CG6199^{LH2a} lethal allele from *Drosophila melanogaster* is a candidate model for investigations on *Ehlers-Danlos* syndrome

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Abstract

CG6199 gene from *Drosophila melanogaster* is a structural orthologous of *PLOD1* human gene, which encodes for a *lysyl hydroxylase* and is involved in *Ehlers–Danlos* syndrome type VI (the ocular type). In order to determine phenotypic consequences of *CG6199* impairing, loss-of-function analysis was initiated and *CG6199*^{LH2a} embryo lethal allele was obtained by P element mutagenesis. This allele harbors an *P{EP}* transposon insertion into the 9th exon of *CG6199*, encoding for an amino acid sequence belonging to the *P4Hc* functional domain of *lysyl hydroxylase*. Phenotypic and molecular analysis of *CG6199*^{LH2a} allele points that *P4Hc* is essential for the normal embryo development of *D. melanogaster*.

Our data support a functional orthology between *CG6199* and *PLOD1* genes, creating premises for the study of *Ehlers-Danlos* syndrome using *D. melanogaster* experimental model.

Keywords: *Ehlers-Danlos* syndrome, *Drosophila melanogaster*, *PLOD1*, *CG6199*, transposon mutagenesis.

Introduction

Ehlers-Danlos syndrome is an autosomal recessive disorder, characterized by hiperelastic skin, severe joints hipermobility and luxations, weakness of tissues and cardiovascular complications [1, 2]. In some cases of *EDS* syndrome type VI (the ocular type), a lysyl hydroxylase deficiency was reported, caused by mutations in *PLOD1* human gene [3]. *PLOD1* gene maps to 1p36.3-36.2 [4], has about 41 kb and 19 exons and encodes for lysyl hydroxylase (*LH1*, *procollagen lysine*, *2-oxoglutarate 5 dioxygenase* 1; EC1.14.11.4). This enzyme belongs to the group of 2-oxoglutarate dioxygenases or 2 OG-Fe(II) oxygenases, which also includes prolyl 4-hydroxylase alpha subunit, isopenicilin synthases and AlkB [5]. *LH1* hydroxylates lysine residues during collagen synthesis, enabling them to serve as attachment sites for carbohydrate units, which confer stability of the intermolecular collagen crosslinks. The hydroxylation reaction catalyzed by lysyl hydroxilase requires Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate as essential cofactors and generates lysyl residues in procollagen polypeptide, CO_2 and succinate [6]. The deficiency of *LH1* leads to a decrease in hydroxylysine content of collagen, resulting in synthesis of collagen that lacks normal structural stability. While the prevalence of this disease is low (1/5000-10000), it is recorded in every continent and affects both sexes [7].

Although there are no evident structural similarities between the primary structure of lysyl hydroxylase subunit and the subunits of prolyl 4-hydroxylase, the most important functional domain for each of them is prolyl 4-hydroxylase alpha subunit homologue (*P4Hc*). Highly conserved histidine residues seem to be important in the binding of a variety of co-factors, such Fe^{2+} ions [8].

Drosophila melanogaster (the fruit fly or the vinegar fly) is an excellent experimental model for medical research. An essential condition for the study of a human genetic disease using this model is to identify specific structural orthologous genes in *D. melanogaster*. Once such a gene is detected, a polyallelic series should be constructed, in order to analyze mutant phenotypes relevant for the genetic disorder. One of the most efficient and versatile mutagenesis method in the fruit fly is excisioinal/insertional mutagenesis with P element derivates [9]. Many of the *D. melanogaster* genes have pleiotropic effects so it is necessary to obtain several different mutant alleles for each analyzed gene, just to be able to reveal its functions. Some of the aberrant phenotypes determined by these alleles are very useful for the medical genetics studies. The sequence of *D. melanogaster* genome was recently deciphered [10], leading to *in silico* identification of many genes. Bioinformatics analysis of *D. melanogaster* and of *Homo sapiens* genomes [11] opened new ways to approach the researches focused on hereditary diseases, making possible the comparison of sequences from fruit fly with the sequence of any gene involved in a genetic disease [12, 13]. If the compared genes show a high degree of sequence homology, there is a theoretical basis for the study of the specific illness in *D. melanogaster* model.

