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Evidence that *Egfr* Contributes to Cryptic Genetic Variation for Photoreceptor Determination in Natural Populations of *Drosophila melanogaster*

Ian Dworkin,[1] Arnar Palsson,[1,2] Kelli Birdsall,[1] and Greg Gibson[1,*]
[1]Department of Genetics
North Carolina State University
Raleigh, North Carolina 27695

Summary

One objective of quantitative genetics is to identify the nucleotide variants within genes that contribute to phenotypic variation and susceptibility [1]. In an evolutionary context, this means characterizing the molecular polymorphisms that modify the penetrance and expressivity of perturbed traits. A survey of association between 267 SNPs in almost 11 kb of the *D. melanogaster Egfr* and the degree of eye roughening due to a gain-of-function $Egfr^{E1}$ allele crossed into 210 isogenic wild-type lines provides evidence that a handful of synonymous substitutions supply cryptic variation for photoreceptor determination. Ten sites exceed Bonferroni threshold for association in two sets of crosses to different $Egfr^{E1}$ backgrounds including a particularly significant cluster of sites in tight linkage disequilibrium toward the 3′ end of the coding region. Epistatic interaction of this cluster with one other site enhances the expressivity of this haplotype. Replication of the strongest associations with an independent sample of 302 phenotypically extreme individuals derived from 1000 crosses of $Egfr^{E1}$ to freshly trapped males was achieved using modified case-control and transmission-disequilibrium tests. A tendency for the rarer alleles to have more disrupted eye development suggests that mutation-selection balance is a possible mechanism contributing to maintaining cryptic variation for *Egfr*.

Results and Discussion

A considerable body of evolutionary theory addresses the impact of variation for mutational effects on the trajectory of new alleles in natural populations, but surprisingly little attention has been given to the potential impact of standing hidden, or cryptic, variation on phenotypic evolution [2]. Cryptic genetic variation is genetic variation for phenotypes not observed under most conditions. However, given specific environmental or genetic backgrounds, phenotypes can be sensitized so as to express phenotypic variation, and this variation has been demonstrated to possess an underlying genetic basis [3–5]. Waddington [6] demonstrated that phenocopies of the *crossveinless* phenotype produced by sensitization with a heat shock could be selected upon, and complementation analysis demonstrated that some of the genetic variation for the phenocopy response was a result of allelic variation in known *crossveinless* genes. Similarly, Gibson and Hogness [7] showed by monitoring marker frequencies in response to artificial selection that variation at the *Ultrabithorax* locus contributes to cryptic variation for bithorax phenocopies. These studies suggest that the effects of mutations are background dependent, and such cryptic variation seems to be prevalent.

To date, there have not been any clear demonstrations of the causative nucleotide variants responsible for cryptic variation. In this paper, we address this issue using cryptic variation for photoreceptor determination. Each of the approximately 800 ommatidia in the *Drosophila* eye are highly stereotypical structures that consist of ~20 cells, including an invariant cluster of eight photoreceptor cells [8]. These differentiate with particular identities as a result of an organized pattern of cell-cell communication and signaling [9]. The organization of the ommatidial array and the eye field in general has proven to be very useful as a highly sensitizable phenotype for finding and characterizing novel genes and resolving genetic interactions through modifier screens [10–12]. One gene in particular, *Epidermal growth factor receptor* (*Egfr*), participates in a number of processes with respect to eye development, including eye versus antennal fate [13] and cell survival and proliferation [14], and also in several steps in the photoreceptor determination sequence [9, 15]. Given that previous studies have demonstrated that some of the cryptic genetic variation can be attributed to genes whose effects are phenocopied, we considered *Egfr* as a candidate for genetic variants affecting photoreceptor number.

