James², Yeowon Jeon¹, Megan R. Johnson², Laine P. Jordan², Zayd Khan¹, Sydney K. Kochensparger³, Fadi J. Koria¹, Ruby M. Krasnow³, Veronica Lilly², Eileen Lim³, Ian T. MacCormack³, Andriy Malesh³, Mikayla G. Mariano², Audrey C. Mentzer², Katelyn H. Messner², Katlyn C. Myers², Emily R. Newman³, Annie M. Richters², Liliana Romero¹, Adam Rotem³, Reese J. Saho², Kaname Sawaki², Ashley N. Selders², Elizabeth Shockney², Farah A. Sobh¹, Isabelle F. Speiser³, Breanna M. Sproul², Veronica J. Sroufe², Antonia Tollkuci³, Cassandra C. Trevino³, Megan A. Vapenik², Erin M. Wagner², Kayla L. Bieser⁴, Jamie L. Siders², Justin R. Thackeray³, Jacob D. Kagey^{1§}

¹University of Detroit Mercy, Detroit, Michigan, United States

²Ohio Northern University, Ada, Ohio, United States

³Clark University, Worcester, Massachusetts, United States

⁴Nevada State University, Henderson, Nevada, United States

[§]To whom correspondence should be addressed: kageyja@udmercy.edu

Abstract

Genetic screens in *Drosophila melanogaster* have long been used to identify genes found in a variety of developmental, cellular, and behavioral processes. Here we describe the characterization and mapping of a mutation identified in a conditional screen for genetic regulators of cell growth and cell division. Within a Flp/FRT system, mutant G.3.2 results in a reduction of mutant tissue and a rough eye phenotype. We find that G.3.2 maps to the gene *cnk*, providing further support that *cnk* is a critical gene in *Drosophila* eye development. This mutant was characterized, mapped and sequenced by undergraduate students within the Fly-CURE consortium.

