The Glucose Transporter (GLUT4) Enhancer Factor is Required for Normal Wing Positioning in Drosophila

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Sequence data from this article will be deposited with the EMBL/GenBank Data Libraries under the following accession nos. (to be included).
ABSTRACT

Many of the transcription factors and target genes that pattern the developing adult remain unknown. In the present study, we find that an orthologue of the poorly understood transcription factor, Glucose Transporter (GLUT4) Enhancer Factor (Glut4EF (GEF)) (also known as the Huntington’s Disease gene regulatory region-binding protein (HDBP) 1), plays a critical role in specifying normal wing positioning in adult Drosophila. Glut4EF proteins are zinc finger transcription factors named for their ability to regulate expression of GLUT4 but nothing is known of Glut4EF’s in vivo physiological functions. Here, we identify a family of Glut4EF proteins that are well-conserved from Drosophila to humans and find that mutations in Drosophila Glut4EF underlie the wing positioning defects seen in stretch mutants. In addition, our results indicate that previously uncharacterized mutations in Glut4EF are present in at least eleven publicly available fly lines and on the widely used TM3 balancer chromosome. These results indicate that previous observations utilizing these common stocks may be complicated by the presence of Glut4EF mutations. For example, our results indicate that Glut4EF mutations are also present on the same chromosome as two gain-of-function mutations of the homeobox transcription factor Antennapedia (Antp) and underlie defects previously attributed to Antp. In fact, our results support a role for Glut4EF in the modulation of morphogenetic processes mediated by Antp, further highlighting the importance of Glut4EF transcription factors in patterning and morphogenesis.
INTRODUCTION

Characterizing the molecular mechanisms that pattern the developing human has been a major focus of biomedical science and studies in model organisms such as the fruitfly, *Drosophila melanogaster*, have proven invaluable for this endeavor. Spontaneous mutations such as *Bithorax, Notch, Antennapedia*, and *wingless* that affect the morphology of the adult fly have been the starting point for the identification of molecular pathways that play essential roles in body plan specification in both invertebrates and vertebrates. More recently, genetic screens have been designed to search for particular changes in morphology in developing and adult Drosophila (RUBIN 1988; ST JOHNSTON 2002). Such screens have led to the identification and characterization of genetic determinants of both development and disease in mammals (BIER 2005; BILEN and BONINI 2005; TOUNTAS and FORTINI 2007).

One adult structure that is particularly useful for identifying the genes required for normal adult morphology are the wings of Drosophila. The characterization of mutations that affect the formation, shape, organization, structure, venation, and positioning of the wings has identified genes conserved both structurally and functionally across diverse phyla. Wingless, for example, the founding member of the phylogenetically-conserved Wnt family of signaling molecules, was first identified as a mutation, *wingless (wg)* (SHARMA and CHOPRA 1976), that prevents wing formation in *Drosophila*. More recently, wingless/Wnts have been found to have widespread effects on both fly and mammalian morphogenesis by coordinating diverse cellular processes (BEJSOVEC 2006; HOPPLER and KAVANAGH 2007).
In the present study we determine the underlying causality of a newly identified wing positioning defect in adult Drosophila, and find that this mutation disrupts a member of a novel family of transcription factors conserved from flies to humans. Our results reveal that the *stretched out* gene corresponds to the Drosophila orthologue of the Glucose Transporter (GLUT4) Enhancer Factor (Glut4EF (GEF)). Glut4EF is a zinc finger transcription factor (Oshel et al. 2000) known to physically associate and activate transcription with the critical muscle regulator myocyte enhancing factor (MEF2) (Knight et al. 2003). Glut4EF transcription factors also contain a CRARF domain that is highly similar to the transcriptional activation and DNA binding domain present in wingless/Wnt signaling effectors, T cell-specific (TCF) HMG box transcription factors (Arce et al. 2006; Hoppler and Kavanagh 2007). Nothing is known, however, of Glut4EF’s in vivo physiological roles. In addition to finding that Drosophila Glut4EF is required for normal adult morphology, our results indicate that previously uncharacterized mutations in *Glut4EF* are present within at least eleven publicly available fly lines including within the widely used TM3 balancer chromosome. Among these fly stocks, our results indicate that mutations in *Glut4EF* are also present on the same chromosome as two dominant gain-of-function *Antennapedia (Antp)* mutations and underlie defects previously attributed to the homeobox transcription factor *Antp*. Furthermore, our results also support a role for *Glut4EF* in the modulation of morphogenetic processes mediated by Antp.
MATERIALS AND METHODS

Genetics: All complementation analysis and genetics were done using standard techniques. All stocks were obtained from the Bloomington Stock Center, except stretch\textsuperscript{NP7418-GAL4} allele (NP7418; Drosophila Genetic Resource Center, Japan), s13308, s103402 (Szeged Stock Center), P\{EPgy2\}stretch\textsuperscript{EY03156} (a kind gift from Hugo Bellen), Evar631 (kind gift from Gunter Reuter), Df(3R)B22-5 (kind gift from Bill Chia), and P\{wH\}2-1 (kind gift from Jim Birchler).

Phenotypic Characterization: The outstretched wing phenotype was quantified by crossing adults at 25°C: adult offspring from these crosses were first sorted as to genotype, placed into vials of five flies each, and then, following recovery from CO₂ anesthesia, examined for outstretched wings. Wings were scored as outstretched if both wings were separated and not overlapping.