Hidden Genetic Variation for EGFR Activity in the Eye

In order to uncover hidden genetic variation affecting photoreceptor determination, we crossed a dominant gain-of-function allele of *Egfr*, $Ellipse^{1}$ ($Egfr^{E1}$), in two different genetic backgrounds to a panel of 210 near-isogenic wild-type lines from populations in North Carolina (NC) and California (CA). $Egfr^{E1}$ has been shown to increase the average number of photoreceptor cells in each ommatidium [15], and crossing this allele to inbred lines reveals considerable genetic variation for this phenotype [16]. The expressivity of the rough-eye phenotype due to extra EGFR signaling in developing ommatidia is strongly modified by genetic factors, resulting in a range of phenotypes from almost complete suppression (normal photoreceptor patterning; a qualitative score of 0) to strong blistering (several ectopic photoreceptors per ommatidium; a score of 5). For each background, the contributions of sex, population, line, and replicate vial to eye-roughness scores were assessed by ANOVA. Extending previous observations [16], we found that (1) males tend to have stronger eye roughening than females; (2) the BL-1564 background produces stronger phenotypes than does BL-5144; (3) in the BL-1564 background, the CA population had a greater variance of

*Correspondence: ggibson@unity.ncsu.edu
[2]Present address: Department of Ecology and Evolution, The University of Chicago, 5801 South Ellis, Chicago, Illinois 60637.

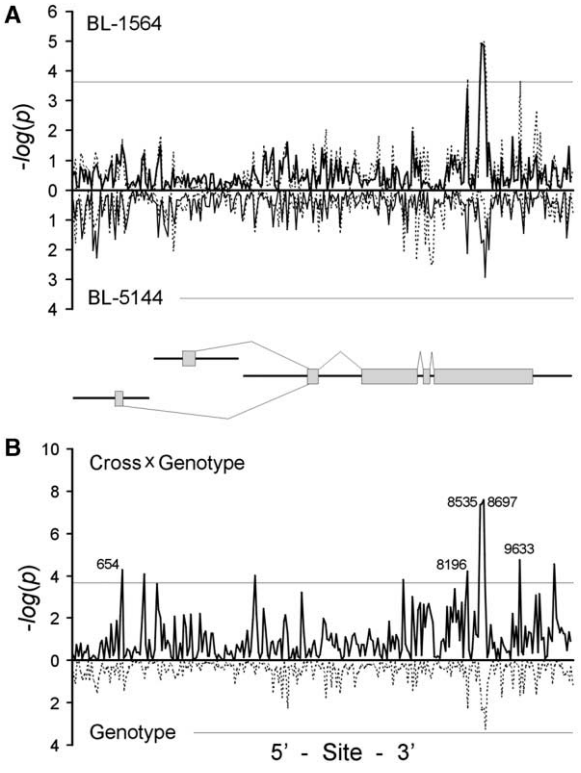

Figure 1. Distribution of Associations by SNP along the *EGFR* Locus

Plots above and below the gene structure in the middle of the figure show the negative logarithm of the p value for various measures of association from 5′ to 3′ end of the 10.9 kb region. Three segments indicated by solid black lines were sequenced, including the two alternate 5′ exons and four common exons in the 3′ segment indicated as shaded boxes. Segments 1 and 2 are separated by 23.6 kb and segments 2 and 3 by 3.2 kb. Due to uneven distribution of SNPs, lengths on the plot only approximately correspond to the length of sequenced DNA. (A) Associations for each sex and cross: females, solid lines; males, dotted; BL-1564, above the abscissa; BL-5144, below it. (B) Associations from full model for the cross × SNP interaction term above the abscissa, and cross term below it. Numbers indicate position of significant SNPs discussed further in the text and highlighted in Figure 2.

eye phenotypes than the NC population; and (4) line differences account for approximately 50% of the phenotypic variation within each sex and population (Table S1). Genetic correlation across sex for both crosses was in the range of 0.7–0.8, while correlation between the crosses was greater than 0.45, further confirming the large heritable component of hidden variation affecting EGFR activity in the eye.

SNPs in *Egfr* Contribute to the Eye-Roughness Phenotype

Do polymorphisms in *Egfr* contribute to hidden variation for eye roughness? To assess this, we monitored the significance of associations between each of 275 biallelic sites and the eye-roughness score. Figure 1A shows a plot of significance against position in a 5′ to 3′ direction for each SNP for the cross to BL-1564 above the abscissa and to BL-5144 below it, with females in solid lines and males, dashed. Significance is indicated as the negative logarithm of the p value associated with the type III sum of squares F ratio for the genotype term in an ANOVA, so that highly significant sites appear as peaks. Terms involving population were included in the model to control for any possible genetic differentiation between the flies in California (CA) and North Carolina (NC). Only one cluster of sites exceeds the Bonferroni threshold of 0.0002 for each cross and sex, but it does so in both sexes for BL-1564, and this cluster also supplies the strongest peak in the much less variable BL-5144 cross. In addition, several of the weaker associations appear in at least one sex of both crosses.