Our investigations targeted *CG6199* gene from *D. melanogaster*, which is located in 68B1 chromosomal region and is structurally orthologous with *PLOD1* human gene (*procollagen lysyl 5-deoxigenases*). Protein *CG6199-PA* (isoform A) contains at its C terminal sequence a *P4Hc* functional domain having a high degree of similarity with the same domain of *PLOD1*. Loss-of-function analysis of *CG6199* was initiated in order to determine the consequences of blocking the activity of this gene in *D. melanogaster*. In a small scale P element mutagenesis screening, we obtained a embryo lethal allele of *CG6199* gene, symbolized *CG6199^{LH2a}*. Phenotypic and molecular analysis of the *CG6199^{LH2a}* allele revealed that *P4Hc* functional domain of lysyl hydroxylase enzyme is essential for normal embryo development of *D. melanogaster*.

Materials and Methods

Genetics: All lines used in our experiment have a *w* background and were as follows: *EP(3)3313*/*TM6TbHu* line, *yw*; *Δ2-3Sb*/*TM2Ubx* transposase source and *TM3SbSer*/*TM6TbHu* double balancer line. Virgin females were collected at maximum 8 hours after emerging from pupae. All genetic crosses were made at room temperature (20^0-25^0 C). Lines were raised on an yeast-cornmeal-agar medium. The genetic scheme for mobilization of the original *P{EP}3133* insertion is described in Fig.1.

Molecular analysis: PCR primers sequences were designed with *FastPCR* [14] and *OligoAnalyzer* [15] software and are as follows:

ED1: 5’acactacggtgacacgtcc3' (forward primer);
ED2: 5’ttccagatcgtgtcggacg3’ (forward primer);
ED4: 5’accaaccagctacgcggac3’(reverse primer);
ED5: 5’acgaaccaaccagctacgc3’(reverse primer);

EDF: 5'gcgagtcactccgaaatgcg 3'(forward primer);

EDR: 5'ccggccgataacgcaccatg 3'(reverse primer).

In addition, the MM11 primer corresponding to both IR ends of P transposable element was used and has the sequence: 5' cgacgggaccaccttatgttatttc 3'. The theoretical size of the specific amplicons is as it follows: ED1+ED5 = 1646 bp; ED1+ED4 = 1642 bp; ED2+ED5 = 1506 bp; ED2+ED4 = 1502 bp; ED1+MM11 = 1598 bp; ED2+MM11 = 1458 bp; ED4+MM11 = 106 bp; ED5+MM11 = 110 bp; EDF+EDR = 618 bp; EDF+MM11 = 409 bp; EDR+MM11 = 259 bp;

For genomic DNA extraction we used an adapted DNA extraction protocol [16]. PCR reactions were performed in a gradient temperature termocycler *Corbett PalmCycler CG1-96* and the final reaction volume was 20 µl, excepting for the purification reaction, where the final reaction volume was 50 µl. PCR reaction components concentrations were: 0,2 µM for each primer, 200 µM dNTP, 0,05 u/µl Taq enzyme, 1X buffer, 1,5 mM $MgCl_2$ (Promega reagents). We used the following PCR program: 95^0C: 5', (95^0C: 30s, 59^0C: 30s, 72^0C: 90s) x 30, 72^0C: 5 min and 4^0C: 5 min. The molecular weight markers are: *OrangeRuler™ 100bp-500bp DNA Leader* (Fermentas) and *100bp* (Promega). Purification of the specific amplicons was made with a *Wizard SV Gel and PCR Clean-Up System* kit (Promega).

Sequencing was first performed with ED1 primer on a ABI Prism 3100 AVANT and then separately repeated with EDL and EDR primers on a BECKMAN CEQ8800 equipment.

Bioinformatics: For various bioinformatics analysis we used the following software and databases: *Apollo* [17], *BLAST* [18], *SMART* [19, 20], *EBI* [5], *FlyBase* [21], *NCBI* [3] and *WormBase* [22].

Results and Discussions

The transgenic line used in our experiments is symbolized *EP(3)3313* and is characterized by an unique insertion/genome of a *P{EP}* element [23, 21]. The original insertion of EP(3)3313 (*P{EP}3313*) is located at about 592 bp downstream to the 3' end of *CG6199* so mobilization of the transposon may affect this gene. So far, three mutant alleles of the *CG6199* gene were reported, namely $CG6199^{EY11195}$ allele which is associated with viability and fertility, $CG6199^{d11691}$ allele with no phenotypic effects described and RNAi induced $CG6199^{GD5882}$ allele which is viable [24]. In order to determine if *CG6199* gene is essential for *D.melanogaster* development and to reveal functional correlations between *CG6199* and *PLOD1* genes, a first step was to obtain loss of function mutations of *CG6199*.

