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A screening method for identification of heterochromatin-promoting drugs using Drosophila

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October 17, 2019

Jove Editorial
1 Alewife Center, Suite 200
Cambridge, MA 02140

Dear Editor,

We are submitting a manuscript titled "**A screening method for identification of heterochromatin-promoting drugs using *Drosophila***" for consideration for publication in *Jove*.

Drosophila is an excellent model organism that can be used to screen compounds that might be useful for cancer therapy. The method described here is a cost-effective *in vivo* method to identify heterochromatin-promoting compounds by using *Drosophila*. The *Drosophila's DX1* strain, which has a variegated eye color phenotype that reflects the extents of heterochromatin, provides a tool for heterochromatin-promoting drug screen. In this screening method, eye variegation is quantified based on the surface area where red pigmentation occupies parts of the eye, and is scored on a scale from 1 to 5. The screening method is straightforward and sensitive and allows for testing compounds *in vivo*. Drug screening using this method provides for a fast and inexpensive way for identifying heterochromatin-promoting drugs that could have beneficial effects in cancer therapeutics. Identifying compounds that promote the formation of heterochromatin could also lead to discovery of epigenetic mechanisms of cancer development.

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Thank you for your help in this matter!

Sincerely,

Lin Zhang, Ph.D.
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TITLE:

A Screening Method for Identification of Heterochromatin-Promoting Drugs Using *Drosophila*

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KEYWORDS:

Heterochromatin; tumor suppression; small-molecule compounds; drug screening; heterochromatin-promoting drug; cell proliferation; *Drosophila* larvae; *Drosophila* cells; adult *Drosophila*; position-effect variegation

SUMMARY:

Drosophila is a widely used experimental model suitable for screening drugs with potential applications for cancer therapy. Here, we describe the use of *Drosophila* variegated eye color phenotypes as a method for screening small-molecule compounds that promote heterochromatin formation.

ABSTRACT:

Drosophila is an excellent model organism that can be used to screen compounds that might be useful for cancer therapy. The method described here is a cost-effective in vivo method to identify heterochromatin-promoting compounds by using *Drosophila*. The *Drosophila's DX1* strain, having a variegated eye color phenotype that reflects the extents of heterochromatin formation, thereby providing a tool for a heterochromatin-promoting drug screen. In this screening method, eye variegation is quantified based on the surface area of red pigmentation occupying parts of the eye and is scored on a scale from 1 to 5. The screening method is straightforward and sensitive and allows for testing compounds in vivo. Drug screening using this method provides a fast and inexpensive way for identifying heterochromatin-promoting drugs that could have beneficial effects in cancer therapeutics. Identifying compounds that promote the formation of heterochromatin could also lead to the discovery of epigenetic mechanisms of cancer development.

INTRODUCTION:

Heterochromatin is a condensed form of DNA that plays a central role in gene expression, in regulating chromosome segregation during cell division, and in protecting against genome instability[1]. Heterochromatin has been considered to be a gene repression regulator and to protect chromosome integrity during cell mitosis[2,3]. It is associated with the di- and tri-methylation of histone H3 lysine 9 (H3K9me) during lineage commitment[4,5]. Moreover, recruitment of Heterochromatin Protein 1 (HP1) chromodomain proteins is also considered to be associated with heterochromatin and epigenetic repression of gene expression[6]. These proteins are essential components and markers of heterochromatin formation.

Since genomic instability enables cells to acquire genetic alterations that promote carcinogenesis, heterochromatin is becoming more recognized in cancer development and may be targeted for cancer treatment[7,8]. Currently, there are no drugs that are well-established in assisting heterochromatin formation. Here, we present a simple and quick yet efficient method for screening small-molecule compounds that promote heterochromatin formation. The screening is done by treating *Drosophila* with a library of small-molecule drugs. This method takes advantage of a variegated eye color phenotype in the *DX1 Drosophila* strain that is influenced by heterochromatin levels. *DX1* flies contain a tandem array of seven *P[lac-w]* transgenes, which have the variegated expression/depression depending on heterochromatinization, therefore, the extent of variegation in the eye color reflect the heterochromatin level. Specifically, increasing heterochromatin could be detected by the rising proportion of variegated eye color (white eye). On the contrary, decreasing heterochromatin would be detected by the rising proportion of the *P[lac-w]* transgene expression (red eye)[9-12].

Therefore, we take advantage of this *Drosophila* transgene system that produces a variegated eye color phenotype since its expression is directly correlated to the amount of heterochromatin present. Upon discovery of compounds that are suspected to promote heterochromatin formation, we may confirm this suspicion using other methods such as western blot. These heterochromatin-promoting substances may be further developed for clinical trials in patients in the future.

