A method for rapid selection of randomly induced mutations in a gene of interest using CRISPR/Cas9 mediated activation of gene expression.

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Abstract

We have developed a CRISPR/Cas9 based method for isolating randomly induced recessive lethal mutations in a gene of interest (GOI) by selection within the F1 progeny of a single genetic cross. Our method takes advantage of the ability to overexpress a GOI using CRISPR/Cas9 mediated activation of gene expression. In essence, the screening strategy is based upon the idea that if overexpression of a wild type allele can generate a phenotype, then overexpression of a newly induced loss-of-function allele will lack this phenotype. As a proof-of-principle, we used this method to select EMS induced mutations of the Drosophila gene *hindsight* (*hnt*). From approximately 45,000 F1 progeny we recovered 8 new EMS induced loss-of-function *hnt* alleles that we characterized as an allelic series of hypomorphic mutations. This new method can, in theory, be used to recover randomly induced point mutants in a GOI and can be applied to any circumstance where CRISPR/Cas9 mediated activation of gene expression is associated with lethality or a visible phenotype.

Introduction

CRISPR/Cas9 based genome editing represents a significant advance within the field of genetics. In general, co-expression of Cas9 and an engineered single guide RNA (sgRNA) forms a sequence homology-dependent endonuclease that creates DNA double-stranded breaks (DSBs) within a specified target sequence (JINEK *et al.* 2012), and this has also been successfully used in Drosophila (BASSETT *et al.* 2013; GRATZ *et al.* 2013). DSBs can be repaired by the cell's endogenous DNA repair machinery, either through error-prone non-homologous end-joining (NHEJ), possibly resulting in mutations – small insertions or deletion, or through homology directed repair (HDR), which requires template DNA containing sequences homologous to the regions flanking the DSB. The CRISPR/Cas9 system is now routinely used to facilitate gene knockout and gene replacement strategies in various model genetic organisms (MA AND LIU 2015).

The CRISPR/Cas9 system is remarkable in that it allows the localization of a protein/RNA complex (Cas9 + sgRNA) to a specific sequence within the genome, as specified through a 17-20 nucleotide region of the sgRNA molecule. Being able to localize a protein to a specific DNA sequence opens up many new opportunities, and Cas9 has been modified to create several new tools and techniques. By mutating the catalytic sites of the Cas9 endonuclease, a catalytically inactive or "dead" Cas9 (dCas9) has been developed (QI *et al.* 2013). Various dCas9 fusion proteins have subsequently been designed, and the dCas9 system can be used for a variety of new applications, including the activation or repression of gene transcription, site-specific chromatin modification, or visualization of specific chromosome sites in living cells - see (PULECIO *et al.* 2017; ADLI 2018) for reviews.

79 For Drosophila researchers, prior to the development of CRISPR/Cas9, targeted
80 gene knockouts or gene replacement by homologous recombination were both possible,
81 but challenging and labor intensive endeavors – see (BIBIKOVA *et al.* 2002; LIU *et al.*
82 2012) for reviews of pre-CRISPR/Cas9 techniques. CRISPR/Cas9 strategies for gene
83 knockout and gene replacement are now common techniques for many Drosophila
84 research groups. Building on the *GAL4*/*UAS* system of inducible gene expression,
85 CRISPR/Cas9 approaches for tissue specific target gene knockdown or overexpression in
86 Drosophila are now also possible; resources, including >1600 transgenic stocks
87 expressing different sgRNAs made by the Drosophila RNAi Screening Center and
88 Transgenic RNAi Project (DRSC/TRiP) are publically available – see (BIER *et al.* 2018)
89 for overview of resources. In the case of targeted gene overexpression, the TRiP
90 transgenic lines were designed to ubiquitously express two sgRNAs targeted to sequences
91 upstream of a gene's transcriptional start site (TSS) (LIN *et al.* 2015; EWEN-CAMPEN *et*
92 *al.* 2017). These lines are known as *TRiP-OE*, or *TOE* lines. Target gene overexpression
93 is achieved by crossing a *TOE* line to a line that carries a *GAL4* driver of one's choice
94 and *UAS-dCas9.VPR*, which is dead-Cas9 fused to the VPR transcriptional activation
95 domain. Other sgRNA lines known as *TRiP-KO*, or *TKO* lines, designed for gene
96 knockout, ubiquitously express a single sgRNA targeted to the coding region of a GOI,
97 and can be used to create germline or somatic mutations.
98 We are interested in the regulation and function of the gene *hindsight* (*hnt*), which
99 is the Drosophila homolog of mammalian *Ras Responsive Element Binding Protein-1*
100 (*RREB-1*) (MELANI *et al.* 2008; MING *et al.* 2013). Most mutations of *hnt* are recessive
101 and embryonic lethal, and fail in the morphogenetic process of germ band retraction (YIP

102 *et al.* 1997). In this study we confirm *hnt* overexpression using a *TRiP-OE sgRNA* with
103 *GAL4* and *UAS-dCas9.VPR*. Moreover, by reducing the number of functional copies of
104 hnt^+ from two to one, we found that known loss-of-function *hnt* mutations heterozygous
105 to a wild type allele (*hnt*/ hnt^+) can suppress the *hnt* overexpression phenotype.
106 Subsequently, we developed a method for screening for new loss-of-function *hnt* alleles.
107 Our method is applicable to any GOI provided two requirements can be satisfied: first,
108 the GOI must be associated with an overexpression phenotype induced by *UAS-*
109 *dCas9.VPR* expression under *GAL4* control along with expression of an appropriate
110 sgRNA; and second, the overexpression phenotype must either be sensitive to the dosage
111 of wild type alleles, or if such is not the case, a refractory allele (an allele that is
112 unresponsive to dCas9.VPR mediated activation of gene expression, but otherwise wild
113 type) must be available. We demonstrate that *TOE-sgRNA* expression with active Cas9 is
114 an efficient way to recover refractory alleles.
115 We present the results of this new screening strategy in which randomly induced
116 recessive lethal alleles are selected in an F1 visible screen. Following EMS treatment of
117 a responsive line, and crossing to a refractory line that carries *TOE-sgRNA* + *GAL4* +
118 *UAS-dCas9.VPR*, we screened for the absence of an overexpression phenotype and, in so
119 doing, selected 8 new alleles of *hnt* from ~45,000 F1 progeny over a period of 2-3 weeks.
120 The recovery of numerous mutant alleles in a GOI is potentially useful for
121 analysis of mutation profiles, or for the rapid recovery of an allelic series for a GOI. The
122 ability to overexpress mutant alleles using dCas9.VPR mediated activation of gene
123 expression can also be useful in classifying alleles as hypomorphic or nullomorphic.
124