After a pilot transposon mutagenesis experiment [25], we initiated new experiments of insertional/excisional mutagenesis using mobilization of *P{EP}EP3313* with *Δ2-3* transposase source [26], in order to obtain lethal alleles of *CG6199* gene. Using the genetic scheme depicted in Fig.1, we performed a screening for both lethal conservative reinsertions and lethal imprecise excisions of the artificial transposon. Individuals with imprecise excisions have white eyes, because *w* genetic background is revealed by losing of *miniwhite* allele of *P{EP}*. On the other hand, individuals with conservative reinsertions have deeper pigmented eyes than the pale orange color specific of *EP(3)3313* line, due to dose effect of *miniwhite*.

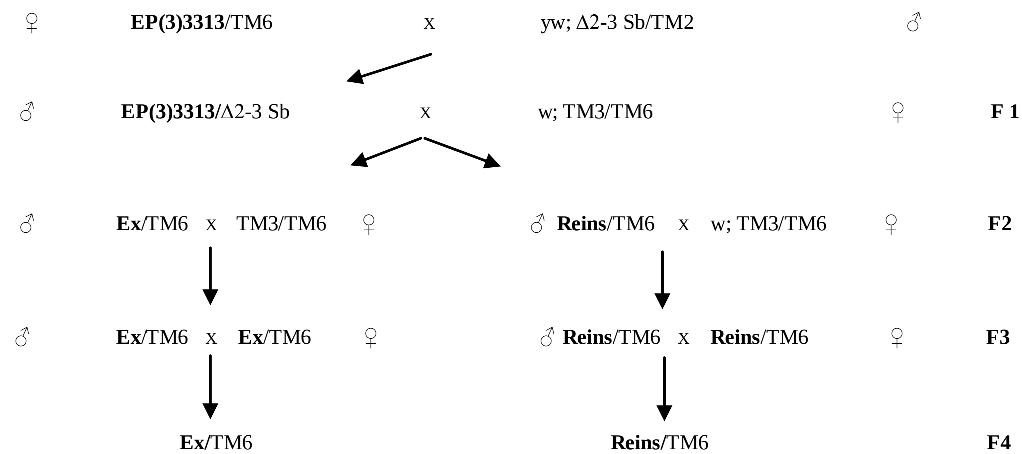

Figure 1. Genetic scheme used for obtaining lethal excisional alleles (**Ex**) and lethal insertional alleles (**Reins**) of *CG6199* gene by mobilization of *P{EP}EP3313* transposon. Only the heterozygous balanced mutants survive in F4 generation.

A total of 16 new lethal lines were obtained; out of them, 13 harbor imprecise excisions and are symbolized LH^{1Mf}, LH^{2Mf}, LH^{3b}, $LH^{3.2}$, LH^{7a}, LH^{7b}, LH^{8a}, LH^{8b}, LH^{10a}, LH^{10b}, LH^{20c}, LH^{21c} and LH^{25c}. The other three lines contain conservative reinsertions of the transposable element and are symbolized LH^{2a} , LH^{3} and $LH^{6.2}$.

In order to perform their molecular analysis, we extracted genomic DNA from each mutant line and designed specific primers as described in *Materials and Methods*. In order to optimize PCR conditions, different primers combinations were used in reactions performed on a DNA template extracted from *Oregon* wild-type strain. All the amplicons calculated theoretically were obtained at the annealing temperature of 60^0C (Fig. 1).

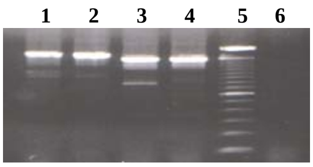

Figure 2: Optimized PCR reactions on Oregon DNA template. Lane 1: ED1 + ED5 = 1646 bp; lane 2: ED1 + ED4 = 1642 bp; lane 3: ED2 + ED5 = 1506 bp; lane 4: ED2 + ED4 = 1502 bp; lane 5: the molecular weight marker (100 bp Promega); lane 6: negative control.

The above mentioned lines were analyzed by triplex PCR with different combinations of primers containing the MM11 primer corresponding to the inverted repeats of the transposon (Fig. 2). According to bioinformatics analysis, a successful triplex PCR reaction performed on the genomic DNA template of *EP(3)3313* line with ED1 + ED5 + MM11 primers should generate three distinct amplicons, namely of 1646 bp (the wild type amplicon), 1598 bp and 110 bp.