PROTOCOL:

1. Drug library preparation

1.1. Identify and prepare a drug library to be screened using appropriate solvent at a desired concentration (e.g., 10 mM in DMSO).

NOTE: A specific example for a drug library is the Oncology Set III from National Cancer Institute (NCI) Developmental Therapeutics Program (DTP). Compounds in this set are provided as 20 µL at 10 mM in 100% DMSO in two 96-well PP U-bottom plates and stored at -20 °C. The NCI Plate maps, and the basic chemical data of each drug could be found at the following website. (https://dtp.cancer.gov/dtpstandard/servlet/PlateMap?searchlist=4740&outputformat=html&searchtype=plate&Submit=Submit).;

https://dtp.cancer.gov/dtpstandard/servlet/PlateMap?searchlist=4741&outputformat=html&searchtype=plate&Submit=Submit).

2. Screen for heterochromatin-promoting drugs using *Drosophila*

2.1. *Drosophila* Breeding strategy (**Figure 1A**)

2.1.1. Day 1: Cross three w^{1118}*/Y; DX1/CyO* male flies and three virgin w^{1118} female flies in a 25 mm x 95 mm food vial with 9 mL of standard *Drosophila* food media (Bloomington recipe). Set up three replicate vials for each drug and one control for the screen.

NOTE: *DX1* flies were kindly provided by James Birchler (University of Missouri)[9,13].

2.1.2. Day 2 and Day 3: Allow the flies to lay eggs for 2 days at room temperature (22 °C).

2.1.3. Day 4: Use a short burst of CO_2 gas to anesthetize the parent flies and dispose of the parent flies in a "fly morgue" (a bottle containing 70% alcohol).

2.2. Feed drugs to *Drosophila* for screening.

2.2.1. Dilute each drug compound from the original stock (20 µL at 10 mM in 100% DMSO in 96-well PP U-bottom plates) to a 10 µM final concentration with 33% DMSO in water. Use 33% DMSO in water without any drug as the control.

NOTE: Some drugs are poorly soluble in water and were dissolved in DMSO. 100% DMSO is toxic to flies. 33% is tolerable.

2.2.2. Day 4: Pipette 60 µL of a 10 µM drug solution or control onto the top of fly food. At this point, ensure that the parent flies have been removed and there are fly eggs and crawling first-instar larvae on the food.

2.2.3. Day 6: Repeat pipetting 60 µL of the same 10 µM drug solution to the food vial.

NOTE: Since drugs were added on to the fly food, the top surface food contains nearly 10 µM concentration and the bottom food could be not be fully penetrated. All the flies lay eggs on the food top and eat the top surface food.

2.3. Observe and score eye color changes.

2.3.1. Two days after the F1 flies emerge (approximately on day 14), examine the flies. Use a short burst of CO_2 to anesthetize the F1 flies and remove the flies from their vial onto a porous dissecting pad with CO_2 sipping through from underneath a filter paper.

2.3.2. Inspect the flies on this pad under a dissection microscope. Examine the whole fly and

identify all *w*^{1118}*/Y; DX1/+* heterozygous males by their lacking the *CyO* dominant curly wing phenotype.

2.3.3. Score the eye color of each of the *w*^{1118}*/Y; DX1/+* heterozygous male flies on a scale of 1 to 5 (**Figure 1B**). The percentage of white eye color was rated as follows:

1. <5% red scattered eye total surface area
2. 6% - 25% red spots eye total surface area
3. 26% - 50% red spots eye total surface area
4. 51% - 75% red spots eye total surface area
5. >75% red spots eye total surface area

NOTE: Alternatively, homogenize fly heads in 100% methanol and use a spectrometer to measure eye pigmentation at OD 450 nm. Select only males because PEV is more pronouced in DX1 males. Score more than 10 males in each vial.

2.3.4. Calculate the mean color index of all the males from each vial scored. Perform triplicates for each compound (**Figure 1C**).

NOTE: Since eye color varies with age, this is a time-sensitive step. The eye color should be calculated at the same age – 2 days old. Usually >10 flies should be scored in each vial.

2.3.5. To confirm that the compounds indeed promote heterochromatin formation, use a different technique such as western blotting to validate (**Figure 1G**).

REPRESENTATIVE RESULTS:

This protocol was successfully used to screen compounds that promote heterochromatin formation in *Drosophila*, which is an efficient and low-cost in vivo system for drug development (**Figure 1**). We screened a small-molecule drug library, Oncology Set III, which is composed of 97 FDA authorized oncology drugs, using the *DX1* strain of *Drosophila melanogaster* (**Figure 1B**). The results for a significant drug were represented in a bar graph (**Figure 1C,D**). According to the screening assay, methotrexate (4-aminopteroylglutamic acid), which is coded as E7 in the library, caused the most variegation in the *DX1* strain, suggesting that methotrexate could be the most promising heterochromatin-promoting drug for future cancer targeted-therapy (**Figure 1E,F**). To further confirm that this is indeed testing the compounds promoting heterochromatin formation, western blot data shows that heterochromatin formation associated protein H3K9me3 is upregulated (**Figure 1G**), further verifying that methotrexate could be the most promising heterochromatin-promoting drug.