125 ## Materials and Methods

126 **Drosophila stocks**

127 All Drosophila cultures were raised on standard medium at 25°C under a 12 hour
128 light/dark cycle regime, unless otherwise indicated. Most stocks used in this study were
129 either obtained from the Bloomington *Drosophila* Resource Center (BDRC), or derived
130 from stocks obtained from the BDRC. The eye specific *GAL4* driver was *GMR-GAL4 =*
131 *P{GAL4-ninaE.GMR}12* (BDRC #1104). The stock ubiquitously expressing sgRNA
132 targeted to *hnt* upstream region was *TOE-GS00052 = y sc* v sev*^{21}; *P{y[+t7.7]*
133 *v[+t1.8]=TOE.GS00052}attP40* (BDRC #67530). The dCas9 transcriptional activator
134 used was *UAS-dCas9.VPR = y w**; *Kr*^{If} */CyO*; *P{y[+t7.7] w[+mC]=UAS-*
135 *3xFLAG.dCas9.VPR}attP2* (BDSC #66562). RNAi knockdown of *hnt* was achieved
136 using *UAS-hnt-RNAi = y sc* v sev*^{21}; *P{y[+t7.7] v[+t1.8]=TRiP.HMS00894}attP2/ TM3,*
137 *Sb* (BDRC #33943). The expression of active Cas9 in the germline used
138 *GAL4-nos.NGT*; *UAS-Cas9.P2 = w**; *P{w[+mC]=GAL4-nos.NGT}40*;
139 *P{y[+t7.7]=UAS-Cas9.P2}attP2* (BDRC #67083). The temperature sensitive *hnt*
140 hypomorphic allele used was *hnt*^{peb} *= peb v* (BDRC #80). The deletion of the *hnt* gene
141 used was
142 *Df(1)ED6727 = Df(1)ED6727*, *w*^{1118}
143 *P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED6727/FM7h* (BDRC #8956).
144 The chromosome used in the mutagenesis screen was *w PBac{RB}e02388b* (Exelixis at
145 Harvard medical School #e02388). Pre-existing *hnt* alleles used included *hnt*^{XE81}, *hnt*^{1142},
146 *hnt*^{308}, and *hnt*^{345x} as previously described (YIP *et al.* 1997; WILK *et al.* 2000; REED *et al.*
147 2001; FARLEY *et al.* 2018). Confocal imaging of pupal eye made use of *Ubi-DE-*

148 *cadherin-GFP* as previously described (REED *et al.* 2004). Complementation crosses
149 were facilitated by the transgenic line *peb*$^{BAC\text{-}CH321\text{-}46J02}$ as described (FARLEY *et al.* 2018).
150 *GAL4* > *UAS* based overexpression of *hnt* used *UAS-GFP-hnt* as previously described
151 (BAECHLER *et al.* 2015). This line *UAS-GFP-Hnt*, however, resulted in pupal lethality in
152 combination with *GMR-GAL4*. To achieve lower levels of *hnt* overexpression not
153 resulting in pupal lethality, the *UAS-GFP-hnt* insertion was mobilized by crossing to the
154 transposase line Δ*2-3* (BDRC #3629). The insertion line *UAS-GFP-hnt*^{J27} was recovered
155 and found to be viable in combination with the same *GMR-GAL4* driver.

156 **EMS Mutagenesis**

157 3-5 day old males of the *w PBac{RB}e02388b* stock were collected and starved
158 for 2 hours by placing in empty vials. Following starvation, male flies were transferred
159 to bottles containing Kimwipe tissues soaked in a 2% sucrose solution containing 25 mM
160 EMS (Sigma-Aldrich) and allowed to feed overnight. They were subsequently placed in
161 vials with 3-5 day old virgin *RGV* females in crowded conditions and allowed to mate for
162 6-8 hours (30-40 virgin females with same number of males). Mated females and males
163 were transferred to bottles for two days at which point they were removed.

164 **Molecular Characterization of Refractory Mutants**
165
166 In order to characterize refractory alleles we extracted genomic DNA from a
167 pooled group of 20-30 isogenic male flies using gSYNC DNA Extraction Kits (Geneaid)
168 according to supplier's instructions. We used 2.5 µL of isolated genomic DNA in 25 µL
169 PCR reactions (2X FroggaMix; FroggaBio) utilizing primers flanking the guide target
170 regions at ~400 bp upstream and downstream of the sgRNA target sequences. PCR

171 products were purified using GenepHlow Gel/PCR Kits (Geneaid) then sent out for
172 Sanger sequencing using both forward and reverse primers.

173 **Complementation crosses**

174 New *hnt* alleles, maintained as balanced lines over *FM7h*, were tested for
175 complementation by crossing to hnt^{peb} males at the restrictive temperature 29°C as well
176 as males of the following genotype: *y w* hnt^{XE81}/*Y*; $peb^{BAC\text{-}CH321\text{-}46J02}$/ *TM6C, Sb*. Failure
177 to complement hnt^{peb} was indicated by a rough eye phenotype, while failure to
178 complement hnt^{XE81} was indicated by the absence of B^{+} *Sb* female progeny carrying
179 neither the *FM7h* balancer or the peb^{BAC} insertion.

180 **Immunostaining and Imaging**

181 Immunostaining of embryos was carried out as described (REED *et al.* 2001).
182 Primary antibodies were used at the indicated dilutions: mouse monoclonal anti-
183 Hindsight (Hnt) 27B8 1G9 (1:25; from H. Lipshitz, University of Toronto), guinea pig
184 polyclonal anti-Hindsight (1:1000; from H. Lipshitz, University of Toronto). Secondary
185 antibodies used were: Alexa Flour® 488 goat anti-mouse and TRITC goat anti-guinea pig
186 (1:500; Cedarlane Labs). In most cases *hnt* mutant embryos were recognized by their u-
187 shaped morphology, which is associated with a failure to complete germ band retraction
188 and premature death of the amnioserosa.

189 Confocal microscopy and confocal image processing were performed as
190 previously described (CORMIER *et al.* 2012). Eye micrographs (Fig. 1A,B) were taken
191 using a Nikon SMZ25 stereomicroscope equipped with Nikon Digital Sight Ri2 16.25MP
192 colour camera and processed using extended-depth-of-focus feature of Nikon NIS-
193 Elements Arv4.50 software. Other eye micrographs were acquired using a compound

194 Olympus microscope with a 5X objective and oblique illumination. Z stacks were
195 collected by manually adjusting the focal plane as images were collected using a mobile
196 phone camera positioned over the ocular lens. Images collected were processed using
197 ImageJ and an extended depth of focus plugin (SCHNEIDER *et al.* 2012).

198 **Data Availability Statement:**

199 All Drosophila stocks used or recovered in this study are available upon request. The
200 authors affirm that all data necessary for confirming the conclusions of the article are
201 present within the article, figures, and tables.

202

203 Results

204 Overall, we present our results as proof-of-principle for the concept that loss-of-function
205 alleles in a GOI can be selected in an F1 screen. By way of example, in the following
206 sections we describe our observations regarding *hnt* overexpression phenotypes, dosage
207 sensitivity, the recovery of refractory *hnt* alleles, and the design and results of our novel
208 screen for new *hnt* loss-of-function alleles.

209

210 Characterization of dCas9.VPR Mediated Activation of *hnt* Expression

211 Previous work demonstrates CRISPR/Cas9 mediated *in vivo* activation of *hnt*
212 expression. In this proof-of-principle example, ectopic *hnt* expression is shown in the
213 third larval instar wing imaginal disc (EWEN-CAMPEN *et al.* 2017). To further investigate
214 CRISPR/Cas9 mediated overexpression of *hnt*, we used the *TRiP-TOE* insertion line
215 *TOE-GS00052*, which ubiquitously expresses two sgRNAs, targeted to 110-129 bps and
216 183-203 bps upstream of the TSS of *hnt* (Fig. 1A). Crossing line *TOE-GS00052* to a line

217 that carries the eye specific *GMR-GAL4* driver and *UAS-dCas9.VPR* resulted in pupal
218 lethality at 25°C, but viable and fertile adults with a strong rough eye phenotype at 18°C.
219 The *hnt* overexpression phenotype, which we have also observed in the context of *GMR-*
220 *GAL4 > UAS-GFP-Hnt*, is distinctive in that the eye margin is consistently more
221 pigmented than the inner region, giving the appearance of dark rings around the eyes
222 (Fig. 1 B,C). We confirmed that the rough eye phenotype in the context of *GAL4>UAS-*
223 *dCas9.VPR + TOE-GSGS00052* is attributable to the overexpression of *hnt* by co-
224 expression of *UAS-hnt-RNAi*, which resulted in a full suppression of the rough eye
225 phenotype (Fig. 1E,F).