The most interesting mutant line was LH^{2a} strain, susceptible to host a reinsertion of *P{EP}* within *CG6199* gene, since PCR reaction performed with ED1 + ED5 + MM11 primers generated an original amplicon of about 300 bp (see Fig. 3, lane 2, the middle amplicon). Generation of this amplicon could be explained if the transposon reinsertion is located within the 9th exon of *CG6199* gene.

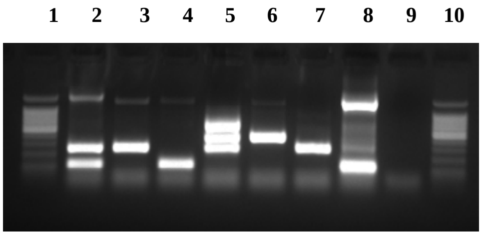

Figure 3. Triplex PCR applied on the genomic DNA of LH^{2a} mutant strain with different combinations of primers (see Materials and Methods for the estimated size of the amplicons); lane 1: the molecular weight marker (100bp-1500bp DNA ladder, Promega); lane 2: ED1+ED5+MM11; lane 3: ED1+MM11 *(the band is estimated to be about 300 bp)*; lane 4: ED5+MM11; lane 5: EDF+EDR+MM1; EDF+MM11; lane 7: EDR+MM11; lane 8: the positive control - *EP{3}3313* with ED1+ED5+MM11; lane 9: the negative control; lane 10: the molecular weight marker (100bp-1500bp DNA ladder, Promega).

PCR was performed again on LH^{2a} DNA template only with ED1 + MM11 primers (annealing temperature = 60^0C) and the specific amplicon of about 300 bp was purified using a *Wizard SV Gel and PCR Clean-Up System purification kit* and sequenced with ED1 primer on an ABI. The sequence of the insertional allele was sent to GenBank/NCBI (accession number *EU240219*).

Afterwards, we repeated PCR on LH^{2a} DNA template with EDL + MM11 primers and, alternatively, with EDR + MM11 primers. A more accurate sequencing procedure of the corresponding amplicons (having about 416 bp and 241 bp) was performed with EDL and with EDR primers using a different equipment (see Materials and Methods) and revealed that *P{EP}* insertion is located at nucleotide position 11192002 of CG6199 (see Fig. 5). Based on the nucleotide sequence analysis we concluded that LH^{2a} strain contains an insertional lethal allele of *CG6199* gene and we symbolize it as $CG6199^{LH2a}$. Bioinformatics analysis using *SMART* database revealed that both human *PLOD1* enzyme and the protein encoded by *CG6199* contain a carboxy terminal conserved domain, symbolized *P4Hc* (*subunit alpha of Prolyl 4-hydroxylases homologue*). In *D. melanogaster* this protein fragment is encoded by the 9^{th} and the 10^{th} exons of *CG6199* gene.

By using *Homophila* database, we obtained the alignment between the amino acid sequence of *P4Hc* domains of *PLOD1* and *CG6199* and the results are presented in Fig. 5.

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  AFCANIRQQDVFMFLTNRHTLGHLLSLDSYRTTHLHNDLWEVFSNPEDWKEKYIHQNYTK
A C ++R   +FM+ +N    GHL++ D + TT    D + +FSN  DW EKYIH NY+
  AMCESLRNAGIFMYASNLRIFGHLVNADDFNTTVTRPDFYTLFSNEIDWTEKYIHPNYSL

  AL-AGKLVETPCPDVYWFPIFTEVACDELVEEMEHFGQWSLGNNKDNRIQGGYENVPTID
 L     ++ PCPDVYWF I ++  CD+LV  ME    WS G+N DNR++GGYE VPT D
  QLNESNKIQQPCPDVYWFQIVSDAFCDDLVAIMEAHNGWSDGSNNDNRLEGGYEAVPTRD

  IHMNQIGFEREWHKFLLEYIAPMTEKLYPGYYTRAQFDLA-FVVRYKPDEQPSLMPHHDA
IHM Q+G ER + KFL  ++ P+ E+ + GY+       L  F+VRY+PDEQPSL PHHD+
  IHMKQVGLERLYLKFLQMFVRPLQERAFTGYFHNPPRALMNFMVRYRPDEQPSLRPHHDS