Genomic Organization, Molecular Analysis, and Transformation Constructs: The insertion site of the P\{PZ\}10477 P element was determined by using Inverse PCR approaches (adapted from a protocol by E. Jay Rehm, Berkeley Drosophila Genome Project) to amplify that portion of genomic DNA flanking the P\{PZ\}10477 insertion site. In brief, genomic DNA from P\{PZ\}10477 flies was digested using Sau3A and subjected to inverse PCR using specific primers (“P-TR2” 5’CGACGGGACCACCTATGTTATTTTCATCATG and “P-Lac” 5’AGCTGGCGTAATAGCGAAGAGGCAGCCCGCA) for the P transposable element.
PZ[ry, lacZ]. The resulting 163bp PCR product was inserted into the pCR2.1-TOPO cloning vector (Invitrogen) and sequenced. The genomic organization of the D-Glut4EF locus was determined, with the aid of the Sequencher 4.6 program (Gene Codes Corp.), using our identified cDNAs, DNA flanking the insertion sites of P elements, and publicly available Drosophila genomic DNA sequences. Proteins, domains, and alignments were identified using Web-based protein domain searching and alignment tools including PFAM, BLAST, and ClustalX and our own molecular analysis. ESTs corresponding to all 4 isoforms (D-Glut4EFa (RE66512), D-Glut4EFb (RE25317), D-Glut4EFc (RH63124), and D-Glut4EFd (GH15792)) were obtained from the Drosophila Genome Resource Center, and sequenced on both strands (Sequences to be deposited in GenBank). The D-Glut4EFd rescue construct was created by cutting D-Glut4EFd from EST GH15792 with two restriction endonucleases (EcoRI (5’) and SalI (3’)) whose sites where present in the 5’ UTR and the 3’ UTR of the GH15792 EST. This 2.2kb fragment containing the D-Glut4EFd open reading frame (ORF) and portions of the 5’ and 3’ UTR was then inserted into compatible restriction sites (EcoRI and XhoI) in the pUAST vector for Drosophila germline transformation. Multiple transformants were obtained.

In Situ Hybridization: RNA in situ analysis on fly larvae imaginal discs were carried out using sense and anti-sense cRNA probes corresponding to the D-Glut4EFd isoform (CADIGAN et al. 1998; HUANG et al. 2007).

Imaging, Quantification, and Statistical Analysis: All images were captured using a Zeiss Discovery Stereomicroscope, or a Leica MZ16 stereomicroscope with an Axiocam
HRc camera and Axiovision software. 3D dimensional reconstruction software (Extended Focus Software, a kind gift from Bernard Lee) was used to construct the images of the adult flies. Brightness, contrast, and color balance of images were adjusted using Adobe Photoshop. Statistical analysis (Chi-square Test) was performed with the aid of the GraphPad InStat software package.
RESULTS

**Adult Drosophila stretched out (stretch) mutants exhibit abnormal wing positioning:** During the process of characterizing mutations mapped to the 85E-85F locus of the Drosophila third chromosome, we obtained a P transposable element insertion line \( P\{PZ\}l(3)10477 \) that was interesting because in contrast to normal flies which hold their wings straight back over their bodies (see Figure 1A), 100% of the flies in this stock held their wings out at a 45 degree angle (see Figure 1B). However, previous work by others on this fly stock (see Figure S1) suggested that there was no association of the wing phenotype with the P transposable element insertion. In the course of designing a screen to make a mutation in the *MICAL* gene (TERMAN et al. 2002), we used this P element containing fly line (\( P\{PZ\}l(3)10477 \)) and recombined a recessive marker (*scarlet* \( st \)) onto the chromosome containing the P element. Recombined \( st, 10477 \) flies were now viable over the original \( P\{PZ\}l(3)10477 \) line, suggesting we had removed a second site lethality. Interestingly, the \( st, 10477/P\{PZ\}l(3)10477 \) flies held their wings out at an angle (Figure 1D), while \( st, 10477 \) flies heterozygous over a wild-type chromosome (\( st, 10477/+ \)) or \( P\{PZ\}l(3)10477 \) flies heterozygous over a wild-type chromosome (\( 10477/+ \)) showed no abnormal wing positioning (Figure 1D; Table S1). These results suggest that the wing positioning defect in these flies is caused by a recessive mutation associated with the presence of the \( P\{PZ\}10477 \) P element.

To determine if the wing positioning defect associated with the \( P\{PZ\}10477 \) P element line was due to an alteration of a gene in the 85E-F region of the Drosophila
genome, we asked if 10477 flies exhibited a wing phenotype when heterozygous to a deficiency removing the 85E-F region. Our results revealed that adult flies heterozygous for both 10477 and Df(3R)by62 (breakpoints 85D10-11; 85F1-8) had an outstretched wing phenotype (Figure 1D). In contrast, adults heterozygous for both 10477 and an adjacent deficiency (Df(3R)by10; deleted region: 85D8-85E13) appeared wild-type (Figure 1D). These results indicate that the recessive mutant wing positioning defect is associated with a gene in the 85E-F region and we have called this mutation stretched out (stretch) based on the wing phenotype.

\textit{stretch mutations are present in a number of fly lines including within the TM3 balancer:} Following our initial characterization of the \textit{stretch} mutation and its localization to the 85E-F region of the Drosophila genome, we re-examined the original stock we had obtained from the Bloomington Stock Center that had an outstretched wing phenotype. In particular, we wondered why the original stock P{PZ}l(3)10477, which was heterozygous for the recessive \textit{stretch}\textsuperscript{10477} mutation, exhibited an 100% outstretched wing phenotype. The original stock was listed as containing a dominant Dichaete allele (CxD) that was responsible for the outstretched wing phenotype. However, our analysis of the stock suggested that CxD was not present within it (see Figure S1). Specifically, the stock had a Stubble (Sb) appearance suggesting that the TM3 Sb balancer was present in the stock. Interestingly, the TM3 balancer is listed as having a chromosomal breakpoint in the 85E area (Lindsley and Zimm 1992), which is the same location that the 10477 P element had been mapped. We therefore wondered if the TM3 balancer disrupted the same locus as the 10477 P element, thereby giving rise to an outstretched
wing defect when the two alleles were present in one fly. Indeed, when we crossed the 10477 P element line to different TM3 balancer lines (e.g., flies containing either TM3 Sb or TM3 Serrate), all 10477/TM3 flies had outstretched wings (Figure 1D). In contrast, when the 10477 line was crossed to another balancer which does not have breakpoints in the 85E-85F region (the TM6B balancer), offspring exhibited a normal, wild-type appearance (Figure 1D). Interestingly, we also found that a progenitor of the TM3 balancer, In(3LR)sep, vvl sep kni sep (Breakpoints: 65D2—3; 85F2—4; (LINDSLEY and ZIMM 1992)), had a previously uncharacterized recessive wings-out phenotype and genetic complementation analysis revealed that it was also a stretch allele (Figure 1D). These data indicate that the TM3 balancer contains a previously undiscovered mutant allele of the gene disrupted by the 10477 P element and should now be listed as containing the stretch mutation which can serve as a 100% penetrant recessive marker.