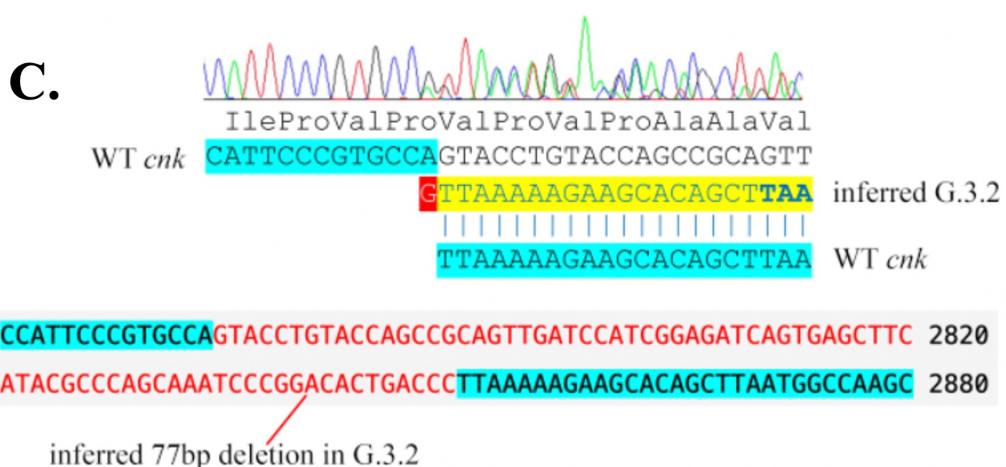
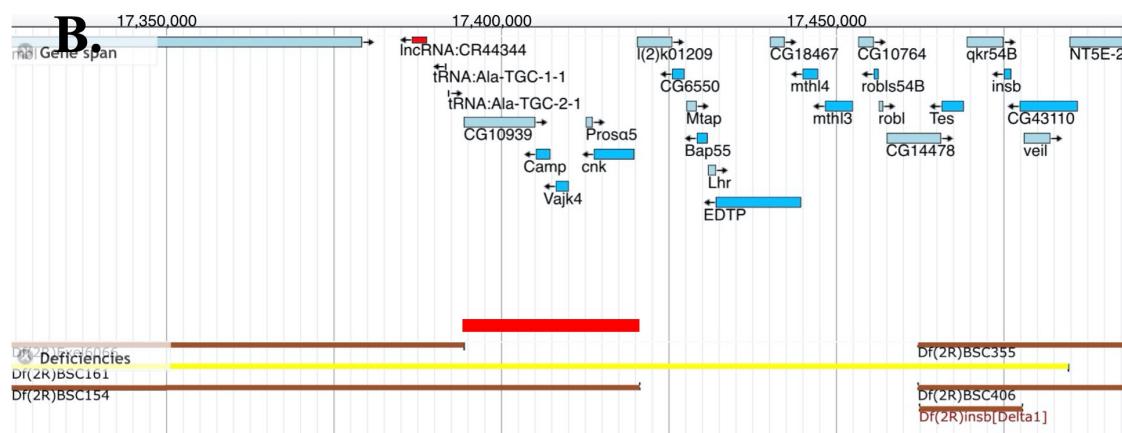
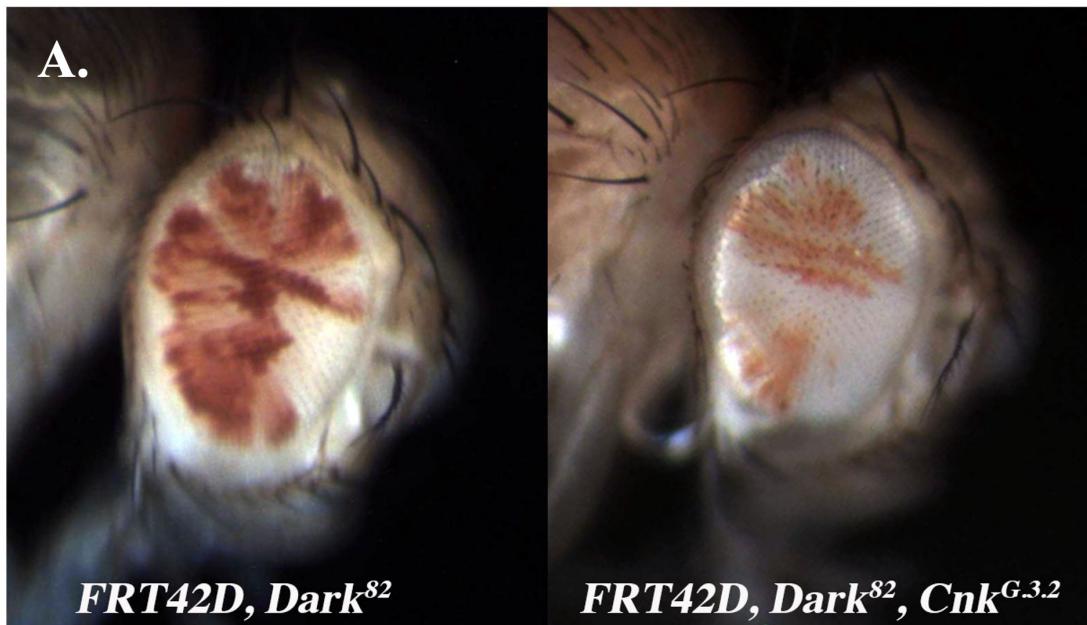


Figure 1. Characterization and mapping of the G.3.2 mutation as a novel allele of *cnk*.

A). Comparison of control mosaic (*FRT42D, Dark*⁸²) and *G.3.2* mosaic (*FRT42D, Dark*⁸², *cnk*^{*G.3.2*}). In both panels the homozygous mutant tissue is *mw*⁺. B). Genetic map of region that failed to complement after mating *G.3.2* to the 2R deficiency kit. *BSC161* is represented in yellow, and failed to complement *G.3.2* and resulted in a region of 2R:17,097,569..17,462,347, red bar represents smallest overlapping area of failure to complement. C). Sanger sequencing of *G.3.2* and *Dark*⁸², identifying a net loss of 76 base pairs within the *cnk* locus, identifying *G.3.2* as a novel allele of *cnk*, *cnk*^{*G.3.2*}. The chromatogram for this region is shown above our interpretation of the combined sequences; beneath that is a segment of the FASTA output for *cnk* within FlyBase, indicating the inferred 77 bp deletion in red. This deleted material is replaced within the *G.3.2* chromosome by a single G, which is highlighted in red within the chromatogram interpretation; the resulting frameshift within *G.3.2* results in a premature stop codon TAA, which is indicated in bold.