226 Through a series of crosses carried out at 18°C, we recovered a stock carrying all
227 three transgenes required for CRISPR/Cas9 mediated activation of *hnt* expression in the
228 pupal eye (i.e. *GMR-GAL4*, *TOE-GS00052*, and *UAS-dCas9.VPR*). When transferred to
229 25°C, this stock was 100% pupal lethal, with no adult escapers. To test the dosage
230 responsiveness of *hnt* overexpression, we crossed males of the overexpression stock
231 (reared at 18°C) to females carrying a balanced deletion of *hnt* (*hnt* is an *X*-linked gene,
232 so the stock was *Df(1)ED6727/FM7*). Interestingly, at 25°C all progeny of this cross are
233 $FM7^{+}$ females and display the distinctive *hnt* overexpression rough eye phenotype,
234 identical to hnt^{+}/hnt^{+} overexpression phenotype at 18°C. We subsequently found that
235 other *hnt* alleles previously characterized as strong loss-of-function alleles (hnt^{XE81},
236 hnt^{1142}, hnt^{345x}) also behave the same way, and only produce $FM7^{+}$ female progeny at
237 25°C (Fig. 1D). Other *hnt* alleles tested, including the hypomorphic semi-lethal allele
238 hnt^{308}, the temperature sensitive hypomorphic allele hnt^{peb}, and the lethal allele hnt^{PL67},
239 do not produce progeny at 25°C.

240

241 **The recovery of *hnt* alleles refractory to CRISPR/Cas9 mediated overexpression.**

242 The above results suggest that suppression of dCas9.VPR mediated activation of
243 gene expression could be used not only to characterize existing alleles, but to select
244 newly induced loss-of-function mutations in a GOI. To be broadly applicable, however,
245 it is necessary to recover GOI alleles that are refractory to CRISPR/Cas9 mediated
246 activation of gene expression, but are in all other respects wild type. In general, such
247 "GOI^{REF}" alleles permit the recovery of heterozygotes in situations where an
248 overexpression phenotype is not sensitive to dosage or temperature. For example, in a
249 CRISPR/Cas9 overexpression background, a GOI^{REF}/GOI^{+} heterozygote might be
250 associated with a visible phenotype or be lethal, whereas a GOI^{REF}/GOI^{null} heterozygote
251 would be incapable of GOI overexpression and would, therefore, have normal viability
252 and no overexpression phenotype.

253 In order to recover hnt^{REF} alleles (*hnt* alleles refractory to CRISPR/Cas9 mediated
254 activation of gene expression – but otherwise wild type), we used the approach of
255 transmitting the ubiquitously expressed TOE-sgRNA transgene *GS00052* through the
256 female germline with active Cas9 expression (crossing scheme is outlined in Fig. 2). In
257 our case, we were able to cross such females to males carrying all three transgenes
258 required for CRISPR/Cas9 mediated activation of gene expression of *hnt* in the pupal eye
259 (*i.e.* the *y w* ; *GMR-GAL4 TOE-GS00052* ; *UAS-dCas9.VPR* stock that is viable at 18°C).
260 Progeny of this cross that carried all three transgenes, but not displaying the
261 overexpression phenotype were found in abundance. In order to recover isogenic
262 refractory lines, single refractory males were crossed with *FM7* females, and through

263 selection and backcrossing, isogenic refractory lines devoid of the autosomal transgenes
264 were established (Fig. 2, crossing scheme 1). Two such *y w* lines were isolated and
265 sequenced and both proved to carry deletions in both sgRNA target sites as compared to
266 the control parental *y w* sequence (since *hnt* and *w* are tightly linked, we selected *y w*
267 hnt^{REF} lines to ensure that we would be comparing the sequence of newly induced hnt^{REF}
268 alleles with the sequence from the parental *y w* line). Other lines were established by
269 backcrossing single refractory males to *y w* ; *GMR-GAL4 TOE-GS00052 / CyO* ; *UAS-*
270 *dCas9.VPR / TM6* females (raised at 18°C). Subsequent crosses performed at 25°C
271 allowed the selection of heterozygous viable hnt^{+}/ hnt^{REF} females, which were again
272 backcrossed, allowing for selection a triple homozygous stock: hnt^{REF}*; GMR-GAL4 TOE-*
273 *GS00052; UAS-dCas9.VPR*, from here on referred to as the "*RGV*" stock (Fig. 2, crossing
274 scheme 2).

275 We found the *RGV* stock to be viable with no overexpression phenotype raised at
276 25°C. We also checked the *RGV* stock by crossing females to hnt^{+} males (responsive to
277 dCas9.VPR mediated activation of gene expression) and confirmed the expected
278 phenotypes of viable female hnt^{+}/hnt^{REF} progeny displaying the overexpression eye
279 phenotype and viable male hnt^{REF} progeny with no overexpression phenotype. Likewise,
280 we confirmed that hnt^{+}/hnt^{+} females crossed to *RGV* males results in only female
281 progeny that display the overexpression phenotype. In addition, we also retested
282 *Df(1)ED6727* as well as the strong *hnt* alleles (hnt^{XE81}, hnt^{1142}, hnt^{345x}) by crossing
283 females of these *FM7* balanced lines to *RGV* males, which confirmed that $FM7^{+}$ female
284 progeny (hnt^{-}/hnt^{REF}) are viable and show no overexpression phenotype. Similar crosses
285 to other *hnt* alleles (hnt^{peb}, hnt^{308}, and hnt^{PL67}) confirmed that female heterozygous

286 progeny (hnt^{-}/hnt^{REF}) are, in these cases, viable but do show the *hnt* overexpression
287 phenotype.
288
289 **An F1 visible screen for recessive lethal mutations of *hnt* using the RGV stock.**
290 As a proof-of-principle, we used the *RGV* stock to screen for new mutant alleles
291 of *hnt* induced by EMS mutagenesis. In the screen (outlined in Fig. 3), *RGV* virgin
292 females were crossed to mutagenized males carrying the [w^{+}] insertion
293 *PBac{RB}e02388b*, which is tightly linked to *hnt* (inserted 6104 bp upstream) and has an
294 unusual and easily scored crescent pattern of [w^{+}] expression in the posterior eye.
295 Recovery of newly induced *hnt* mutations was performed as an F1 visible screen for
296 female progeny lacking the *hnt* overexpression phenotype. We screened approximately
297 45,000 progeny, from which we isolated 39 F1 females lacking the *hnt* eye
298 overexpression phenotype. Of the 39 rare female progeny, 15 lines bred true and were
299 established as balanced stocks over *FM7h,w* lacking the autosomal [w^{+}] transgenes
300 *GMR-GAL4* and *UAS-dCas9.VPR* from the *RGV* stock.
301
302 **Characterization of new *hnt* alleles.**
303 Balanced lines recovered in the screen were retested for their refractory nature by
304 crossing to *RGV* males and also tested for complementation with hnt^{XE81} (see Materials
305 and Methods for description of complementation cross). Following retesting, we
306 recovered eight new mutant alleles of *hnt* (all lethal alleles) and seven lines that carried
307 new hnt^{REF} (hnt^{+}) alleles. Of note, $hnt^{REF\text{-}BHR7}$ was found to be mildly responsive to
308 overexpression in males in the *RGV* retest, but completely refractory in females. All new