In light of our identification of stretch mutations on the TM3 balancer chromosome we wondered how prevalent stretch mutations might be in other stocks and looked for additional alleles of stretch. We obtained publicly available fly stocks with breakpoints (deletions, inversions) or insertions assigned to the 85E-85F region. Of the large number of fly lines we tested using genetic complementation analysis (Figure S1; Table S1; data not shown), we found six additional lines, including four additional P element stocks that exhibited a recessive wing phenotype indicating they were also stretch alleles (Figure 1D-F). Interestingly, wing positioning defects had previously been seen in some of the stretch alleles we identified including A1 (Mapped Insertion: 85E), In(3R)AntpR (Breakpoints: 84B1; 85F; (GARBER et al. 1983)), and In(3R)AntpR (Breakpoints: 84B1-2; 85E; (DUNCAN and KAUFMAN 1975; KAUFMAN et al. 1980;
LINDSLEY and ZIMM 1992)) but their causality had either been assigned as a dominant allele (A1), or characterized to mutations in other genes (In(3R)Antp\textsuperscript{R}, In(3R)Antp\textsuperscript{B}; see below). Furthermore, it is likely that the In(3LR)4f fly line (Breakpoints: 79D3-D4; 85E; (CARPENTER 1994)), which we have been unable to obtain, also disrupts stretch since the chromosomal breakpoints map to this location and the In(3LR)4f fly line has been indicated to have a wings-out phenotype. Lastly, we also noticed from our complementation analysis that the wing positioning defect in several weaker stretch alleles was more severe in males (Table S1).

**stretch mutations disrupt Drosophila Glut4EF - a member of a novel phylogenetically conserved family of transcription factors:** To determine the gene disrupted in these stretch mutants we sequenced the genomic DNA flanking several stretch P element alleles and found that all of these P element mutations were inserted in a novel Drosophila gene that covered more than 115 kb of genomic DNA (Figure 2A). This single gene had previously been annotated as three separate genes and given the names CG12418, CG12802, and CG33975 (previously CG11676 and CG32469) (Figure 2B). Our indepth analysis of ESTs and cDNAs in that region of the genome revealed at least four different transcripts sharing overlapping exons (Figure 2B-C). The longest of these splice variants (Glut4EF\textsubscript{d}; Figure 2B-C) showed a high degree of amino acid identity and conserved domain organization to the mammalian Glucose Transporter 4 Enhancer Factor (Glut4EF (GEF)) (Figure 3, 4A; (O'SHEL et al. 2000)). Glut4EF (also called the Huntington’s Disease gene regulatory region-binding protein (HDBP) 1 (TANAKA et al. 2004), and SLC2A4RG) was originally characterized and named for its
ability to bind to the enhancer of the Glucose Transporter 4 gene (OSHEL et al. 2000). Glut4EF proteins are also highly similar to Papillomavirus Binding Factor (PBF (BOECKLE et al. 2002); also called HDBP2 (TANAKA et al. 2004), osteosarcoma antigen (TSUKAHARA et al. 2004), and ZNF395 (STOECKMAN et al. 2006)) (Figure 4A), and in our search of the database we found a previously uncharacterized human EST ZNF704 (similar to the mouse EST Zfp704 (BLACKSHAW et al. 2004); also called mouse glucocorticoid-induced gene 1 (Gig1)) that was highly related to Glut4EF proteins and represents a third mammalian family member (Figure 4A). Therefore, the gene disrupted in stretch mutants represents the Drosophila member of a new family of transcription factors conserved from Drosophila to mammals. We propose to name this new family of proteins the Glucose Transporter 4 Enhancer Factor (Glut4EF (GEF)) family after the original member (OSHEL et al. 2000).

Drosophila Glut4EF (D-Glut4EFd), like Glut4EF, PBF and ZNF704, contains a nuclear localization signal (NLS), and a classical C2H2 zinc finger DNA-binding domain (Figures 3, 4A). At their C-terminus, D-Glut4EFd, Glut4EF, PBF, and ZNF704 also contain a CR (CRARF/RKKCIRY) domain that is highly similar to the transcriptional activation and DNA binding domain present in “E” variants of TCF transcription factors (Figures 3-4; (ARCE et al. 2006; HOPPLER and KAVANAGH 2007)). Our analysis suggested that the CRARF domain may also be important for D-Glut4EF function in proper wing positioning since a P element insertion (EY04651) situated upstream of the CRARF domain but downstream of the NLS and the C2H2 zinc finger DNA-binding domain resulted in an outstretched wing phenotype similar to that seen in other stretch alleles (Figures 2A; 3).
Expression of Glut4EFd rescues the wing positioning defect observed in *stretch* mutants: To confirm that disruption of D-Glut4EF was responsible for the wing positioning defect present in *stretch* mutants we sought to express D-Glut4EF in a *stretch* mutant background. Initially, we decided to focus our rescue experiments on the D-Glut4EFd isoform since it was well-conserved to mammalian Glut4EF (Figure 4A) and our P element insertion site mapping revealed that D-Glut4EFd was likely to be disrupted by each of the *stretch* P element mutations (Figure 2B). We therefore restored the expression of D-Glut4EFd in *stretch* mutants using the GAL4-UAS system (Brand and Perrimon 1993) and a GAL4 enhancer trap line (NP7418GAL4 (D-GlutEF-GAL4)) that we identified as being inserted within *D-Glut4EF* (Figures 1D-F, 2A-B). Expressing one copy of the *D-Glut4EFd* cDNA (UAS-D-Glut4EFd) using the *D-Glut4EF-GAL4* driver rescued the wing positioning defect we observed in *stretch* mutants (Figure 1C-D; Table S1). These results reveal that expression of D-Glut4EFd is sufficient to rescue the wing positioning defect present in *stretch* mutants, and uncover an important role for Glut4EF proteins in specifying normal adult morphology.