FIGURE AND TABLE LEGENDS:

Figure 1: An illustration of the drug screening in *Drosophila*. (**A**) *Drosophila* screen methodology. An overview of scheme for heterochromatin-promoting compounds screening by using the *Drosophila* model. Three *w*^{1118}*/Y; DX1/CyO* males and three virgin *w*^{1118} are crossed at room temperature. Then, remove the adult flies at day four and add 60 µL of the 10 µM drug dissolved in 33% DMSO solution to the top of the fly food at day four and day six, respectively.

33% DMSO solution was used as a control. The eye color (white to red) ratio of the F1 male generation were observed. (**B**) Eye color phenotype analysis and score. Representative images of eye color showing variegated phenotype. The percentage of variegation eye color was rated as following: 1, 1-5% red spots in total surface area; 2, 6-25% red spots in total surface area; 3, 26-50% red spots in total surface area; 4, 51-75% red spots in total surface area; and 5, >75% red spots in total surface area. (Scale Bar = 200 μm) (**C**) Each drug was assayed as the mean values from three independent experiments ± s.d. (standard deviation). Red bar shows the control. (**D**) Results of the scale performed in drug E7 and control. Differences between E7 and control groups were considered significant if P-values were <0.05 (*) by Student's t-Test. (**E**) The molecular structure of a chemical compound which termed E7 used in the example drug library. (**F**) Representative images of the fly eyes in E7 and control treatment group. (Scale Bar, 200 μm) (**G**) The western blot was performed with antibodies specific for $H_3K_9me_3$, H_3, or α-Tubulin by using the 3rd instar larvae total protein without or with methotrexate treatment at the indicated concentrations. This figure has been modified from Loyola et al[14].

DISCUSSION:

Heterochromatin is a condensed form of DNA that plays a central role in regulating gene expression. It is becoming increasingly more recognized in cancer and may serve as a potential target for cancer therapy[15-18]. Small-molecule compounds are commonly used in drug development due to advantages in manufacture, preservation, and metabolism in human bodies. To identify heterochromatin-promoting small-molecule compounds, an efficient method was designed and presented by using the *DX1 Drosophila* strain, which has been proven to affect flies' eye color in a heterochromatin-dependent manner (**Figure 1**).

Our screening protocol is a simple, inexpensive and easy-to-follow in vivo system for drug development. However, one limitation of the screening strategy is determining an effective drug concentration. The female fruit fly lays her eggs on the surface of the semisolid fly food. For the drug treatment, we pipet the drug solution to the surface of the food, which is supposed to contain a predetermined concentration. However, since different drugs could have different efficiencies in diffusing to the bottom of the food, the drug concentration is not guaranteed to be consistent below the surface or towards the bottom of the food. At 10 μM concentration, lethality was seldom observed in larvae treated with any of the drugs in the library we screened. However, most of the drugs caused severe lethality to the larvae at 100 μM concentration, suggesting that drug concentration could also be considered as an important factor for the screen.

Here, we notice that tandem repeats such as those used here can trigger PEV by a mechanism that differs in some regards from that seen in pericentric heterochromatin. A drug that impacts PEV during embryonic and larval development (the test described here) will only identify drugs that impact the maintenance of heterochromatin in somatic cells and will not identify drugs that impact the initial formation of heterochromatin, which most likely occurs during blastoderm (nuclear cycles 10-14). Nonetheless, this could be a useful assay for a quick starting screen.

This protocol is reliable and cost-effective due to the sensitivity of this transgene cluster (*DX1*) to heterochromatin formation, reflected by the amount of red pigmentation present in the eye. Additionally, the short lifespan of *Drosophila* makes this protocol more efficient than other model organisms such as zebrafish or human cells. While there likely might be a reporter gene system present in zebrafish that is just as sensitive to heterochromatin levels, their lifespan is much longer than *Drosophila* and would take longer to obtain results. Additionally, using human cells would not be possible since this protocol specifically focuses on using phenotypic changes from epigenetic regulation to determine heterochromatin levels. Thus, this protocol provides an efficient in vivo method to determine which small drug molecule could potentially serve as a candidate for suppressing oncogenes in cancer therapeutics. While the *Drosophila* drug screening method is a slow approach to identifying compounds when compared with some in vitro screening methods, it is relatively sensitive and allows us to test compounds in vivo. In combination with the cell screening method, which is relatively inexpensive, easy to perform and is high throughput, the *Drosophila* screening method could also be used to confirm hits as a supplementary method.