Description

Drosophila melanogaster is a commonly used model organism that is an excellent tool for genetic research (Tolwinski 2017). Genetic model systems of this type are particularly amenable to genetic screens, which provide an unbiased mechanism to identify novel genes associated with many different pathways (St Johnston 2002). Here we report on the mapping and characterization of a novel mutation (*G.3.2*) isolated from a Flp/FRT EMS screen in the adult *Drosophila* eye (Kagey *et al.* 2012). This screen utilized an allele of *Dark* (*Dark*⁸²) on chromosome 2R, prior to mutagenesis (Akdemir *et al.* 2006), ensuring that there was a block in the canonical apoptotic pathway within the homozygous mutant mosaic cells. The overarching hypothesis from this screen was that there is a class of mutants that would have been missed in the first generation of Flp/FRT screens due to the residual apoptosis in the background. Previous mutants from this screen have been characterized and mapped by undergraduate students that are part of the Fly-CURE consortium, where students conduct this research within the structure of an undergraduate laboratory course (Merkle *et al.* 2023).

G.3.2 was isolated from the Flp/FRT screen described above. Students from the University of Detroit Mercy, Ohio Northern University, and Clark University collaborated to characterize and map this mutant as part of the Fly-CURE consortium. Mosaic *G.3.2* eyes and control (*Dark*⁸²) mosaic eyes were generated through crosses of ♀ *Ey-Flp; FRT42D* (BDSC 5616) mated to ♂ *FRT42D, Dark*⁸², *G.3.2/CyO* or *FRT42D, Dark*^{82/CyO}, respectively. Straight-winged F1 progeny with mosaic eyes were submerged in 70% ethanol and visualized at 40x magnification. The *Dark*⁸² ratio of red to white tissue was in alignment with previously published mutants from this screen [6]. Images were taken with AM Scope (AM 550). We find that the *Dark*⁸² mosaic eye is comprised of ~60% homozygous mutant cells (*mw*⁺) while the *Dark*⁸², *G.3.2* mosaic eye has less mutant tissue (~30%) and results in smaller rough eye (Figure 1A, *mw*⁺ is mutant tissue in both images). This suggests that the *G.3.2* mutation introduces a growth/development deficiency into the mosaic eye phenotype.

To identify the location of the *G.3.2* mutation we utilized the homozygous lethal nature of the mutation to conduct deficiency mapping with the 2R deficiency kit from the Bloomington *Drosophila* Stock Center (Cook *et al.* 2012). In parallel, unmated ♀ from *FRT42D, Dark*⁸², *G.3.2/CyO* were mated to ♂ from *Df(2R)/CyO*. This resulted in 86 deficiency crosses covering the region between the FRT42D location and the distal end of chromosome 2R. Complementation was scored if at least 10 straight-winged F1 progeny emerged, while a failure to complement was scored by reaching 100 curly winged flies without the presence of any straight winged flies. Any crosses that did not produce enough F1 progeny to determine complementation were repeated. All complementation testing was done in independent replicates by the students at each institution. From these experiments we find that mutant *G.3.2* failed to complement *Df(2R)BSC161* while complementing the overlapping stocks of *Df(2R)Exel6066* and *Df(2R)BSC355* (Figure 1B; see table 1 for breakpoints and full genotypes of deficiency stocks). These results identify the non-complementing region as 2R:17,097,569..17,462,347 (Figure 1B) (Ozturk-Colak *et al.* 2024). To narrow this region down further we used additional complementation crosses with deficiencies and single-gene lethal alleles (see table 1 for genotypes). From these experiments we found that *G.3.2* fails to complement a previously characterized lethal allele of the gene *cnk*, *cnk*^{E-2083}, indicating that *G.3.2* is a novel allele of *cnk* that we now therefore refer to as *cnk*^{*G.3.2*} (Therrien *et al.* 1998; Therrien *et al.* 1999).

Next, we utilized Sanger Sequencing to identify the specific lesion in *cnk*^{*G.3.2*} that is driving the mutant phenotype. Genomic DNA was isolated from single, heterozygous *FRT42D, Dark*⁸² *cnk*^{*G.3.2*}/*CyO* and *FRT42D, Dark*⁸²/*CyO* adults using the method described by Gloor *et al.* (Gloor *et al.* 1993). A 5.9 kb genomic region encompassing the *cnk* open reading frame was amplified in seven overlapping reactions, each around 1kb long; these overlapping regions included all introns and about 500 bp upstream of the initiating methionine and 500 bp downstream of the stop codon. Sanger sequencing of each PCR product was carried out using three internal primers, all reading in a 5' to 3' direction (Psomagen). The chromatogram from one