309 *hnt*^{REF} alleles were sequenced by direct PCR sequencing (Fig. 4). The line *hnt*$^{REF\text{-}BHR7}$
310 was found to have a single base pair change A >T within the first sgRNA target
311 sequence, and *hnt*$^{REF\text{-}WN72}$ was found to have a single base pair change of C >T within the
312 PAM motif of the second sgRNA target sequence. Interestingly, these two *hnt*^{REF} alleles,
313 where each is associated with a single base pair change affecting one sgRNA target
314 sequence or the other, suggests that dCas9.VPR activation of *hnt* expression is only
315 effective when both guide target sequences are fully intact and functional. All other
316 *hnt*^{REF} lines were found to contain an identical deletion spanning from the first guide
317 target to the second. Since each of these five lines was isolated from the same bottle, this
318 likely corresponds to a "premeiotic cluster", resulting from a mutation induced in a
319 germline stem cell. Overall, therefore, we conclude that our screen resulted in the
320 recovery of eight new *hnt* mutant alleles and only three new *hnt*^{REF} alleles.
321 All new *hnt* mutant alleles were also confirmed by failure to complement the
322 temperature sensitive hypomorphic allele *hnt*^{peb} at the restrictive temperature of 29°C.
323 Using *Ubi-DEcadherin-GFP*, we imaged pupal eyes of all *hnt*^{peb}/*hnt*^{new} heteroallelic
324 combinations at 25°C, which we found to be a more sensitive background for the *hnt*^{peb}
325 phenotype. The severity of each allele was ranked by quantification of cones cells, which
326 invariably number four per ommatidium in wild type (Fig. 5A). Mutant ommatidia
327 frequently contain fewer than four cone cells per ommatidium (Fig. 5B-D, white
328 arrowheads). Occasionally mutant ommatidia were found to contain 5 or 6 cone cells;
329 these likely result from ommatidial fusion as they also contain three rather than two
330 primary pigment cells (Fig. 5C, blue arrowheads). According to this analysis, none of the
331 newly induced alleles is as severe as the *Df(1)ED6727* control, suggesting that none

332 correspond to a null allele. We subsequently performed immunostaining on all lines
333 using both monoclonal and polyclonal anti-Hnt antibodies. All alleles were found to be
334 anti-Hnt positive using the polyclonal antibody (Figure 6, Table 1). We subsequently
335 found that the strong hypomorphic EMS induced allele hnt^{XE81}, previously described as
336 antibody-null (using the same monoclonal antibody 27B8 1G9), also stains positive for
337 Hnt using the polyclonal antibody (Fig. 6E). Interestingly, two alleles (hnt^{BHR49} and
338 hnt^{WN52}) were monoclonal negative but polyclonal positive, and although the polyclonal
339 signal was expressed with correct tissue specificity, it did not show the normal nuclear
340 localization of Hnt (Fig. 6D). These results further support the notion that none of these
341 new *hnt* alleles corresponds to a null allele and that most EMS induced alleles of *hnt*
342 produce non-functional protein.
343

344 **Discussion**

345 Overall, we have invented a new way to recover randomly induced mutations in a
346 GOI based on CRISPR/Cas9 mediated activation of gene expression. As a proof-of-
347 principle we applied this method to the gene *hnt* and recovered 8 new loss-of-function
348 alleles. In addition to newly induced loss of function alleles, we also recovered newly
349 induced refractory chromosomes in which the sgRNA target sequences had undergone
350 mutation. While the recovery of new refractory alleles represents a source of false
351 positives for this screening method, such false "hits" are easily recognized by
352 complementation tests and sequencing the sgRNA target sites.
353 Considering the 8 new *hnt* alleles recovered, all showed a strong refractory
354 phenotype in the cross to the *RGV* stock. However, we also isolated many F1 progeny

355 that showed intermediate phenotypes. We were very interested in the possibility of
356 recovering weak loss-of-function *hnt* alleles from these "hits", but none of these lines
357 bred true as X-linked mutations. We were also aware of the possibility of recovering
358 dose-dependent second site modifiers. Two such lines were indeed recovered that
359 consistently resulted in an intermediate dCas9.VPR mediated *hnt* overexpression
360 phenotype, but do not map to the X chromosome. Further analysis of these lines may
361 allow us to identify genes that are required for the full penetrance of the *hnt*
362 overexpression phenotype. Such genes may ultimately shed light on the pathways and
363 target genes regulated by Hnt.
364
365 **General Comments on Setting up an Overexpression Screen**
366 As mentioned above, it is not an absolute requirement that a GOI show dosage
367 sensitivity in order to design an overexpression screen, but it is useful to determine if this
368 is the case. If the overexpression phenotype for a GOI is visible, with good viability and
369 fertility, then one can determine if it is sensitive to gene dosage by crossing the
370 overexpression background (*sgRNA + GAL4 driver + UAS-dCas9.VPR*) to a known loss-
371 of-function allele or a deletion of the GOI and assaying for suppression of the visible
372 phenotype. If such is the case, a screening strategy can be developed without using an
373 allele that is refractory to dCas9.VPR mediated gene expression. If, however, the GOI is
374 associated with a visible overexpression phenotype that is not sensitive to gene dosage, or
375 if overexpression of a single responsive allele proves to be lethal, then an allele of the
376 GOI that is refractory to dCas9.VPR mediated activation of gene expression is required.
377 In this latter case, a refractory allele heterozygous to a loss-of-function or deletion of a

378 GOI in the dCas9.VPR overexpression background will be viable and have no
379 overexpression phenotype (lethal or visible).
380
381 **Can these Techniques be Generalized?**
382 The generalization of our screening method will likely depend on the ability to
383 recover refractory alleles of the GOI – that is, alleles whose only phenotype is that of not
384 being responsive to CRISPR/Cas9 mediated activation of gene expression. Two
385 questions remain to be answered before this technique can be generalized. First, is it
386 always possible to create a refractory allele for a GOI, or is this only possible for certain
387 genes? And second, if the overexpression phenotype for a GOI is not sensitive to
388 temperature or gene dosage, would it still be possible to recover a refractory allele? The
389 first question cannot be answered until CRISPR/Cas9 mediated activation of gene
390 expression is used on more genes. In regards to the second question, it is clear that the
391 development of our system was greatly enhanced by the striking temperature and gene
392 dosage sensitivity of the *hnt* overexpression phenotype in the pupal eye. But what if just
393 one copy of the GOI is sufficient to generate a lethal phenotype in the background of
394 CRISPR/Cas9 mediated activation of gene expression (*sgRNA* + *GAL4* + *UAS-*
395 *dCas9.VPR*)? Assuming the structure of a gene is such that it can be mutated to create a
396 refractory chromosome, it should still be possible to select viable refractory alleles. This
397 could be accomplished by a series of crosses in which a chromosome carrying a wild type
398 allele in the GOI is put in the background of the TOE-sgRNA and germline expression of
399 active Cas9. The potentially mutated chromosome can then be recovered as a stock
400 without the active Cas9 but still carrying the TOE-sg-RNA transgene. Crossing such

401 stocks to a background carrying a *Df* for the GOI along with the appropriate *GAL4* +
402 *UAS-dCas9.VPR* to drive overexpression will allow selection of refractory alleles.
403 Further studies will be required to determine if such techniques are feasible.
404