In summary, with an interest in identifying compounds for further research and targeting heterochromatin for cancer therapy, although the mechanism in which this is occurring is not yet fully understood, a simple screen was performed in order to identify heterochromatin-promoting drugs. Discovering a low concentration at which a drug can promote heterochromatin formation could offer more unique treatments that may result in fewer side effects compared to modern chemotherapy treatments.

ACKNOWLEDGMENTS:

We thank J. Birchler, E. Bach and the Bloomington Drosophila Stock Center for various Drosophila strains; the National Cancer Institute (NCI) Developmental Therapeutics Program for the Oncology Set small-molecule drug library; UCSD undergraduate students including Amy Chang, Taesik You, Jessica Singh-Banga, Rachel Meza, and Alex Chavez. Research reported in this publication was supported by a research grant from American Thoracic Society to J.L. and funding from NIH: R01GM131044 to W.X.L.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

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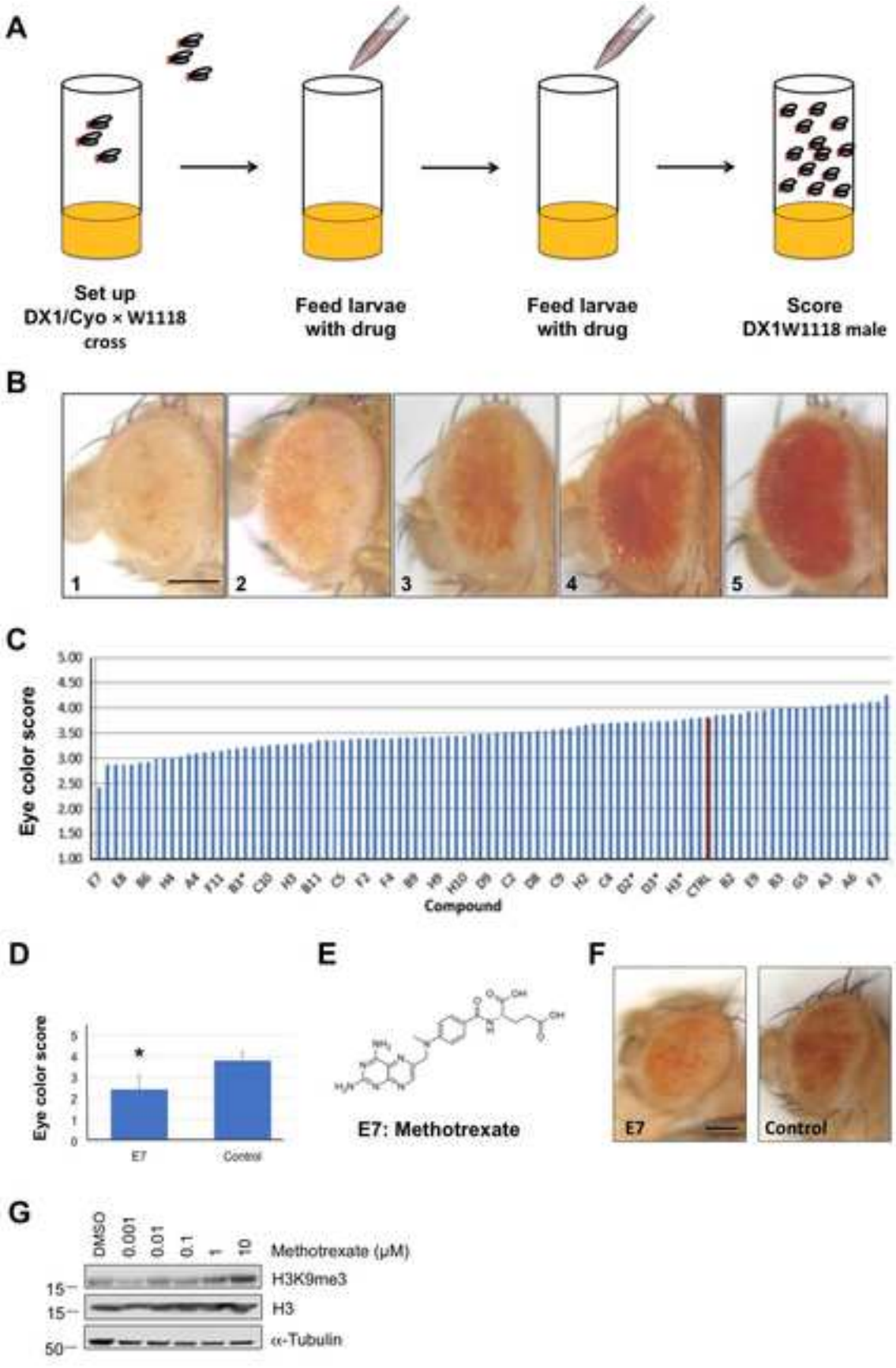
Figure