Mutations in *D-Glut4EF* are present in some mutant lines of the homeobox transcription factor *Antennapedia* (*Antp*): Our results highlight the importance of Glut4EF in normal adult morphology but they also reveal that previously uncharacterized mutations in *stretch* are present in a number of different genetic backgrounds including on the TM3 balancer. Interestingly, two previously characterized dominant gain-of-function (GOF) mutant alleles of the homeobox gene *Antennapedia* (*Antp^B* and *Antp^R*)
exhibit outstretched wing positioning defects (Figure 5B, D) that had previously been attributed to disruptions in *Antp* (Gutiérrez et al. 2003; Vazquez et al. 1999). However, both of these *Antp* mutants (*In(3R)AntpB* and *In(3R)AntpR*) also have chromosomal breakpoints that are in the vicinity of *D-Glut4EF* (85E-F; Duncan and Kaufman 1975; Garber et al. 1983; Kaufman et al. 1980; Lindsley and Zimm 1992; Scott et al. 1983). Strikingly, our genetic complementation and mapping studies indicated that both previously characterized *Antp* mutants (*In(3R)AntpB* and *In(3R)AntpR*) also contained mutations in *D-Glut4EF* (Figures 1D-F; 2A; 5B, D; Figure S1; Table S1). For example, our results with the *In(3R)AntpB* allele were in line with previously published results on the chromosomal breakpoints observed in this mutant (Breakpoints: 84B1-2; 85E; Duncan and Kaufman 1975; Kaufman et al. 1980; Lindsley and Zimm 1992) and indicated that the right chromosomal breakpoint of *In(3R)AntpB* falls to the right of the 85E13 region of the Drosophila third chromosome and disrupts the *stretch* gene (Figures 1F, 2A). In addition, phenotypic and complementation analysis indicated that heterozygous *In(3R)AntpB/+* adults position their wings normally (Figure 5A, D) but exhibit 100% penetrant wing-positioning defects when heterozygous with *D-Glut4EF* mutants (*In(3R)AntpB/stretch<sup>NP7418-GAL4</sup>; *n=57*; Figure 5D; see also Figure 1F and Table S1). In contrast, we find that *Antp* alleles that do not have published breakpoints in the vicinity of *D-Glut4EF* (*Antp<sup>Ns</sup>, *Antp<sup>17</sup>, *Antp<sup>73b</sup>, *Antp<sup>Yu</sup>, *Antp<sup>50</sup>, *Antp<sup>RM</sup>, *Antp<sup>Cix</sup>, *Antp<sup>Pw</sup>*, and the *Antp P2* promoter mutants (*Antp<sup>1</sup> and *Antp<sup>23</sup>*); Lindsley and Zimm 1992) exhibit normal wing positioning in combination with *D-Glut4EF* mutants (Figure S1; Table S1). Lastly, we were able to rescue the wing positioning defects observed in adults heterozygous for both *In(3R)AntpB* and *D-Glut4EF* by expressing D-Glut4EFd under the
Therefore, our results indicate that both of these previously characterized Antp mutants \((\text{In}(3R)\text{Antp}^B \text{ and In}(3R)\text{Antp}^R)\) contain mutations in both \textit{Antp} and \textit{D-Glut4EF} but it is \textit{D-Glut4EF} that is needed for proper positioning of the wings.

\textit{D-Glut4EF modifies the morphogenetic function of Antp:} Dominant \textit{Antp} alleles, like \textit{Antp}^B \text{ and Antp}^R, produce a range of homeotic transformations of anterior regions, most notably of antenna to mesothoracic leg (Reviewed in (KAUFMAN \textit{et al.} 1990; LINDSLEY and ZIMM 1992)). These mutant phenotypes are due to inappropriate expression of \textit{Antp} in the eye-antennal disc (FRISCHER \textit{et al.} 1986; JORGENSEN and GARBER 1987; SCHNEUWLY \textit{et al.} 1987). Since our results indicate that mutations in both \textit{Antp} and \textit{D-Glut4EF} are present in these two previously characterized dominant \textit{Antp} mutants \((\text{In}(3R)\text{Antp}^B \text{ and In}(3R)\text{Antp}^R)\), we wondered if \textit{D-Glut4EF} might be genetically modifying the phenotypes associated with mis-expression of \textit{Antp}. In line with this hypothesis, we found that when we used D-Glut4EFd to rescue the outstretched wing defects present in the \textit{Antp}^B mutant, we also observed an increase in the severity of the antenna defects (Figure 5C). This suggested that D-Glut4EFd might be a positive modifier of \textit{Antp} function. To better address this possibility we examined the expression of \textit{D-Glut4EFd} and found that high levels of the \textit{D-Glut4EFd} transcript were present in the regions that specify the antennae, the eye-antenna disc (Figure 6A-C). To further examine whether D-Glut4EFd genetically modifies \textit{Antp} function we looked at a dominant \textit{Antp} allele that does not contain breakpoints in the vicinity of \textit{D-Glut4EF} \((\text{Antp}^N_s); \text{(LINDSLEY and ZIMM 1992)}\). The \textit{Antp}^N_s allele is a dominant allele in which the
antenna are transformed to legs and the whole head is disrupted (reviewed in (LINDSLEY
and ZIMM 1992); Figure 6D-E, G-H). Removing one copy of the D-Glut4EF gene
dramatically suppressed these severe morphogenetic defects and returned the fly to a
more wild-type appearance (Figure 6F-H). These results are consistent with the model
that a Glut4EF transcription factor family member positively modulates the
morphogenetic function of the homeobox transcription factor Antp.
DISCUSSION