9/26/2024 - Open Access

reaction transitioned to double-peaks about 200 nucleotides into the read, suggesting an indel event had occurred at that point that made the sequence from the balancer chromosome different from the *G.3.2*-bearing chromosome. After reading both bases in each double peak the CyO (wildtype for *cnk*) sequence was subtracted, revealing the inferred sequence of the *G.3.2* chromosome. This contains a 77 bp deletion of *cnk* that removes bases 2,774-2850, combined with a single base insertion of a G (Figure 1C). This predicts a frame-shifting loss of 76 nucleotides, as shown in figure 1C, causing a truncation of *cnk* after P671, and a premature stop codon after the addition of six novel amino acids. This removes more than half of the *cnk* amino acid sequence, including the PH domain, and is therefore very likely to be a null allele. The sequencing data further supports our genetic mapping data, thus solidifying *G.3.2* as a novel allele of *cnk*, *cnk*^{*G.3.2*}. Further confirmation of *G.3.2* as an allele of *cnk* could be completed by utilizing rescue experiments with a wild type *cnk* allele.

Cnk (Connector enhancer of KSR) is a multi-domain containing scaffold protein identified as a positive regulator of the RAS pathway and plays a crucial role in the signaling pathway in *Drosophila* (Therrien *et al.* 1999). Previous studies have shown that an eye entirely mutant for *cnk* prohibits normal eye development in a similar manner to that observed in loss-of-function mutations in *Ras1* or *ksr*. Our data, showing a reduction in mutant mosaic tissue representation, further supports this finding and suggests the role in eye development of *cnk* is autonomous in nature. Additionally, *cnk* disruption has been shown to impact pathways downstream of Ras including *Egfr* signaling. Previously, the Fly-CURE has isolated an allele of *Egfr* from this same screen (*Egfr*^{*L.3.1*}). The *Egfr* allele and *cnk* alleles had similar mosaic phenotypes suggesting a similar disruption of *Drosophila* eye development (Stamm *et al.* 2019).

Reagents

Stocks from BDSC 2R Deficiency Kit			
Genotype	BDSC #	Region	Complementation Results
<i>Df(2R)BSC161</i>	9596	2R:17304783..17484828	Failure to complement
<i>Df(2R)Exel6066</i>	7548	2R:17097569..17394642	Complement
<i>Df(2R)BSC355</i>	24379	2R:17462347..17536673	Complement
Additional Deficiency stocks from BDSC			
<i>Df(2R)BSC154</i>	9541	2R:17304783..17420811	Failure to complement
Single genes tested for complementation			
Gene	BDSC	Allele	Complementation Results
<i>camp</i>	17063	<i>camp</i> ^{<i>ep2499</i>}	Complement
<i>CG10939</i>	12034	<i>CG10939</i> ^{<i>06373</i>}	Complement
<i>cnk</i>	6577	<i>cnk</i> ^{<i>E-2083</i>}	Failure to complement
<i>Bap55</i>	21174	<i>Bap55</i> ^{<i>EY15967</i>}	Complement
<i>l(2)k01209</i>	63391	<i>l(2)k01209</i> ^{<i>0540-G4</i>}	Complement

<i>mthl3</i>	84533	<i>TI{RFP[3xP3.cUa]=TI}mthl3^{attP}</i>	Complement
<i>robl</i>	28484	<i>robl^{G7617}</i>	Complement

Acknowledgements:

Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study.

References

Akdemir F, Farkas R, Chen P, Juhasz G, Medved'ová L, Sass M, et al., Abrams JM. 2006. Autophagy occurs upstream or parallel to the apoptosome during histolytic cell death. *Development* 133(8): 1457-65. PubMed ID: [16540507](#)

Cook RK, Christensen SJ, Deal JA, Coburn RA, Deal ME, Gresens JM, Kaufman TC, Cook KR. 2012. The generation of chromosomal deletions to provide extensive coverage and subdivision of the *Drosophila melanogaster* genome. *Genome Biol* 13(3): R21. PubMed ID: [22445104](#)

Cordes CN, Gouge MM, Morgan H, Porter C, Siders J, Bacab E, et al., Kagey JD. 2023. Genetic mapping of the p47 (L.3.2) mutation in *Drosophila melanogaster*. *MicroPubl Biol* 2023. PubMed ID: [37799208](#)