Click here to access/download;Figure;Figure 1.psd



Name of Material/Equipment	Company	Catalog Number
Dimethyl sulfoxide (DMSO)	Sigma	D2650
Drosophila		*DX1* strain
Drosophila food media	UCSD fly kitchen	
Methotrexate	NCI drug library	

	Comments/Description
DX1	flies were kindly provided by James Birchler (University of Missouri)

UC San Diego
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November 22, 2019

Jove Editorial
1 Alewife Center, Suite 200
Cambridge, MA 02140

Dear Editor,

We appreciate the referees and editors for their time. We are grateful the reviewers for their constructive comments. Several points were not clear in our original manuscript. We would like to apologize for the lack of clarity and have now revised the paper to explain the protocol better. Below we will respond to the comments point by point.

Editorial comments 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Reply: We have proofread the manuscript thoroughly for spelling and grammar issues to the best we can.

Editorial comments 2. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Reply: We have reviewed the all text in the protocol section thoroughly and made sure the text is written in the imperative tense. There is no phrase such as "could be," "should be," and "would be" throughout the Protocol.

Editorial comments 3. Please reword lines 75-77, 104-105, 111-112, 116-121 as it matches with previously published literature.

Reply: We have reworded lines 75-77, 104-105, 111-112, 116-121.

Editorial comments 4. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Reply: To make the protocol be clearer and easier to follow, we have added more details to step 1, 2.1.1, 2.1.3, 2.2.1, 2.2.2, 2.2.3, 2.3.1, 2.3.2, 2.3.3, 2.3.4, 2.3.5.,

Editorial comments 5. Please include a citation for the drug library.

DEPARTMENT OF MEDICINE
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Reply: The detailed drug information including drug library origin, the NCI Plate maps, the Basic Chemical Data of each drug and detailed protocol have been added to line 80-90.

Editorial comments 6. 2.1.1. Please include details on how you make standard food media. How many drugs and controls are tested in this case? How many replicates per drug?

Reply: Food media (Bloomington recipe) were purchased from the University of California at San Diego (UCSD) fly kitchen and the detailed food media information has been added to JoVE_Table_of_Materials. We tested 97 drugs and 1 control (33% DMSO in water) in this case, 3 replicates have been done per drug. We have added this information in the protocol 1 and 2.2.1.

Editorial comments 7. 2.1.3: How do you remove the flies? Do you anesthetize? Do you just transfer the files to another vial? Please provide details.

Reply: To remove the parent flies from the vial, we use CO_2 to anesthetize the parent flies. And the details have been added to 2.1.3.

Editorial comments 8. 2.2.1: How did you come up with the final concentration value? Citation if any? Why 33% DMSO?

Reply: Some drugs are poorly soluble in water and were dissolved in DMSO. 100% DMSO is toxic to flies. 33% is tolerable. We added this as a note to line 127-128.

Editorial comments 9. 2.2.2. and 2.1.2 are performed on the same day? Please write steps in the order of it being performed.

Reply: 2.2.2. and 2.1.2 are performed on different days. We corrected it in the text of 2.2.2.

Editorial comments 10. 2.2.3 and 2.1.3 are performed on the same day?

Reply: 2.2.3. and 2.1.3 are also performed on different days. We corrected it in the text of 2.2.2.

Editorial comments 11. 2.2.4, 2.2.5 How do you do this? Do you anesthetize the flies? Do you view them under microscope? Is determining the color scale done by a blinded person? Do you click pictures and use some software to do so? Please provide all the specific details. Is the color scale study performed only on curly wing males? Why?

Reply: We added detailed protocol in 2.2.3 and 2.2.4. We use CO_2 to anesthetize the flies. Specifically, to view the flies' phenotypic change, remove flies from their vial onto a porous dissecting pad with CO_2 sipping through from underneath a filter paper . Inspect the flies on this pad under a dissection microscope. Score eye color only on straight-winged males, which are of DX1/+, according to the color scale of 1 to 5. It is helpful to remove all other flies – males with curly wings and females. {To determine the color scale, perform a blind or blinded experiment. Examine the whole fly by eye and select the eye area and magnify. Hold the camera lens against the microscope eyepiece and record pictures. Use Image J to quantify the eye color. Deleted all within {} – we did NOT take pictures and use Image J; this would be too tedious!}

Editorial comments 12. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Reply: We have highlighted 2.75 pages or less of the Protocol with light yellow.

Editorial comments 13. For the result section, please include validation with some other molecular technique (Western blot etc.) to confirm that this is indeed testing the compounds promoting heterochromatin formation.

Reply: We added the Western blot data to confirm that the compound indeed promotes heterochromatin formation.