  STFTINIALNRVGVDYEGGGCRFLRYNCSIRAPRKGWTLMHPGRLTHYHEGLPTTRGTRY
ST+TINIA+NR G+DY+GGGCRF+RYNCS+   +KGW LMHPGRLTHYHEGL  T GTRY
  STYTINIAMNRAGIDYQGGGCRFIRYNCSVTDTKKGWMLMHPGRLTHYHEGLLVTNGTRY

IAVSFVDP
I +SF+DP
IMISFIDP
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Figure 5. Analysis of the structural homology (adapted from *Homophila*) between a partial amino acid sequence encoded by *PLOD1* (the upper sequence in each row) and a fragment of the amino acid sequence encrypted by *CG6199* gene (the lower sequence in each row). The underlined region represents the amino acids encoded by the 9^{th} (in bold and italics also) and the 10^{th} exon of *CG6199*. The *P{EP}* reinsertion is located between two codons codifying for glutamic acid and for glycine (the shadowed **EG** fragment) in $CG6199^{LH2a}$ allele. Overlapping between the *P4Hc* domain of *PLOD1* (in bold) and the amino acid sequence encoded by the 9^{th} and the 10^{th} exon of *CG6199* gene is shown, where each **+** in the middle rows stands for an equivalent amino acid substitution.

The *P{EP}* insertion defining $CG6199^{LH2a}$ affects the *P4Hc* functional domain of lysyl hydroxylase, involved in collagen biosynthesis. Using a balancer chromosome harboring the GFP (Green Fluorescent Protein) marker we noticed that the lethality of $CG6199^{LH2a}$ is induced at the final stage of the homozygous embryo's development. Embryo lethality determined by *P4Hc* disruption in *D.melanogaster* is by itself remarkable, proving that *CG6199* is an essential gene.

We inferred from our results that lysyl hydroxylase is essential for the normal body development in *D.melanogaster* and mutations affecting *P4Hc* functional domain determine severe phenotypes, including lethality. In *C.elegans* there is also a lysyl hydroxylase, symbolized *let-268*, which has a *P4Hc* domain with approximately 70% sequence similarity with the *P4Hc* domain of *CG6199*. Mutations in *let-268* determine lethality in the larval stage of development, probably due to a wrong secretion and incorporation of type IV collagen into the basement membrane during embryogenesis [22].

In *D. melanogaster* a cluster of ten different genes encoding for prolyl 4-hydroxylase were described and six of them are expressed in a tissue-specific pattern in the embryo, revealing their importance for the normal development process [27]. Studies performed on *Caenorhabditis elegans* revealed that the double mutants for *dpy-18* and *phy-2* genes, both encoding for prolyl 4-hydroxylase, are also lethal [28].

All this data demonstrate that both lysyl hydroxylase and prolyl 4-hydroxylase are key players in collagen metabolism in animals. In *D. melanogaster* there are two neighboring genes encoding for collagen IV, namely *viking* and *Cg25C* [21], which qualify fruit fly as a model for collagen disorders.

Some human patients with *EDS* type VI present either deletions affecting the 17th exon of *PLOD1* gene in heterozygotic condition [29] or homozygotic deletions of this exon [30]. These deletions are associated with severe forms of *EDS* caused by low activities of lysyl hydroxylase enzyme. *P4Hc* functional domain is encoded by the terminal exons 16, 17, 18 and 19 of *PLOD1* gene and mutations affecting this domain are probably the main determinants of some forms of the the disease.

Interpretation of our sequencing data suggests a functional orthology between *CG6199* and *PLOD1* genes, creating premises for the study of *EDS* in *D.melanogaster* experimental model. The transposon reinsertion specific to *CG6199*^{LH2a} is located into the 9th exon and may be functionally assimilated with the deletion of this exon, revealing an analogy with previously described deletions specific to *EDS*. A more detailed analysis of this allele during fruit fly development is expected to reveal some basic aspects of collagen dynamics which are damaged in collagen disorders.

Conclusions

CG6199^{LH2a} is an embryo lethal allele of *CG6199* gene, a *D.melanogaster* structural orthologous of human *PLOD1* gene, involved in *EDS*. Phenotypic and molecular analysis of the *CG6199*^{LH2a} revealed that *P4Hc* is essential for the normal *D.melanogaster* embryo development. The insertional mutation is basically equivalent with deletions affecting *P4Hc* in some *EDS* patients.

We regard *CG6199*^{LH2a} allele as a promising model candidate for further functional studies concerning *EDS*. Obtaining of new alleles of *CG6199* which determine milder, mutant phenotypes, would open new ways to understand the fundamental mechanisms responsible for disorders in collagen biosynthesis.

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