Gloor GB, Preston CR, Johnson-Schlitz DM, Nassif NA, Phillis RW, Benz WK, Robertson HM, Engels WR. 1993. Type I repressors of P element mobility. *Genetics* 135(1): 81-95. PubMed ID: [8224830](#)

Kagey JD, Brown JA, Moberg KH. 2012. Regulation of Yorkie activity in *Drosophila* imaginal discs by the Hedgehog receptor gene patched. *Mech Dev* 129(9-12): 339-49. PubMed ID: [22705500](#)

Merkle JA, Devergne O, Kelly SM, Croonquist PA, Evans CJ, Hwalek MA, et al., Kagey JD. 2023. Fly-CURE, a multi-institutional CURE using *Drosophila*, increases students' confidence, sense of belonging, and persistence in research. *J Microbiol Biol Educ* 24(3). PubMed ID: [38107988](#)

Öztürk-Çolak A, Marygold SJ, Antonazzo G, Attrill H, Goutte-Gattat D, Jenkins VK, et al., FlyBase Consortium. 2024. FlyBase: updates to the *Drosophila* genes and genomes database. *Genetics* 227(1). PubMed ID: [38301657](#)

St Johnston D. 2002. The art and design of genetic screens: *Drosophila melanogaster*. *Nat Rev Genet* 3(3): 176-88. PubMed ID: [11972155](#)

Stamm J, Joshi G, Anderson MA, Bussing K, Houchin C, Elinsky A, et al., Kagey J. 2019. Genetic mapping of EgfrL.3.1 in *Drosophila melanogaster*. *MicroPubl Biol* 2019. PubMed ID: [32550448](#)

Therrien M, Wong AM, Kwan E, Rubin GM. 1999. Functional analysis of CNK in RAS signaling. *Proc Natl Acad Sci U S A* 96(23): 13259-63. PubMed ID: [10557308](#)

Therrien M, Wong AM, Rubin GM. 1998. CNK, a RAF-binding multidomain protein required for RAS signaling. *Cell* 95(3): 343-53. PubMed ID: [9814705](#)

Tolwinski NS. 2017. Introduction: *Drosophila*-A Model System for Developmental Biology. *J Dev Biol* 5(3). PubMed ID: [29615566](#)

Funding:

Supported by National Science Foundation (United States) 2021146 to K. Bieser J. Kagey.

Supported by National Institutes of Health (United States) UL1GM118982, TL4GM118983, and RL5GM118981 to H. El Dana F. Koria.

Author Contributions: Hanadi Chammout: data curation, formal analysis, investigation, writing - review editing. Delia L. Adkins: data curation, formal analysis, investigation, writing - review editing. Aleece K. Al-Olimat: data curation, formal analysis, investigation, writing - review editing. Zeinab Alsaad: data curation, formal analysis, investigation, writing - review editing. Beatrice M. Altopp: data curation, formal analysis, investigation, writing - review editing. Tuqa Amer: data curation, formal analysis, investigation, writing - review editing. Feyi O. Apampa: data curation, formal analysis, investigation, writing - review editing. Gwendolyn R. Avery: data curation, formal analysis, investigation, writing - review editing. Isaac I. Bazzi: data curation, formal analysis, investigation, writing - review editing. Emilia D. Beck: data curation, formal analysis, investigation, writing - review editing. Elise L. Beier: data curation, formal analysis, investigation, writing - review editing. B.