A full ANOVA model including cross, sex, and interaction terms as described in the Experimental Procedures section confirmed the potential significance of several of these sites. Owing to the difference in variance of the phenotype between the two crosses, much of the genotype effect is absorbed into the cross × genotype interaction effect. After Bonferroni correction [17], ten sites with $-log_{10}(p) > 3.7$ are nominally significant at the 0.05 level for cross × genotype interaction, including the cluster of three sites in very strong linkage disequilibrium at positions 8535, 8541, and 8697 in the sequenced region (Figure 1B). We also performed the analysis for each population (CA and NC) separately and observed the same qualitative pattern for these sites. This analysis identifies a set of candidate sites that may contribute to variation for photoreceptor determination and suggests that significance in the BL-5144 cross is masked by the general suppression of $Egfr^{E1}$ in this background. Only one of the significant sites encodes a replacement polymorphism, S654I. For ease of interpretation, we focus the remaining analysis solely on the more variable BL-1564 cross.

Single-site tests fail to account for correlations in the data due to a number of causes; for example, to linkage disequilibrium between pairs of sites or between each site and other loci that contribute to the variation. Linkage disequilibrium (LD) in the *Egfr* locus of *D. melanogaster* rarely extends beyond several SNPs in more than a few hundred base pair region (A.P., I.D., and G.G., unpublished data), but even weak LD can induce some similarity in association statistics. Figure 2A shows the relationship between LD and association significance at the 3′ end of *Egfr*. Sites 8535, 8541, and 8697 are in almost complete LD, and there is no resolution to determine which of the SNPs in this block is causative with respect to the association with phenotypic variation (if indeed it is a single site). On the other hand, site 8196 is 350 bp 5′ to this significant cluster and shows a moderate amount of LD with it. Interestingly, there are four sites at intermediate frequency in between 8196 and the cluster at 8535 that show neither association nor significant LD. Thus, it is unlikely that LD (and the association) is maintained solely due to physical proximity to the cluster. The significance of the site 8196 association drops considerably when the test is conditioned on the association with site 8697, suggesting that it does not affect eye roughness alone. By contrast, site 9633 is almost 1 kb 3′ to site 8697 and shows no LD with it, nor are its effects conditional upon those of the other significant sites.

We also tested for an epistatic interaction between

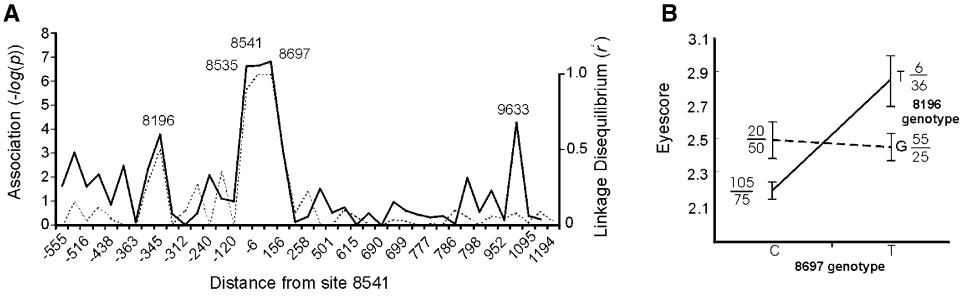

Figure 2. Relationship between Linkage Disequilibrium and Association in the 3′ Exon

(A) Association as the negative logarithm of the p value for the cross × SNP interaction term in the full model is shown as a solid line and linkage disequilibrium as r^2 as a dotted line. The absicssa shows the distance in base pairs of each SNP from the focal site at position 8541 in our sequence. Four of the SNPs between 8196 and 8535 have both alleles at a frequency greater than 0.18, but the relationship between LD and association does not hold in this region. Site 9633 shows significant association but is in linkage equilibrium with the focal cluster.
(B) Epistatic interaction between sites 8196 and 8697. The mean eye-roughness score for the four biallelic two-locus haplotypes are shown with plus or minus one standard error. Averaged across all haplotypes, at site 8196, G > T, and at site 8697, T > C, but the most enhancing haplotype is actually TT. Numbers beside each haplotype represent the observed over the expected number of haplotypes in the sample given Hardy-Weinberg equilibrium (n = 186, due to incomplete genotyping). Coupling phase linkage disequilibrium ensures that the two less-enhancing haplotypes at site 8697 (TC and GT) are overrepresented in the sample, which results in an observed average eye-roughness score of 2.36 as opposed to 2.43 expected under Hardy-Weinberg equilibrium.