Identifying and characterizing the transcriptional regulators and target genes that pattern the developing organism is a critical component to our understanding of human birth defects and later onset diseases. Here, we identify a new family of transcriptional regulators conserved from Drosophila to humans and find that Glut4EF family transcription factors play an essential role in the specification of a normal fly. In particular, our results reveal that Glut4EF is necessary for proper wing positioning in Drosophila. Future work will be aimed at understanding the mechanisms underlying these wing positioning defects. As an initial step in this direction, we find no abnormalities in somatic muscle formation or nervous system development in Drosophila embryos carrying mutations in D-Glut4EF (unpublished observations). Interestingly, the proper positioning of the wings in insects is known to be controlled by a selective set of muscle groups, the direct flight muscles (DFM; (BATE 1993; HEIDE and GOTZ 1996; MILLER 1950), and future work will explore a role for Glut4EF in the patterning of these muscles.

Importantly, our results provide new insights into the Glut4EF family of transcription factors and are the first demonstration of a role for Glut4EF in vivo. Glut4EF was originally named for its ability to bind to the enhancer of the Glucose Transporter 4 gene, the major glucose regulator in skeletal muscle and principally responsible for insulin-mediated glucose uptake in muscle and adipose tissue (OSHEL et al. 2000). More recently, Glut4EF has been found to associate with the myocyte enhancing factor (MEF2A) to activate transcription of GLUT4 (KNIGHT et al. 2003).
However, the functional significance of these interactions is poorly understood. Likewise, Glut4EF/HDBP1 also regulates expression of the Huntington’s disease (HD) gene in neuronal cells in culture but the in vivo functional significance of this interaction is also not known (TANAKA et al. 2004). Members of the D-Glut4EF family of transcription factors bind to DNA sequences within a number of genes including ACCGG within GLUT4 (KNIGHT et al. 2003; OSHEL et al. 2000), GCCGGCG within the human Huntington’s Disease Gene (TANAKA et al. 2004), and CCGG in the E2 binding site of papillomavirus genomes (BOECKLE et al. 2002). D-Glut4EFd, Glut4EF, PBF, and ZNF704 also contain a CR (CRARF/RKKCIRY) domain at their C-terminus that is highly similar to the transcriptional activation and DNA binding domain present in “E” variants of the wingless/Wnt signaling effectors, TCF transcription factors (ARCE et al. 2006; HOPPLER and KAVANAGH 2007). In TCF, the CRARF domain is necessary for transcriptional activation mediated through both β-Catenin and the CREB binding protein (CBP)/p300 (ATCHA et al. 2003; HECHT and STEMMLER 2003). This activity is important during development but its misregulation leads to overactive Wnt signaling and drives TCFs/lymphoid enhancing factors (LEFs) to transform cells (ARCE et al. 2006; POLAKIS 2007). In both Glut4EF/HDBP1 and PBF/HDBP2, the CR/CRARF domain also plays a role in DNA binding and the regulation of transcription (TANAKA et al. 2004). However, despite the similarity between Glut4EF and TCF, it remains to be determined if Glut4EF and TCF transcription factors function together in any cellular processes. Along these lines, it is interesting that mutations in one of the Drosophila Wnts, D-Wnt-2, gives rise to a “stretch-like” outstretched wing phenotype (KOZOPAS and NUSSE 2002).
Our results also indicate that previously uncharacterized mutations in *Glut4EF* are present in at least eleven publicly available fly lines including within the TM3 balancer chromosome. The TM3 balancer has been used extensively in fly work since its discovery by E.B. Lewis in 1955 (LINDSLEY and ZIMM 1992) and should now include the *stretch* mutation which can serve as a 100% penetrant recessive marker. Interestingly, a progenitor of the TM3 balancer discovered by H.J. Muller (LINDSLEY and ZIMM 1992), *In(3LR)sep*, also has a previously uncharacterized recessive wings-out phenotype and is also a *stretch* allele. Our results also raise the possibility that previous observations utilizing these common stocks may be complicated by the presence of *Glut4EF* mutations. For example, the TM3 balancer (a *D-Glut4EF* allele) genetically enhances defects in the homeodomain transcription factor *mirr* in both egg morphology, epithelial morphogenesis, and expanded gene expression of the Notch signaling regulator fringe (*fng*; (ZHAO *et al.* 2000)). Furthermore, our results indicate that mutations in *Glut4EF* are also present in two dominant gain-of-function *Antp* mutants and underlie defects previously attributed to *Antp* (GUTIERREZ *et al.* 2003; VAZQUEZ *et al.* 1999). Interestingly, a third dominant gain-of-function mutation in *Antp*, *Antp^{LC}* (LINDSLEY and ZIMM 1992), also exhibits a wings-out phenotype, but we have been unable to obtain the line to determine if the outstretched wing phenotype present in the *Antp^{LC}* allele is also due to mutations in *Glut4EF*.