405 **Gene Overexpression as New Method for Characterizing Alleles**

406 In general, the approach of dCas9.VPR mediated expression of mutant alleles can
407 be considered as a new method for characterizing alleles. In a way, this is similar to
408 Muller's classical test for hypomorphic alleles through the synthesis of duplications
409 containing a third copy of the mutant allele being tested. In this case, a third copy of a
410 hypomorphic allele is expected to ameliorate the mutant phenotype compared to the
411 phenotype of the homozygous euploid mutant (MULLER 1932). In the case of
412 dCas9.VPR mediated activation of gene expression, if an overexpression phenotype is
413 suppressed in mutant heterozygote (*m*/+) relative to the wild type homozygote (+/+),
414 then two possibilities arise: 1) the mutant allele may not encode a functional protein; or
415 2) the mutant allele may be refractory to overexpression through mutation or deletion of
416 its sgRNA target sequences. Although these two possibilities may not be mutually
417 exclusive, sequencing the sgRNA target sequences of a mutant allele could rule out the
418 second possibility and thereby suggest that a particular allele is associated with a
419 disruption of the protein-coding region of the GOI. The characterization of mutant alleles
420 can be further improved if a refractory allele of the GOI is available ($+^{REF}$) and the
421 mutant is known to have intact sgRNA target sequences. In this situation, any phenotype
422 resulting from CRISPR/Cas9 mediated activation of gene expression in the heterozygote
423 ($+^{REF}/m$), would suggest that the mutant allele is able to express a protein that is fully or

424 partially functional. In this case, the mutant is more likely to be hypomorphic in nature,
425 and could be associated with reduced gene expression or reduced protein function.
426 Consistent with this, we found that the allele *hnt*^{308} was fully responsive to dCas9.VPR
427 mediated activation of gene expression. This allele, which is associated with a *P* element
428 insertion 226 base pairs upstream of the TSS of *hnt*, was previously characterized as
429 hypomorphic and displays a lower level of *hnt* expression (REED *et al.* 2001). The
430 dCas9.VPR activation of *hnt*^{308} expression is consistent with the hypomorphic nature of
431 this allele, but also indicates that the sgRNA target sequences on the *hnt*^{308} chromosome
432 are intact and that the protein coding region can produce Hnt product that is sufficient to
433 generate the overexpression phenotype. The same interpretations also apply to the allele
434 *hnt*^{PL67}, which is an enhancer trap line associated with embryonic lethality but is largely
435 uncharacterized.
436

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445
446

447

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513

514

515 **Figure 1.**

516 **Overexpression of *hnt* disrupts eye development and the CRISPR/Cas9 mediated**

517 ***hnt* overexpression phenotype is sensitive to temperature and gene dosage.**

518 (**A**) Schematic showing the sgRNA target sequences of *TOE-GS00052* relative to the TSS

519 of *hnt*. (**B**) Wild type eye. (**C**) The *hnt* overexpression phenotype of *GMR-GAL4 > UAS-*

520 *GFP-Hnt*^{J27}. (**D**) Nomenclature and associated Punnett square diagram of a balanced *hnt*

521 allele (females) crossed to the CRISPR/Cas9 *hnt* overexpression stock (males). (**E**)

522 Overexpression phenotype of *TOE-GS00052* + *GMR-GAL4>UAS-Cas9.VPR*. (**F**)

523 Suppression of the overexpression phenotype *of TOE-GS00052* + *GMR-GAL4>UAS-*

524 *Cas9.VPR* by co-expression of *UAS-hnt-RNAi*.

525

526 **Figure 2.**

527 **Creating chromosomes refractory to CRISPR/Cas9 mediated overexpression of *hnt*.**

528 Flow chart showing (**1**) the crossing scheme used to generate isogenic *hnt*^{REF} lines and

529 the sgRNA target site sequences for two such lines; and (**2**) crossing scheme for the

530 recovery of the *RGV* stock (*hnt*^{REF}/*hnt*^{REF}; *GMR-GAL4 TOE-GS00052*; *UAS-*

531 *dCas9.VPR*). Selection of isogenic lines in crossing scheme (**1**) permitted the sequence

532 of *hnt*^{REF} lines to be compared with the parental *y w* chromosome as *hnt* is tightly linked

533 to *w*. Note that in crossing scheme (**2**) that the background markers on the *X*

534 chromosome are not known and this stock is best described as (*y*) (*w*) *hnt*^{REF} (*v*). See

535 Materials and Methods for full descriptions of stock genotypes.

536

537

538 **Figure 3.**

539 **Crossing scheme and screen for the selection of EMS induced recessive lethal**

540 **mutations of *hnt* as an F1 visible screen.**

541 Virgin females of the *RGV* stock crossed to EMS treated males results in the vast

542 majority of females showing the *hnt* overexpression phenotype. Rare females not

543 showing the *hnt* overexpression phenotype are selected as balanced stocks (*w*

544 *PBac{RB}e02388b hnt** / *FM7h,w*) and tested to determine if they are new *hnt* loss-of-

545 function alleles or newly induced refractory chromosomes.

546

547 **Figure 4.**

548 **EMS-induced hnt^{REF} alleles are associated with mutations in the *sgRNA* target**

549 **sequences of *TOE-GS00052*.**

550 Sequencing results for seven newly recovered EMS induced hnt^{REF} alleles shown in

551 alignment with the reference genomic sequence (topmost). The *TOE-GS00052* sgRNA

552 target sequences are highlighted in yellow. The PAM motifs for the two sgRNA targets

553 sequences of *TOE-GS00052* are underlined. Single base pair changes of $hnt^{REF\text{-}BHR7}$ and

554 $hnt^{REF\text{-}WN72}$ are shown in red font. The bottom five hnt^{REF} allele sequences are identical,

555 indicative of a premeiotic mutation and represent a single mutagenic event.

556

557 **Figure 5.**

558 **New *hnt* alleles can be arranged into an allelic series of strong hypomorphs**

559 **according to their pupal eye phenotype when heterozygous to hnt^{peb}.**

560 (**A**) *Ubi-DEcadherin-GFP* expression in control *hnt*^{peb}/+ pupal eye showing the normal
561 ommatidial structure with four cone cells per ommatidium. (**B**) Homozygous
562 *hnt*^{peb}/*h*nt^{peb} reared at 25°C showing abnormal ommatidia having fewer than four cone
563 cells (white arrowheads). (**C**) *hnt*^{peb}/*Df(1)ED6727* raised at 25°C showing abnormal
564 ommatidia with more (blue arrowheads) or less than four cone cells (white arrowheads).
565 (**D**) *hnt*^{peb}/*hnt*^{WN47} reared at 25°C showing abnormal ommatidia having fewer than four
566 cone cells (white arrowheads). (**E**) Stacked bar graph showing the distribution of
567 ommatidia cone cell numbers in wild type, and new *hnt* alleles heterozygous to *hnt*^{peb}
568 reared at 25°C. Scale bar represents 20 μm.
569
570 **Figure 6.**
571 **Examples of immunostaining of *hnt* mutants using anti-Hnt monoclonal and**
572 **polyclonal antibodies.**
573 Shown are representative anti-Hnt monoclonal (**A-D**) and anti-Hnt polyclonal (**A'-D', E**)
574 confocal micrographs. (**A, A'**) Wild type (wt) showing normal pattern of anti-Hnt
575 immunostaining in a stage 15 embryo. (**B,B'**) *hnt*^{WN29} shown in same frame as wt sib
576 showing reduced anti-Hnt signal using both monoclonal and polyclonal antibody. The
577 strong oenocyte signal observed in wild type is not seen in the *hnt*^{WN29} mutant
578 (arrowheads). (**C,C'**) *hnt*^{BHR49} u-shaped mutant embryo showing weak monoclonal
579 (arrowhead) but strong polyclonal signal. (**D,D'**) *hnt*^{WN52} u-shaped mutant embryo
580 showing absence of signal with the monoclonal antibody and weak non-nuclear signal
581 with the polyclonal antibody. (**E**) *hnt*^{XE81} u-shaped mutant with partially degenerated
582 amnioserosa (arrowhead) showing strong anti-Hnt signal using the polyclonal antibody.