pairs of sites and found evidence for a highly significant synergistic interaction between sites 8196 and 8697 (p = 0.004, ANOVA) in the BL-1564 cross. As can be seen in Figure 2B, the effect of substituting T for C in site 8697 is highly dependent upon whether a C or G is present at site 8196. The two-SNP haplotype formed by this pair of sites yielded the most significant haplotype association between the *Egfr* and eye roughness in the BL-1564 cross ($p = 3.7 \times 10^{-7}$). Furthermore, the LD between these two sites has the effect of reducing the number of flies with perturbed eye development, since the two less-active haplotypes, TC and GT, are observed in 160 lines rather than the 100 that would be expected under linkage equilibrium.

Replication of the Associations in a Large Outbred Population

These results demonstrate that the molecular basis for hidden variation can be dissected to the nucleotide level in highly inbred lines raised under controlled laboratory conditions. Rather than directly replicating the study with a new set of lines, we chose to test for association in a large outbred population. One thousand freshly caught wild-type males from the NC location were mated to virgin females of the BL-1564 $Egfr^{E1}$/SM1,*Cy* strain. Non-*Cy* (straight-winged) male progeny of this cross were scored for eye roughness, and a single male with a score of 0 or 5, corresponding approximately to the top and bottom 15% of the phenotypic distribution, was selected for genotyping along with the father of the cross that produced the extreme offspring. Case-control [18] and transmission-disequilibrium tests [19] were then performed on this data.

The case control test assesses whether there is a difference in allele frequency between suppressed and enhanced F1 males. Figure 3 shows the difference in frequency of the rare allele for each of the four sites in the 700 bp fragment that was sequenced in the progeny. The transmitted allele was inferred by subtraction from the $Egfr^{E1}$ haplotype present on the opposite chromosome in all individuals. Site 8697 shows a significant difference between the two phenotype classes (Figure 3). Although site 8196 was not significant in this analysis, the magnitude and direction of the effect (T average score = 2.22, G average score = 2.44) is the same as that observed in the near isogenic lines. Similarly, the effects of the other three sites are consistent with the original data. A log-linear analysis confirmed the significance of the site 8697 association (p = 0.02) but failed to find any evidence for interaction between the sites or for an enhanced haplotype effect.

The transmission-disequilibrium (TDT) test assesses whether heterozygous parents transmit the two alleles with equal frequency to affected (or unaffected) progeny [19, 27]. Since fewer than half of the parents of affected individuals are expected to be heterozygotes, sample

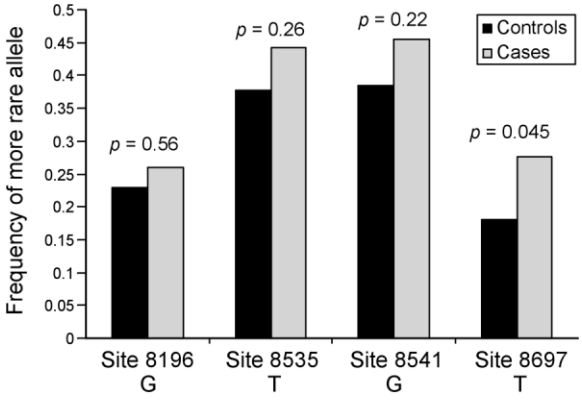

Figure 3. Replication of Significant Associations with a Case-Control Approach

Bars show the frequency of the more rare allele in the control (low eye phenotype score, black) and case (high eye phenotype score, light shading) samples. In each case, the frequency of the more rare allele (identified below the site position) is elevated in the case sample, and this difference is significant for site 8697. The direction of the effect is as predicted from the initial experiment in all cases.