Editorial comments 14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Reply: Based on the copyright permission policy from Scientific Reports: "The author of articles published by Springer Nature do not usually need to seek permission for re-use of their material as long as the journal is credited with initial publication." (https://www.nature.com/nature-research/reprints-and-permissions/permissions-requests#permission-requests-from-authors). We added "This figure has been modified from [citation] to the legend.

Editorial comments 15. Please expand the journal titles in the reference section.
Reply: We use EndNote to format the reference by its JoVE EndNote style file and expanded the journal titles.

Editorial comments 16. Figure 1: Please include a scale bar.

Reply: We added a scale bar for Figure 1 B and Figure 1 F.

Editorial comments 17. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

Reply: We have added more information to the JoVE_Table_of_Materials.

Reviewer #1:
Major Concerns: 1. As per author's own admission, they used DX1 fly strain that contains certain level of PEV in the eye color and they assumed that exposure to small molecule possibly directly affect the levels of PEV that is proxy to levels of heterochromatin. But Authors must be aware that, heterochromatin may not be necessarily directly involved in the mechanism for more or less PEV, so authors need to take extra precaution in showing representative results for the screen.

Reply: : We confirmed with Western blot for levels of H3K9 methylation, a known heterochromatin marker.

Major Concerns: 2. Authors need to provide at least one control experiment to provide evidence that indeed change in PEV is directly linked to change in heterochromatin status. One can envision doing a ChIP assay for DX1 transgene region in flies with or without drug exposure to see H3K9 methylation levels.

Reply: We added the Western blot data for H3K9 methylation levels in flies with or without drug exposure.

Major Concerns: 3. It is imperative to show independent validation of the PEV levels across the population by doing eye pigment assay with group of flies raised on the same food with or without drugs. In fact, it would be much

more objective and quantifiable approach to measure levels of heterochromatin under any condition. Also it is fast, reproducible and inexpensive method to supplement visual scoring.

Reply: We agree that measuring eye pigmentation is more objective. But our screen was done by examining eye color by human eye in the original paper. Nonetheless, we added a Note in the protocol "Alternatively, homogenize fly heads in 100% methanol and use a spectrometer to measure eye pigmentation at O.D. 450 nm"

Major Concerns: 4. They absolutely need to show more than one promising candidate hit and possibly provide rationale behind potential mechanism.

Reply: Here we screened a 97-compounds drug library and only identified one promising candidate probably due to the drug library size limitation. We would like to introduce such a screening method for identification of heterochromatin-promoting drugs by using Drosophila and further research will be focused on scaling up the drug library size, in order to find more heterochromatin-promoting drugs.

Major Concerns: 5. As the DX1 transgene is 2nd chromosome linked; it is not clear why they only chose to show pigment levels in straight wing 'male' flies. In fact, it would be interesting to know if there seem to be any sex specific bias in the heterochromatin levels due to the drugs exposure.

Reply: The parent flies were DX1/CyO. So curly F1 flies won't inherit the DX1 transgene.

Major Concerns: 6. While the authors eluded to the concentration and drug diffusion conundrum that is a big question for this screen, they certainly have not tested other alternatives to administer drug. One can envision administering food containing drug in containers that have more surface area like a petri plate set up. They also do not explain why they need second dose of drug exposure.

Reply: We crossed flies in commercial 25x95 mm food vials each with 9 ml of standard Drosophila food media. Since drugs were added on to the fly food, the top surface food contains nearly 10 μM concentration and the bottom food could be not be fully penetrated. We suppose all the flies lay eggs on the food top and eat the top surface food. We should test other alternatives to administer drug like a petri plate. But it is not practical to raise flies in petri dishes. Regarding to the drug does, we also test higher concentration (100 μM) and it caused severe lethality to the larvae during the early development.

Minor Concerns: 1. In the discussion where the authors discuss the potential of the screen hits to enter into clinical trials right after few validation steps, it becomes too far reaching speculation given that exact concentration of drug is not known, there is limited range of concentrations flies can tolerate and more importantly, tolerance and tissue toxicity limits for flies and humans would be completely different.

Reply: We agree with the reviewer's point and have deleted this sentence. We are currently testing whether the promising candidate which we selected would be efficient in cancer cell lines as a next step, we'll validate its role in vitro and in vivo.

Minor Concerns: 2. The representative images of eye PEV patterns are not striking enough to convince one of the efficiency of this method. Please try to provide better resolution pictures with more evident differences between control and test scenarios.

Reply: The images were from Loyola et al (reference).

Reviewer #2:
Major Concerns: 1. Why was the drug added on the food-surface when it could be dissolved with food uniformly? An explanation must be provided.

Reply: We added a detailed explanation as a Note in protocol 2.2.3.