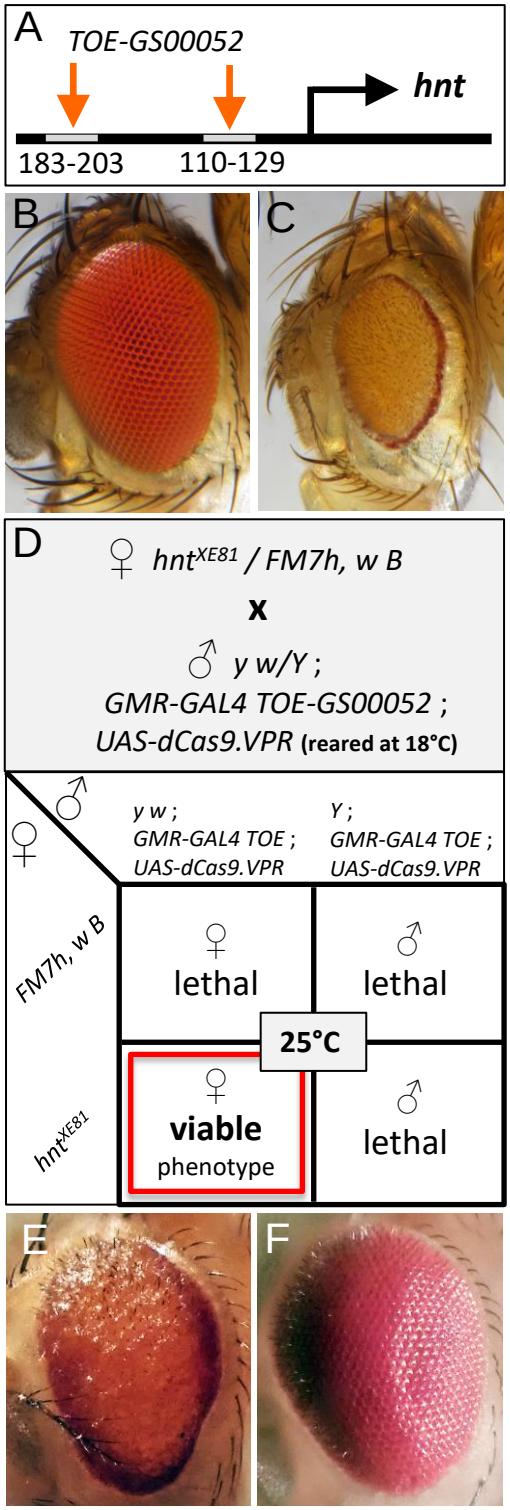
583

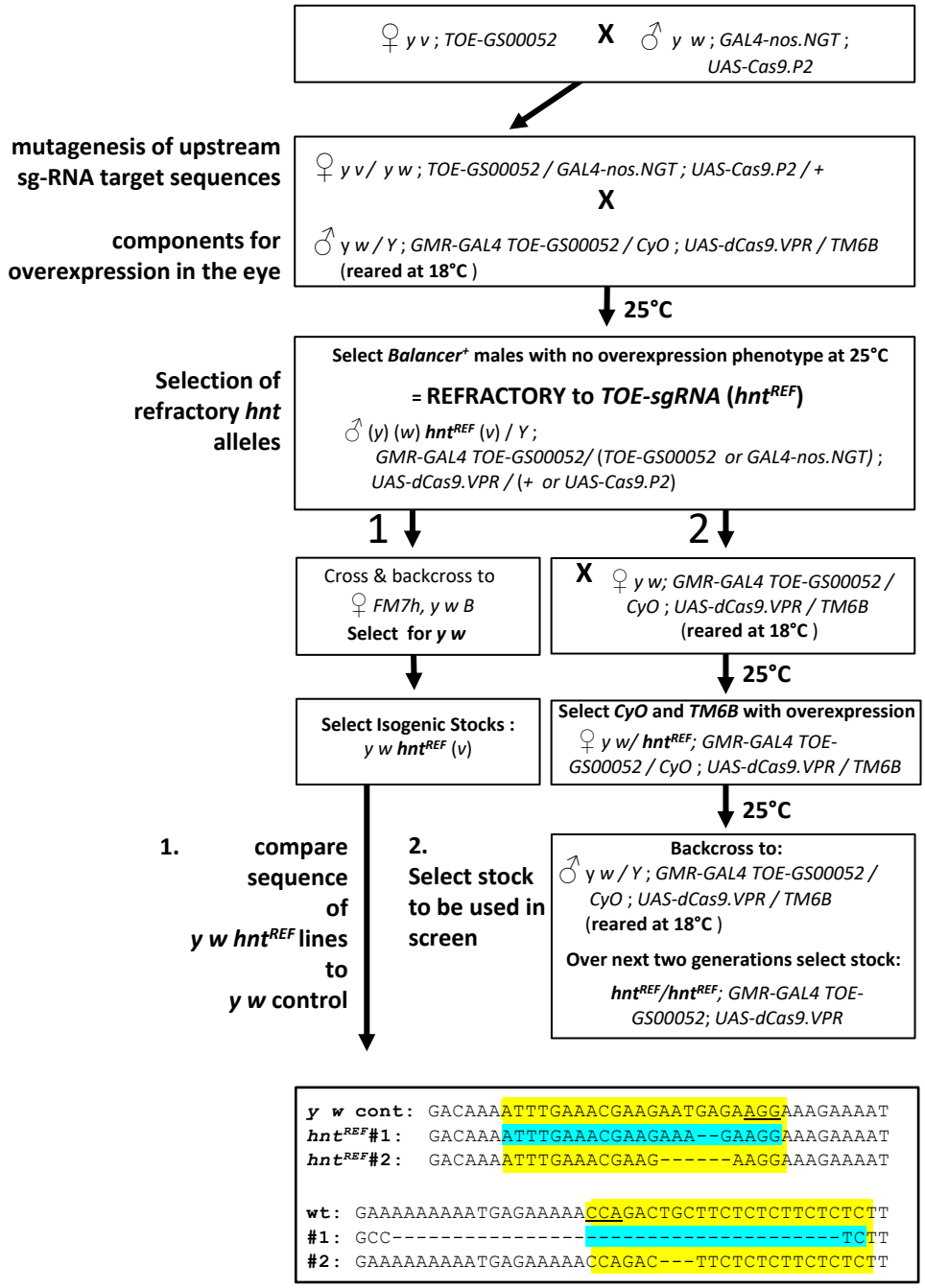
584 **Table 1.**

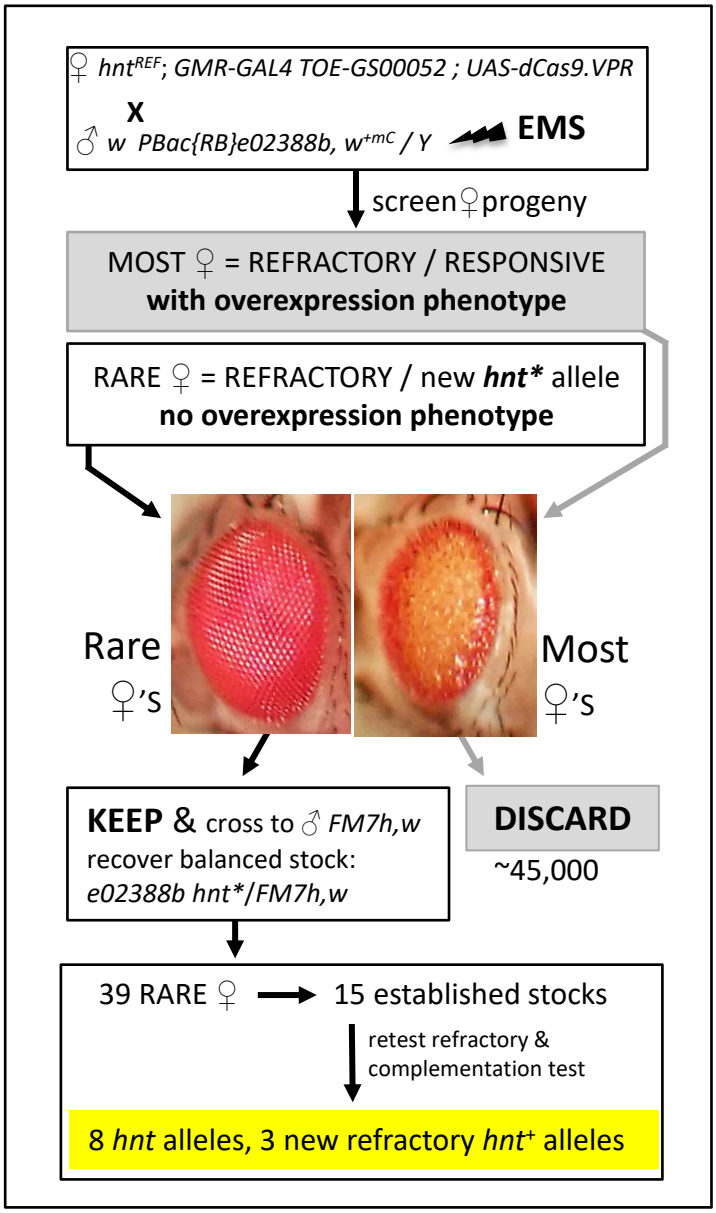
585 **Summary of immunostaining results using anti-Hnt monoclonal and polyclonal**

586 **antibodies as well as terminal phenotype analysis of new *hnt* alleles.**

587 * Anti-Hnt signal was not localized to the nuclei.

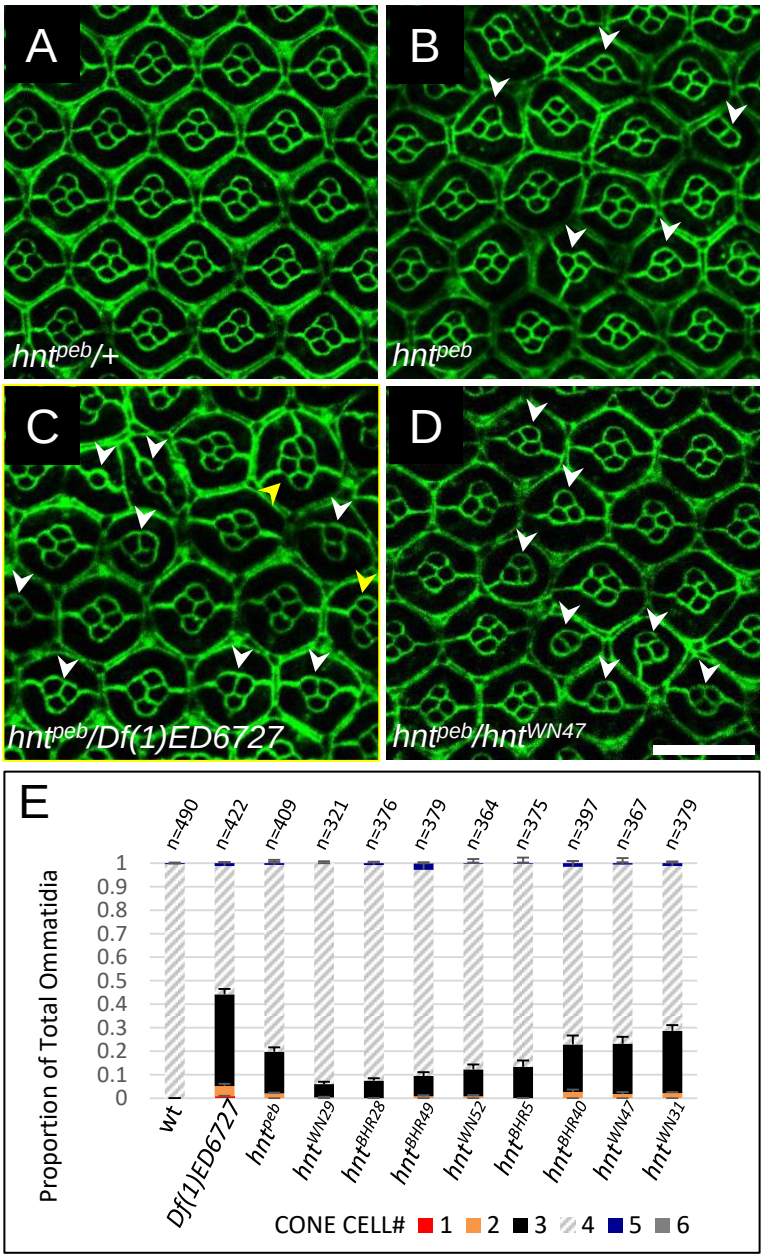

A
TOE-GS00052
hnt
183-203
110-129
B
C
D
♀ hntXE81 / FM7h, w B
x
♂ y w/Y ;
GMR-GAL4 TOE-GS00052 ;
UAS-dCas9.VPR (reared at 18°C)
♂
♀
y w ;
GMR-GAL4 TOE ;
UAS-dCas9.VPR
Y ;
GMR-GAL4 TOE ;
UAS-dCas9.VPR
FM7h, w B
hntXE81
♀
lethal
♂
lethal
25°C
♀
viable
phenotype
♂
lethal
E
F


♀ y v ; TOE-GS00052
X
♂ y w ; GAL4-nos.NGT ; UAS-Cas9.P2
mutagenesis of upstream sg-RNA target sequences
components for overexpression in the eye
♀ y v / y w ; TOE-GS00052 / GAL4-nos.NGT ; UAS-Cas9.P2 / +
X
♂ y w / Y ; GMR-GAL4 TOE-GS00052 / CyO ; UAS-dCas9.VPR / TM6B
(reared at 18°C)
25°C
Selection of refractory hnt alleles