9/26/2024 - Open Access

Shafer Belisle: data curation, formal analysis, investigation, writing - review editing. Lane Benton: data curation, formal analysis, investigation, writing - review editing. Madison M. Bolyard: data curation, formal analysis, investigation, writing - review editing. Olivia E. Brain: data curation, formal analysis, investigation, writing - review editing. Eldon T. Buckner: data curation, formal analysis, investigation, writing - review editing. Shria Roy Chowdhury: data curation, formal analysis, investigation, writing - review editing. Jennifer R. Cifranic: data curation, formal analysis, investigation, writing - review editing. Liam Cleary: data curation, formal analysis, investigation, writing - review editing. Tyler R. Clum: data curation, formal analysis, investigation, writing - review editing. Autumn M. Cruz: data curation, formal analysis, investigation, writing - review editing. Meghan V. DeGray: data curation, formal analysis, investigation, writing - review editing. Isabel L. Echeverry: data curation, formal analysis, investigation, writing - review editing. Haya El dana: data curation, formal analysis, investigation, writing - review editing. Sarah K. Elkadri: data curation, formal analysis, investigation, writing - review editing. Paige L. Estep: data curation, formal analysis, investigation, writing - review editing. Luke R. Falke: data curation, formal analysis, investigation, writing - review editing. Hannah J. Foor: data curation, formal analysis, investigation, writing - review editing. Anika S. Gullapalli: data curation, formal analysis, investigation, writing - review editing. Sandro S. Hakim: data curation, formal analysis, investigation, writing - review editing. Hussein B. Hazime: data curation, formal analysis, investigation, writing - review editing. Lauren E. Heininger: data curation, formal analysis, investigation, writing - review editing. Emma G. Hoeft: data curation, formal analysis, investigation, writing - review editing. Lauren M. James: data curation, formal analysis, investigation, writing - review editing. Yeowon Jeon: data curation, formal analysis, investigation, writing - review editing. Megan R. Johnson: data curation, formal analysis, investigation, writing - review editing. Laine P. Jordan: data curation, formal analysis, investigation, writing - review editing. Zayd Khan: data curation, formal analysis, investigation, writing - review editing. Sydney K. Kochensparger: data curation, formal analysis, investigation, writing - review editing. Fadi J. Koria: data curation, formal analysis, investigation, writing - review editing. Ruby M. Krasnow: data curation, formal analysis, investigation, writing - review editing. Veronica Lilly: data curation, formal analysis, investigation, writing - review editing. Eileen Lim: data curation, formal analysis, investigation, writing - review editing. Ian T. MacCormack: data curation, formal analysis, investigation, writing - review editing. Andriy Malesh: data curation, formal analysis, investigation, writing - review editing. Mikayla G. Mariano: data curation, formal analysis, investigation, writing - review editing. Audrey C. Mentzer: data curation, formal analysis, investigation, writing - review editing. Katelyn H. Messner: data curation, formal analysis, investigation, writing - review editing. Katlyn C. Myers: data curation, formal analysis, investigation, writing - review editing. Emily R. Newman: data curation, formal analysis, investigation, writing - review editing. Annie M. Richters: data curation, formal analysis, investigation, writing - review editing. Liliana Romero: data curation, formal analysis, investigation, writing - review editing. Adam Rotem: data curation, formal analysis, investigation, writing - review editing. Reese J. Saho: data curation, formal analysis, investigation, writing - review editing. Kaname Sawaki: data curation, formal analysis, investigation, writing - review editing. Ashley N. Selders: data curation, formal analysis, investigation, writing - review editing. Elizabeth Shockney: data curation, formal analysis, investigation, writing - review editing. Farah A. Sobh: data curation, formal analysis, investigation, writing - review editing. Isabelle F. Speiser: data curation, formal analysis, investigation, writing - review editing. Breanna M. Sproul: data curation, formal analysis, investigation, writing - review editing. Veronica J. Sroufe: data curation, formal analysis, investigation, writing - review editing. Antonia Tollkuci: data curation, formal analysis, investigation, writing - review editing. Cassandra C. Trevino: data curation, formal analysis, investigation, writing - review editing. Megan A. Vapenik: data curation, formal analysis, investigation, writing - review editing. Erin M. Wagner: data curation, formal analysis, investigation, writing - review editing. Kayla L. Bieser: data curation, formal analysis, investigation, methodology, supervision, validation, writing - review editing. Jamie L. Siders: data curation, formal analysis, investigation, writing - review editing. Justin R. Thackeray: data curation, formal analysis, investigation, writing - review editing. Jacob D. Kagey: conceptualization, data curation, formal analysis, funding acquisition, investigation, project administration, supervision, validation, writing - review editing.

Reviewed By: Anonymous**Nomenclature Validated By:** Anonymous**History:** Received July 19, 2024 **Revision Received** August 26, 2024 **Accepted** September 23, 2024 **Published Online** September 26, 2024 **Indexed** October 10, 2024**Copyright:** © 2024 by the authors. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International (CC BY 4.0) License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.**Citation:** Chammout, H; Adkins, DL; Al-Olimat, AK; Alsaad, Z; Altopp, BM; Amer, T; et al.; Kagey, JD (2024). G.3.2 is a novel allele of the gene *connector enhancer of ksr* (*cnk*) in *Drosophila melanogaster*. microPublication Biology. [10.17912/micropub.biology.001290](https://doi.org/10.17912/micropub.biology.001290)