Major Concerns: 2. Why only the w1118; DX1/+ males were selected for the screening assays? Appropriate explanation may be incorporated.

Reply: We added a Note: Select only males because PEV is more pronounced in DX1 males

Major Concerns: 3. The manuscript includes almost identical or same figures/images those are already published in Loyola et al. 2019 (Scientific Reports). Image duplication and novelty policies of the journals may be referred before publication.

Reply: Here we would like to introduce a general screening method for identification of heterochromatin-promoting drugs by using Drosophila. We have obtained explicit copyright permission to reuse the figures from the previous publication. We also cited the figure appropriately in the Representative Results (line 297) and Figure Legend (line 346).

Reviewer #3:
Major Concerns: 1. The major problem with the assay is that it is not clear what concentration of drug is being tested, or how reproducible the results are, as applying the drug to the food surface means that the concentration will be very uneven in the vial, as acknowledged. Reproducibility for this assay may be affected by the food used, local humidity etc. At a minimum, a standard food recipe should be provided or referenced. The drug is to be solubilized in 33% DMSO, which is necessary for many compounds, but problematical; as I recall, the presence of uniform DMSO in fly media at greater than 0.1% causes detrimental effects in developing flies (see Levinson & Cagan 2016 Cell Reports 16: 3052-61). The drugs to be tested will also vary in toxicity. Thus only flies that ingest a) the appropriate concentration to have an impact while b) avoiding concentrations that would be lethal and c) avoiding concentrations of DMSO that would be deleterious will provide the change in PEV being scored. This will be a minority of the adults assayed, but might be sufficient. To convince us, the authors need to demonstrate reproducibility. Given their results, this could be done using their system with methotrexate, the drug they identify as having an impact. This should be done showing us not only a numerical average, but pictures of a representative field of eyes. One would anticipate striking change in a minority of the population - is this what is observed?

Reply: We have provided a standard food recipe. We have tested different concentrations of DMSO and have found that 33% is tolerable in the food we used. We indeed tested several candidate drugs that increased PEV and found only one that showed consistency.

Major Concerns: 2. It should be noted that follow-up tests looking at the impact of the drug over a range of concentrations are normally required to establish an effect. Given the uneven administration of the drug in this assay, it is not clear whether the same protocol can be used, or a different approach will be needed. Again, this could be tested with methotrexate.

Reply: To confirm that the compounds indeed promote heterochromatin formation, we used Western blotting (Fig. 1G). Western blot was performed to test H3K9 methylation levels by using the 3rd instar larvae total protein without or with methotrexate treatment at different concentrations.

Major Concerns: 3. The Discussion should point out that a user might want to check the impact of a given drug on other PEV reporters to establish the generality of the effect. Tandem repeats such as those used here can trigger PEV by a mechanism that differs in some regards from that seen in pericentric heterochromatin. And it should be pointed out that a drug that impacts PEV during embryonic and larval development (the test described here) will only identify drugs that impact the maintenance of heterochromatin in somatic cells, and will not identify drugs that impact the initial formation of heterochromatin, which most likely occurs during blastoderm (nuclear cycles 10-14). Nonetheless, this could be a useful assay for a quick starting screen.

Reply: We really appreciate the reviewer's suggestions and added this information to the discussion (line 367-373).

Minor Concerns: 1.1. Please provide a reference for the library. Were the compounds supplied in dry form or in a DMSO solution?

Reply: We added the details in line 83-90.

Minor Concerns: 2.1.1 Why did the authors decided perform a cross between DX/CyO and +/+? This step does not change the PEV reporter copies, but adds significantly to the labor required to do the screen. Does it impact the PEV phenotype? Note that the phenotype of most variegating lines can be made more uniform by inbreeding with selection for either high or low levels of pigment. (In this case, one would want higher levels of white expression to facilitate identification of increased heterochromatin formation.) By reducing noise, this could result in more consistent observations and increase sensitivity.

Reply: We performed an outcross in order to minimize background PEV modifiers due to inbreeding. We agree that it increased labor.

Minor Concerns: 2.1.1 to 2.2.5 The time line description is confusing, in that it implies that the drug is first added on Day 2, when the adult flies are still in the vial. Is this correct? It would be easier to add the drug after the adults are removed, as implied by the description in Fig 1 (line 142).

Reply: We clarified the drug administration timeline in 2.1.1, 2.1.2, 2.1.3, 2.2.2, 2.2.3.

Minor Concerns: 2.2.5 The determination of PEV level (degree of expression of the white reporter gene) as presented is very subjective and cumbersome. For quantitation, one could use a pigment extraction assay. If you wish to score the pattern, use ImageJ software on an I-phone to get a quantitative assay.

Reply: To determining the color scale, perform a blind or blinded experiment. For quantitation, we also used a pigment extraction assay (data not shown). We use Image J to quantify the eye color.