Select Balancer+ males with no overexpression phenotype at 25°C
= REFRACTORY to TOE-sgRNA (hntREF)
♂ (y) (w) hntREF (v) / Y ;
GMR-GAL4 TOE-GS00052/ (TOE-GS00052 or GAL4-nos.NGT) ;
UAS-dCas9.VPR / (+ or UAS-Cas9.P2)
1
2
Cross & backcross to
♀ FM7h, y w B
Select for y w
X ♀ y w; GMR-GAL4 TOE-GS00052 / CyO ; UAS-dCas9.VPR / TM6B
(reared at 18°C)
25°C
Select Isogenic Stocks :
y w hntREF (v)
Select CyO and TM6B with overexpression
♀ y w/ hntREF; GMR-GAL4 TOE-GS00052 / CyO ; UAS-dCas9.VPR / TM6B
25°C
1. compare sequence of y w hntREF lines to y w control
2. Select stock to be used in screen
Backcross to:
♂ y w / Y ; GMR-GAL4 TOE-GS00052 / CyO ; UAS-dCas9.VPR / TM6B
(reared at 18°C)
Over next two generations select stock:
hntREF/hntREF; GMR-GAL4 TOE-GS00052; UAS-dCas9.VPR
y w cont: GACAAAATTTGAAACGAAGAATGAGAAGGAAAGAAAAT
hntREF#1: GACAAAATTTGAAACGAAGAAA--GAAGGAAAGAAAAT
hntREF#2: GACAAAATTTGAAACGAAG------AAGGAAAGAAAAT
wt: GAAAAAAAAATGAGAAAAACCAGACTGCTTCTCTCTTCTCTCTT
#1: GCC----------TCTT
#2: GAAAAAAAAATGAGAAAAACCAGAC---TTCTCTCTTCTCTCTT