The *Antp* gene has two alternative promoters, P1 and P2, and the dominant gain-of-function antenna-to-leg transformation results from the misregulation of the *Antp* P2 promoter (Reviewed in (KAUFMAN *et al.* 1990; LINDSLEY and ZIMM 1992)). More recently, published work suggested that the outstretched wing phenotype in the
In(3R)Antp\textsuperscript{B} and In(3R)Antp\textsuperscript{R} alleles was also due to a disruption of P2 promoter activity of the Antp gene \cite{Gutierrez2003, Vazquez1999}. It was concluded that reduced expression of Antp, through a decrease in Antp P2 promoter activity in the imaginal wing disc, causes some Antp mutants to extend their wings out from the body \cite{Gutierrez2003, Vazquez1999}. Instead, our results in combination with previously published breakpoint mapping studies \cite{Duncan1975, Garber1983, Kaufman1980, Lindsley1992, Scott1983} indicate that the wing phenotype in these two Antp lines (In(3R)Antp\textsuperscript{B} and In(3R)Antp\textsuperscript{R}) is due to mutations in Glut4EF. Furthermore, we find that Antp mutations that disrupt the Antp P2 promoter (Antp\textsuperscript{1} and Antp\textsuperscript{23}) do not exhibit a wing phenotype in combination with Glut4EF. However, our genetic interaction analysis indicates that Glut4EF genetically interacts with Antp and this may be through regulation of the P2 promoter activity of Antp.

Notably, our results reveal a critical role for a Glut4EF transcription factor family member in Drosophila wing positioning but the molecular mechanisms of action of Glut4EF remain poorly understood. As a step toward this understanding, our genetic results support a role for Glut4EF in modulating the function of the homeobox transcription factor Antp, a well-known regulator of morphology \cite{Lemons2006}. Provocatively, a re-examination of the results observed by others using the In(3R)Antp\textsuperscript{B} mutation may also provide new insights into the molecular mechanisms of Glut4EF action. The stretched out wing phenotype present in the Antp mutant In(3R)Antp\textsuperscript{B} has been used previously in a screen to identify several Antp-interacting genes including components of the SWI/SNF chromatin remodeling complex.
(Gutiérrez et al. 2003; Vázquez et al. 1999). Since our results indicate that the wing phenotype in the In(3R)AntpB mutant is due to defects in D-Glut4EF, work is underway to test the likely possibility that D-Glut4EF is interacting with these genes. It is also intriguing that D-Glut4EF (previously CG11676; see Figure 2B) was recently found in a transcriptional targeting screen to be a target of the Notch signaling regulator/transcriptional repressor Hairy/Enhancer of Split (Bianchi-Frias et al. 2004). Mutations within several regulators of Notch signaling, including deltex, Hairless, and the Notch ligand Delta, are known to result in a “stretch-like” wings-out phenotype (Nagel et al. 2000; Vassin et al. 1987). Taken altogether, our results provide new insights into the importance of Glut4EF family transcription factors in patterning of the developing organism. In light of the possibility that Glut4EF family transcription factors may play roles in type 2 diabetes (McGee and Hargreaves 2006), Huntington’s disease (Tanaka et al. 2004), and in several human cancers (Boeckle et al. 2002; Tsukahara et al. 2004), future work will be directed at further understanding the molecular mechanisms of Glut4EF’s action.
ACKNOWLEDGMENTS

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LITERATURE CITED


CARPENTER, A. T. C., 1994 Excerpt from stocklist II.


**FIGURE LEGENDS**

**Figure 1.** Adult *Drosophila stretched out* (stretch) mutants exhibit abnormal wing positioning. (A) The wings of a fly heterozygous for a *stretch* mutation (*stretch*<sup>NP7418-GAL4/+</sup>) are wild-type in appearance and are both positioned over the dorsal abdomen. 

(B) The wings of a fly homozygous for a mutation in *stretch* (*stretch*<sup>NP7418-GAL4/stretch<sup>TM3 balancer</sup>) are stretched-out from the body in an abnormal position. (C) In a rescue line (*UAS-D-Glut4EFd; stretch<sup>NP7418-GAL4/stretch<sup>TM3 balancer</sup>*, expression of D-Glut4EFd driven by a GAL4 insertion in D-Glut4EF restores normal wing positioning to a *stretch* mutant. 

(D) Percentage of wing positioning defects in adults heterozygous for the *stretch* mutation, 10477 (P[PZ]stretch<sup>10477</sup>, l(3)10477<sup>10477</sup>), and also heterozygous for either wild-type (+; n=159); st, 10477 (n=63); Df(3R)by62 (n=160); Df(3R)by10 (n=70); TM3 balancer (n=148); TM6B balancer (n=100); In(3LR)sep (n=37); A1 (n=34); NP7418 (n=50); EY03156 (n=84); EY04651 (n=59); Df(3R)swp2 (n=133); In(3R)Antp<sup>R</sup> (n=35); or In(3R)Antp<sup>B</sup> (n=27) (for full nomenclature see below). In the D-Glut4EFd rescue experiment, flies homozygous for a mutation in *stretch* (*stretch*<sup>NP7418-GAL4/stretch<sup>TM3 balancer</sup>*) exhibit normal wing positioning when expressing Glut4EFd using the *stretch*<sup>NP7418-GAL4</sup> driver (*UAS-D-Glut4EFd; stretch*<sup>NP7418-GAL4/stretch<sup>TM3 balancer</sup>; n=61*).