Table 1. Transmission-Disequilibrium Tests

	Site 8196		Site 8535		Site 8542		Site 8697	
	G	T	C	T	C	T	C	T
High-Phenotype TDT								
OBS	15	16	18	23	22	16	11	19
EXP	15.5	15.5	20.5	20.5	19	19	15	15
p	0.50		0.26		0.21		0.10	
Low-Phenotype TDT								
OBS	16	29	27	20	20	27	14	11
EXP	22.5	22.5	23.5	23.5	23.5	23.5	12.5	12.5
p	0.04		0.19		0.19		0.34	

The top panel represents the transmission of alleles to offspring with the roughest-eye phenotypes. The bottom panel represents the transmission of alleles to progeny with almost wild-type phenotypes. Abbreviations: OBS, observed number of progeny; EXP, expected number of alleles under the null model of equal transmission; and p, probability for a one-sided Fisher's exact text on the binomial for a proportion of 0.5.

sizes for each test were quite small even though we started with 1000 crosses. Despite this, all of the transmission ratios in the smooth and rough-eye progeny were in the expected direction, and site 8196 was marginally significant in the smooth-eyed sample, as shown in Table 1. Almost twice as many flies with suppressed EGFR signaling received the T allele at site 8196 than the G allele, whereas equal numbers of flies with enhanced activity had these two alleles. Thus, the TDT and case-control tests complement one another with respect to which sites show significance and were always consistent with respect to the direction of effects.

Our approach to replication demonstrates that tests that are commonly used in human epidemiological genetics can be adapted for use in evolutionary and quantitative genetics. However, the benefits of the various tests should be considered with respect to such issues as population structure, sample size, and genotyping costs. In model systems such as *D. melanogaster*, genetic manipulations can be used to control for both unwanted environmental and genetic variation [20, 21]. However, approaches involving several generations of crosses may not be suitable or desirable for studying natural genetic variation in many systems. They are expensive due to the large amount of animal or plant husbandry involved, and few organisms afford the opportunity for inbreeding or establishment of large-scale controlled crosses. The case-control and TD tests provide alternatives that can be performed both with field and laboratory populations. The family of TDTs are particularly useful with regard to controlling for population structure [22, 23]. These tests are not necessarily practical for initial screening, but once a set of candidate SNPs are in hand, they can provide independent verification with minimal effort and cost.

A Test for Mutation-Selection Balance

Under a mutation-selection balance model, an excess of the rare alleles should be deleterious, which in our assay would correspond to a tendency to promote ectopic photoreceptor formation. As a weak test of this hypothesis, we performed a sign test on the number of the less-common alleles that were associated with an increase or decrease in eye roughness. For BL-1564, females showed a significant deviation (χ^2 test, $p = 0.002$) from the null model in favor of perturbed eye development. This result was not replicated in males, but after adding 275 rare polymorphisms (mainly singletons and doubletons) to the collection of 275 common polymorphisms that were tested for association, both sexes were significant at $p < 10^{-7}$. The case-control data shown in Figure 3 is also consistent with this prediction for the significant sites, as the frequency of the less-common allele is always higher among individuals from the "rough" eye class as compared to the "wild-type" class. One concern with this test is that it does not take nonindependence due to LD into account. Finally, three lines in the NC population were found to have a *pogo* element inserted 253 bp upstream of exon 1, and a significant cross $\times$ genotype term ($p = 0.0008$, ANOVA) for this polymorphism suggests strong enhancement of the effect of the BL-1564 background on the eye phenotype (mean *pogo* eye score 3.26 versus 2.32 for BL-1564, 1.92 versus 1.62 for BL-5144). There was no evidence to suggest that the main associations were due to LD between the *pogo* element and the putative causal SNPs given both the rapid breakdown of LD and the low frequency of the element.

SNPs and Phenotypic Evolution

Since LD is minimal in *Egfr* and given that we have sampled every polymorphic site in the sequenced region, our experimental design promotes the interpretation that the sites showing highly significant associations are the actual sites responsible for the effect. Identification of the exact sites that contribute to phenotypic variation allows us to ask further questions with regard to the evolutionary forces acting upon this gene and this phenotype. One question that is especially pertinent is whether the genetic architecture of cryptic variation is similar to that observed for other morphological traits [24]. We previously used QTL mapping [25] to identify a large-effect modifier of the *Ultrabithorax* homeotic phenotype but show here that relatively small effect modifiers of developmental mutations can also be uncovered. An analysis of association between SNPs in *Egfr* and natural genetic variation for wing shape in the same inbred lines described here identified a distinct set of polymorphisms that show association for these different traits (A.P. and G.G., unpublished data). Whether

this is due to the fact that *Egfr* operates differently in development of the eye and wing or is in fact due to differences in genetic architecture is not as of yet clear.