♀ hnt^REF; GMR-GAL4 TOE-GS00052 ; UAS-dCas9.VPR
X
♂ w PBac{RB}e02388b, w+mC / Y
EMS
screen♀progeny
MOST ♀ = REFRACTORY / RESPONSIVE
with overexpression phenotype
RARE ♀ = REFRACTORY / new hnt* allele
no overexpression phenotype
Rare ♀'s
Most ♀'s
KEEP & cross to ♂ FM7h,w
recover balanced stock:
e02388b hnt*/FM7h,w
DISCARD
~45,000
39 RARE ♀
15 established stocks
retest refractory &
complementation test
8 hnt alleles, 3 new refractory hnt+ alleles

```
hnt^wildtype   GACAAAATTTGAAACGAAGAATGAGAAAGGAAAGAAAATCCAAGCTGCGCTGCC
hnt^REF-BHR7   GACAAAATTTGAATCGAAGAATGAGAAAGGAAAGAAAATCCAAGCTGCGCTGCC
hnt^REF-WN72   GACAAAATTTGAAACGAAGAATGAGAAAGGAAAGAAAATCCAAGCTGCGCTGCC
hnt^REF-BHR17  GACAAAATTTGAAACGAAGAATG------------------------------
hnt^REF-WN30   GACAAAATTTGAAACGAAGAATG------------------------------
hnt^REF-WN31   GACAAAATTTGAAACGAAGAATG------------------------------
hnt^REF-WN35   GACAAAATTTGAAACGAAGAATG------------------------------
hnt^REF-WN36   GACAAAATTTGAAACGAAGAATG------------------------------

hnt^wildtype    TCCGTTGAAAAAAAAATGAGAAAAACCAGACTGCTTCTCTCTTCTCTCTTTTC
hnt^REF-BHR7    TCCGTTGAAAAAAAAATGAGAAAAACCAGACTGCTTCTCTCTTCTCTCTTTTC
hnt^REF-WN72    TCCGTTGAAAAAAAAATGAGAAAAACTAGACTGCTTCTCTCTTCTCTCTTTTC
hnt^REF-BHR17   --------------------------------GCTTCTCTCTTCTCTCTTTTC
hnt^REF-WN30    --------------------------------GCTTCTCTCTTCTCTCTTTTC
hnt^REF-WN31    --------------------------------GCTTCTCTCTTCTCTCTTTTC
hnt^REF-WN35    --------------------------------GCTTCTCTCTTCTCTCTTTTC
hnt^REF-WN36    --------------------------------GCTTCTCTCTTCTCTCTTTTC
```


A
hnt^peb/+
B
hnt^peb
C
hnt^peb/Df(1)ED6727
D
hnt^peb/hnt^WN47
E
Proportion of Total Ommatidia
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
n=490
n=422
n=409
n=321
n=376
n=379
n=364
n=375
n=397
n=367
n=379
wt
Df(1)ED6727
hnt^peb
hnt^WN29
hnt^BHR28
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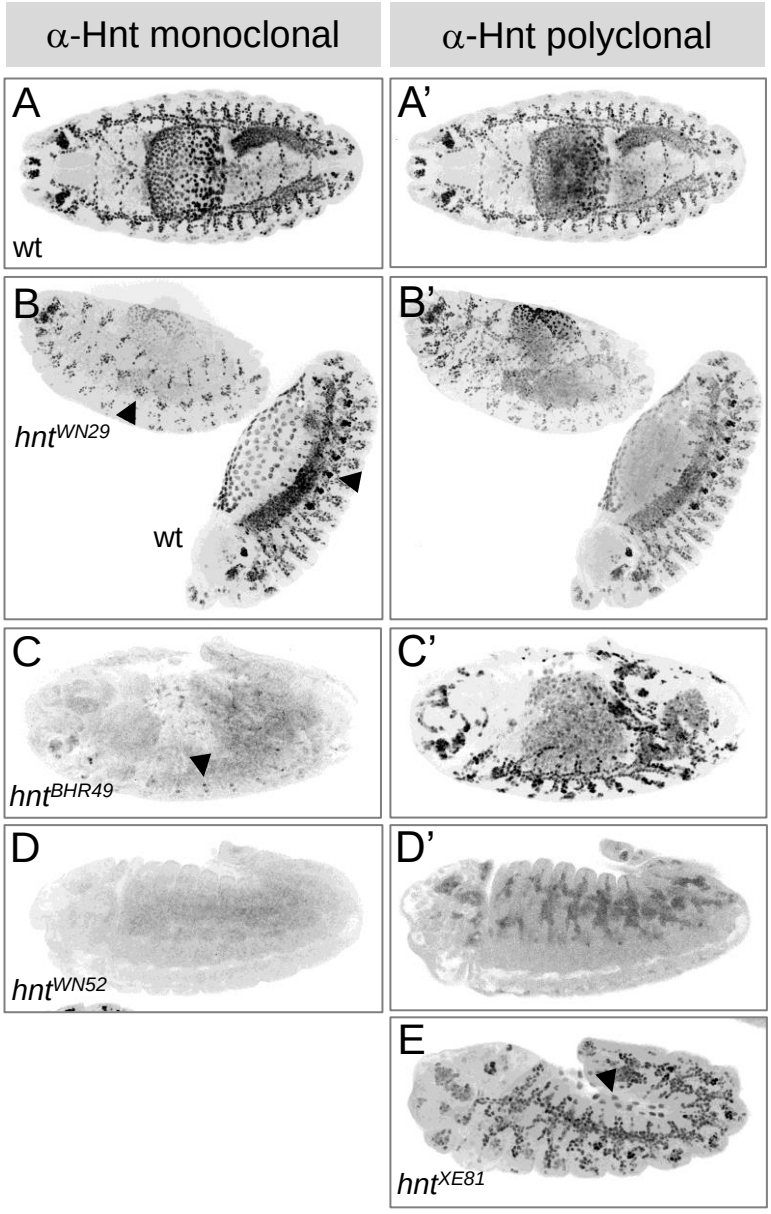
hnt^WN47
hnt^WN31
CONE CELL# 1 2 3 4 5 6


α-Hnt monoclonal
α-Hnt polyclonal
A
wt
A'
B
hntWN29
wt
B'
C
hntBHR49
C'
D
hntWN52
D'
E
hntXE81

Allele	α-Hnt monoclonal	α-Hnt polyclonal	GBR phenotype
hnt^{WN29}	++	++	retracted
hnt^{BHR28}	+	+++	tail-up
hnt^{BHR49}	+	+++	unretracted
hnt^{WN52}	-	+*	unretracted
hnt^{BHR5}	+	+	unretracted
hnt^{BHR40}	+	+++	unretracted
hnt^{WN47}	++	+++	unretracted
hnt^{WN31}	-	+*	unretracted
Df(1)ED6727	_	_	unretracted