Asterisk = a statistically significant difference in the number of *stretch* mutant flies (*stretch*<sup>NP7418-GAL4/stretch<sup>TM3 balancer</sup>) exhibiting out-stretched wings is observed when Glut4EFd is expressed in these *stretch* mutants using the *stretch*<sup>NP7418-GAL4</sup> driver (*UAS-D-Glut4EFd; stretch*<sup>NP7418-GAL4/stretch<sup>TM3 balancer</sup>; n=61*) (Chi-Square Test; p<0.0001; Degrees of Freedom=1; see also Table S1). (E-F) Genetic complementation and
molecular analysis was used to identify a number of different *stretch* alleles. +, viable; −, lethal; wing, wing phenotype was exhibited in all of the resultant flies; * = not completely penetrant (>50%); N/D, not determined. See also Table S1. Interestingly, in line with previously published chromosomal breakpoints for the *In(3R)Antp* mutant (breakpoints 84B1-2; 85E), we find that the *In(3R)Antp* allele is viable over *Df(3R)by10* (deleted region: 85D8-85E13) while it is lethal over *Df(3R)by62* (breakpoints 85D10-11; 85F1-8). These data position the right breakpoint of *In(3R)Antp* to the right of 85E13 (see also Figure 2A). Furthermore, the *In(3R)Antp* allele is viable over *Df(3R)swp2* (a deficiency that removes *stretch* and several adjacent genes to the right of *stretch*; see also Figure 2A) but gives a wing phenotype in combination with *Df(3R)swp2*. Therefore, these results and the others from the complementation matrix indicate that the *stretch* gene is disrupted in the *In(3R)Antp* fly line. These results also suggest that *In(3R)Antp* may disrupt an adjacent gene(s) to the left of *stretch* since it is lethal when in combination with *Df(3R)by62* but viable when in combination with *Df(3R)swp2* (see also Figure 2A). The new nomenclature for these *stretch* mutant lines are as follows: 10477 (*P[PZ]*stretch**10477**, l(3)10477**10477**); st, 10477 (st, *P[PZ]*stretch**10477**); A1 (*P[Dpse82]*stretch**A1**); NP7418 (*P[GawB]*stretch**NP7418-GAL4**); EY03156 (*P[EPgy2]*stretch**EY03156**); EY04651 (*P[EPgy2]*stretch**EY04651**); TM3 balancer (*In(3LR)TM3, knir* **p** *sep* **l** stretch**TM3 balancer** l(3)89Aa1 Ubx**bx-34e** e1); In(3LR)sep (*In(3LR)sep, vvlsep knir* **p** *sep* **l** stretch**sep**); *In(3R)Antp* (*In(3R) Antp*, *Antp* stretch**Antp-R**); and *In(3R)Antp* (*In(3R)Antp*, *Antp* stretch**Antp-B**).
Figure 2. **Stretch mutations disrupt a large novel Drosophila gene that codes for at least 4 overlapping transcripts.** (A) Genetic organization of the *D-Glut4EF* locus. The large *D-Glut4EF* locus covers ~115kb of DNA and is adjacent to the *MICAL* locus which covers ~40kb of DNA. The position of several P elements and deficiencies (solid lines) are indicated based on our complementation analysis, mapping, and molecular data (see Figure 1F, Figure S1; *Df(3R)GB104* (deleted region: 85D12-85E10); *Df(3R)by10* (deleted region: 85D8-85E13); *Df(3R)by62* (breakpoints 85D10-11; 85F1-8); *Df(3R)swp2* (TERMAN et al. 2002)). Dashed lines indicate that the end points of deficiencies are not molecularly defined. Sizes are in kilobases (kb); non-continuous sequence is indicated by “//”.

(B) The *D-Glut4EF* genomic locus (black). cDNAs encoding four splice variants were identified that contained overlapping exons and were named *D-Glut4EFa*, *D-Glut4EFb*, *D-Glut4EFc*, and *D-Glut4EFd*. The position of several P elements are indicated. Flies containing the 5’ most P elements (EP3681 and BG02024) do not show a wing positioning defect (Table S1, Figure S1; data not shown). Flies containing any of the other P elements (10477, EY03156, NP7418\textsuperscript{GAL4}, or EY04651) exhibit a wing positioning defect. This single gene had been previously annotated as CG12418, CG12802, CG11676, and CG32469. CG11676 and CG32469 have now been combined and annotated into CG33975 (which we find to be *D-Glut4EFd*). (C) D-Glut4EF protein splice variants. cDNAs containing four different splice variants of D-Glut4EF were isolated. Each contained overlapping exons coding for a region of the protein (black region). Three of the splice variants contained the gray region. Each splice variant had a unique C-terminal tail. The nuclear localization signal (NLS) was mostly intact in isoforms b, c, and d. Only the d isoform contains the Zinc Finger C2H2 region and the CRARF/CR domain. Interestingly, a stretch of amino acids in the N terminus of
isoforms a and b (brackets) showed similarity to human Glut4EF orthologues (PBF (HDBP2)) suggesting similar isoforms may also exist in mammalian Glut4EF family members.

**Figure 3. Sequence of the D-Glut4EFd isoform.** The DNA sequence of the *D-Glut4EFd* isoform with the amino acid sequence shown. The nuclear localization signal (NLS) is underlined, the Zinc Finger C2H2 domain is indicated with a dashed line, and the CRARF/CR domain is indicated with a double underline. The insertion position of the EY04651 P element (the *stretch* allele *P*/EPgy2/stretch^{EY04651}) in relation to the amino acid sequence is indicated (black triangle).

**Figure 4. The Glut4EF Family of Transcription Factors.** (A) The GLUT4 Enhancer Factor (Glut4EF) family of proteins. Amino acid identities are indicated among human members and Drosophila D-Glut4EFd (percents within domains). Our analysis of the database revealed a third human family member (EST ZNF704). (B) All Glut4EF proteins contain a highly conserved C-terminal CRARF/CR domain that is known to be important for transcriptional activation. An alignment of the CRARF/CR domain’s present within Glut4EF family members and TCF/LEF HMG box transcription factors. Consensus is indicated.