It is a curiosity that allelic variation for a trait that is likely not observed under most environmental conditions can still show the imprint of purifying selection. The reason for this is unknown, but we conjecture that it may be due to the pleiotropic functions of the *Egfr* gene. Thus, we may be observing the results of stabilizing selection on a trait correlated to photoreceptor determination. More theoretical work is required both to assess the conditions under which deleterious selection can affect a correlated cryptic trait [26] and to explore the power of empirical tests to detect this phenomenon. However, our results suggest a new consideration for evolutionary models of the maintenance of genetic variation. Association studies may not only reveal genes that are involved with natural variation of phenotypic traits, but also may help unravel the evolutionary forces underlying the ability of naturally segregating modifiers to affect the evolutionary trajectory of novel mutations of relatively large effect.

Experimental Procedures

Genetics

Inbred lines were generated from a panel of iso-female lines collected near Davis, California ([CA], courtesy of S. Nuzhdin) and West End, North Carolina (NC). Inbreeding was performed by sib mating for 15–20 (NC) or 50 generations (CA). Crosses were performed using the *Egfr* allele *Ellipse*^{1} (*Egfr*^{E1}, A887T [14]). To control for genetic background, two strains harboring the *Egfr*^{E1} allele were used, BL-1564 and BL-5144. Two virgin females from each of the *Egfr*^{E1} stocks were crossed to two males of each inbred line. Eye roughness was scored on ten individuals of each sex from each of two replicate vials according to a previously described scale [16]. A single experimenter assigned a score to each specimen along the preset qualitative scale of eye roughness, ranging from complete suppression (0) to extreme enhancement (5). For the case-control and TDT tests, 1000 males from NC were collected and crossed to BL-1564 virgin females bearing the *Egfr*^{E1} allele. Approximately 15% of crosses produced at least one male with apparently wild-type eyes, and 15% produced progeny with blistered eyes due to strong enhancement of the mutation. One male was selected from each of these cases and control crosses, along with his father, for genotyping.

Polymorphisms in *EGFR* were identified by sequencing 10.9 kb corresponding to the six exons and flanking noncoding regions of each of the inbred lines (A.P., A. Rouse, R. Riley-Berger, I.D., and G.G., unpublished data). A total of 245 SNPs and 30 indels with the more rare allele at a frequency of 0.05 or higher were tested for their effect on eye roughness. A table of polymorphisms and traits is available as Supplemental Information.

Statistical Analyses

All statistical analyses were performed using SAS Version 8.2 (Cary, NC), fitting models that included fixed effects of population ([*P*], NC or CA), sex (*S*), Cross ([*C*]: BL1564 or BL5144), and genotype for each SNP (*G*), and random effects of line (*L*) and Replicate vial (*R*). The dependent variable, *y*, was the qualitative eye roughness score. Line means by sex, denoted *Y* below, were assessed for each *Egfr*^{E1} strain separately, using the least square mean option in PROC GLM with the model $y = L + S + L \times S + R(L \times S) + \epsilon$.

Associations between sequence variants in *Egfr* and eye roughness were tested using PROC MIXED for each SNP on least square mean phenotype estimates, with the model $Y = P + S + C + G + G \times C + G \times P + L(G \times P) + \epsilon$. The random effect $L(G \times P)$ controls for the correlation between sexes and crosses due to line effects that are nested within genotype and population. A number of submodels were also considered, including cross separately and sex and cross separately using PROC GLM. Where possible, results were confirmed using a nonparametric permutation procedure in Tassel (www.maizegenetics.net), which allows for the control of population admixture [22, 23]. To correct for multiple comparisons, we used a Bonferroni procedure [17]. Implementation of the case-control and TDT tests is described in detail in the Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data including three tables, two Excel files containing summary data, and an enhanced experimental procedures section are available at http://www.current-biology.com/cgi/content/full/13/21/1888/DC1/.

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