Minor Concerns: 2.2.6. How many males were scored for each drug/vial? What is the standard deviation, as well as the mean? Show some measure of variation in Figure 1C. The fly eye images provided are of very low quality; sharpen the focus for Fig 1B and Fig 1F.

Reply: Figure 1D shows mean and standard deviation for one drug we tested multiple times. We sharpened the images.

Minor Concerns: Discussion, line 189. The authors claim that the assay is very sensitive - by what criteria? This needs to be shown.

Reply: We changed "very sensitive" to "relatively sensitive" in the text. The word choice is based on the knowledge that a genetic screen for PEV modifiers identified many mutations including HP1, Su(var)3-9 etc heterochromatin components.

Reviewer #4:
Major Concerns: The protocol as it is currently described could benefit from a bit more detail, as well as more proof-of-principal. The authors demonstrate that the drugs affect the eye color phenotype, but do not provide evidence that this is due to modulation of heterochromatin, so that may give pause to potential users of the protocol. I recommend the authors include evidence on the chromatin status, perhaps modified from (Loyola et al 2019) about this point. Another option might be to explain how, if a drug is identified in this screen, what would be the next steps/recommended experiments to demonstrate it is a chromatin modulator and not affecting fly eye color in some other way. Aside from that, I have minor suggestions, as detailed below.

Reply: We added more details to the protocol and included confirmation results (Western blots, Figure 1G).

Minor Concerns: 1) The introduction, especially the first sentence, is somewhat vague in how the authors link heterochromatin and cancer. For example, they say "Heterochromatin … affects genome instability". If they are trying to say it protects against instability, or ensures stability, that would be more clear.

Reply: We revised accordingly in the introduction.

Minor Concerns: 2) Line edit for clarity: "has been considered [to be a] gene repressing regulator and [to protect] chromosome integrity…"

Reply: We modified this sentence as suggested.

Minor Concerns: 3) More details on how P[lac-w] affects eye color, eg, through expression of the white mini-gene would be helpful in the introduction for non-experts. They should also include references about the genetic evidence that variegation relates to chromatin status.

Reply: We added evidence in reference 9-12.

Minor Concerns: 4) For section 1.2, please include information on storage of compounds in DMSO- eg, how long and under what conditions can diluted compounds be stored, or make general recommendations if it is case-specific.

Reply: We added the detailed storage information in line 84-85.

Minor Concerns: 5) In part 2.1.1. the authors should include information on how many vials they recommend be set up per drug (triplicate?)

Reply: We added the detailed information in 2.1.1.

Minor Concerns: 6) The timing is confusing in sections 2.1 and 2.2 and in the figure, in part because there is more than one mention of Day 2 and day 4. In the protocol, it says to remove the parents at day 4, then add the drug at day 2 and day 4. Presumably, that means the timing starts again after removing the adults, therefore adding drugs at day 6 and 8 from the start of the experiment- but it is not clear. In figure 1 legend, it states "remove the adult flies at day four and add 60 µl 10 µM drug … at day four and day six, respectively." So, that is also different from my

interpretation of the main text. The authors must clarify the timing of steps in this section.

Reply: We clarified the timeline in 2.1.1, 2.1.2, 2.1.3, 2.2.2, 2.2.3, 2.3.1.

Minor Concerns: 7) For part 2.2.4, since eye color varies with age, the authors may want to caution/remind readers that this is a time-sensitive step.

Reply: We added the information in line 214-215.

Minor Concerns: 8) For part 2.2.5-6, how many males should be screened?

Reply: Usually >10 in each vial. We added this to Notes.

Minor Concerns: 9) While the figure 1B is helpful, it is not entirely clear what's red compared to pink/orange. The authors should consider using a close-up picture in which individual ommatidia are clearer, and I would encourage them to outline the "red" cells in a few examples, especially the lower scoring ones, to give a better feeling of how to make a call for the percentages affected. I also wonder if this could be done in an automated way, eg, with images that are scored by a computer. Perhaps the authors could comment.

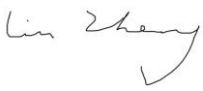
Reply: We provided details in the scoring section.

Minor Concerns: 10) As the authors explain in the conclusions, it is not clear how much drug the larvae are exposed to. Thus, perhaps the authors could suggest how to set up a dose-response curve or other titration experiments.

Reply: We used different doses on larvae and provided Western blot results (Fig. 1G). It is difficult to test dose on PEV because flies won't survive high dose.

In the end, we would like to thank the referees and editors for evaluating our manuscript again! We have tried to address all the reviewers' concerns in a proper way and believe that our paper has improved considerably. We would be happy to make further corrections if necessary and look forward to hearing from you soon.

Respectfully,



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