**Figure 5. Mutations in D-Glut4EF are also present in some mutant stocks of the homeobox transcription factor Antennapedia (Antp).** (A) Adult flies heterozygous for the *Antennapedia* mutation, In(3R)Antp^{B}, have normal wing positioning. (B) Adults flies heterozygous for In(3R)Antp^{B} and *D-Glut4EF* (In(3R)Antp^{B}/stretch^{NP7418-GAL4}) exhibit an 100% defect in wing positioning. (C) The wing positioning defects observed in adult
flies heterozygous for both \textit{In(3R)Antp}^{B} and \textit{D-Glut4EF} (\textit{In(3R)Antp}^{B}/\textit{stretch}^{NP7418-GAL4}) are rescued by expressing \textit{UAS-D-Glut4EFd} using the \textit{D-Glut4EF-GAL4} driver (\textit{UAS-D-Glut4EF}; \textit{In(3R)Antp}^{B}/\textit{stretch}^{NP7418-GAL4}). \textbf{(D)} Percentage of wing positioning defects and transformed antennae in adults heterozygous for \textit{In(3R)Antp}^{B} (\textit{In(3R)Antp}^{B}/+; \textit{n}=80), adults heterozygous for both \textit{In(3R)Antp}^{B} and \textit{D-Glut4EF} (\textit{In(3R)Antp}^{B}/\textit{stretch}^{NP7418-GAL4}; \textit{n}=57), or adults heterozygous for both \textit{In(3R)Antp}^{B} and \textit{D-Glut4EF} and also expressing \textit{D-Glut4EFd} using the \textit{D-Glut4EF-GAL4} driver (\textit{UAS-D-Glut4EFd}; \textit{In(3R)Antp}^{B}/\textit{stretch}^{NP7418-GAL4}; \textit{n}=73). Note that expression of \textit{D-Glut4EFd} rescues the wing positioning defects but not the antenna transformation defects in \textit{In(3R)Antp}^{B} mutants. Asterisk $=$ a statistically significant difference in the number of flies heterozygous for both \textit{In(3R)Antp}^{B} and \textit{D-Glut4EF} (\textit{In(3R)Antp}^{B}/\textit{stretch}^{NP7418-GAL4}) that exhibit an out-stretched wing phenotype is observed when Glut4EFd is expressed in these \textit{In(3R)Antp}^{B}/\textit{stretch}^{NP7418-GAL4} mutants using the \textit{stretch}^{NP7418-GAL4} driver (\textit{UAS-D-Glut4EF}; \textit{In(3R)Antp}^{B}/\textit{stretch}^{NP7418-GAL4}; \textit{n}=61) (Chi-Square Test; \textit{p}<0.0001; Degrees of Freedom=1). A new, more-complete nomenclature should now be adopted for both the \textit{In(3R)Antp}^{R} and the \textit{In(3R)Antp}^{B} mutants to indicate the presence of mutations in both \textit{Antp} and \textit{D-Glut4EF}: \textit{In(3R)Antp}^{R} (\textit{In(3R)Antp}^{R}, \textit{Antp}^{R} \textit{stretch}^{Antp-R}); and \textit{In(3R)Antp}^{B} (\textit{In(3R)Antp}^{B}, \textit{Antp}^{B} \textit{stretch}^{Antp-B}).

\textbf{Figure 6.} \textit{D-Glut4EF modifies the morphogenetic function of Antp.} (A) Drawing of the Drosophila eye and antenna disc. (B-C) Drosophila eye and antenna disc following in situ hybridization with anti-sense (B) and sense (C) probes to \textit{D-Glut4EFd}. Note that
high levels of expression of *D-GLut4EFd* are seen in the eye and antenna disc. (D) The eyes and antennae (arrows) in a wild-type (+/+) adult fly. (E) The *Antp<sup>Ns</sup>* allele is a dominant allele in which the antennae are transformed to legs and the head is disrupted. Abnormal eyes and antennae are present in an *Antp<sup>Ns</sup>* heterozygote adult. Note the leg-like protrusions emanating from the antennae (arrows) and smaller eyes in these flies. (F) Removing one copy of the *D-GLut4EF* gene dramatically suppresses this severe defect and returns the fly to a more wild-type appearance. Normal appearing eyes and antennae (arrows) are present when *Antp<sup>Ns</sup>* heterozygotes are also heterozygous for *D-GLut4EF* (*Antp<sup>Ns</sup>/stretch<sup>Df[3R]swp2</sup>). (G-H) The percentage (%) of adult flies heterozygous for either *Antp<sup>Ns</sup>* (*Antp<sup>Ns</sup>/+) or heterozygous for both *Antp<sup>Ns</sup>* and *stretch* (*Antp<sup>Ns</sup>/stretch<sup>Df[3R]swp2</sup>) in which either two (G) or one (H) antenna were abnormal in appearance. Asterisk in G-H = statistically significant differences between the two groups (Chi-Square Test; p<0.0001; Degrees of freedom=1). Scale bar in B applies to C (25µm).
**stretch** Alleles & Deficiencies

- stretch\textsuperscript{10477}
- Df(3R)by62
- stretch\textsuperscript{TM3 balancer}
- stretch\textsuperscript{sep}
- stretch\textsuperscript{A1}
- stretch\textsuperscript{NP7418-GAL4}
- stretch\textsuperscript{EY03156}
- stretch\textsuperscript{EY04651}
- Df(3R)swp2
- stretch\textsuperscript{Antp-R}
- stretch\textsuperscript{Antp-B}

**Genetic Complementation Analysis to Identify Different stretch Alleles**

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Figure 1 (Yazdani et al. (Terman))
Figure 2 (Yazdani et al (Terman))
**A** Glut4EF Family of Proteins

- **D-Glut4EFd** (521aa)
- **H-Glut4EF** (387aa)
- **H-PBF** (513aa)
- **H-ZNF704** (412aa)

**B** CRARF/CR Domain Containing Proteins

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Figure 5 (Yazdani et al (Terman))
Figure 6 (Yazdani et al (Terman))