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Genetic Mapping and Phenotypic Characterization of the *Drosophila AIF^{e04281}* Allele Reveals Mutant Clone Loss in Mosaic Eyes

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Abstract

As part of the Fly-CURE consortium, a mutant allele of *Apoptosis inducing factor* (*AIF*) was characterized using complementation mapping, genomic sequencing, and mosaic phenotypic analysis to investigate its role in cell growth control in *Drosophila melanogaster*. The *AIF^{e04281}* mutation dramatically reduced homozygous mutant clone size and caused morphological defects in genetically mosaic eyes. Sequencing confirmed a transposon insertion that truncates the AIF protein preceding conserved domains essential for mitochondrial function and apoptosis. The observed clone loss indicates a cell-autonomous requirement for *AIF* and supports the use of *AIF^{e04281}* as a loss-of-function background for genetic modifier screens on chromosome arm 2L.

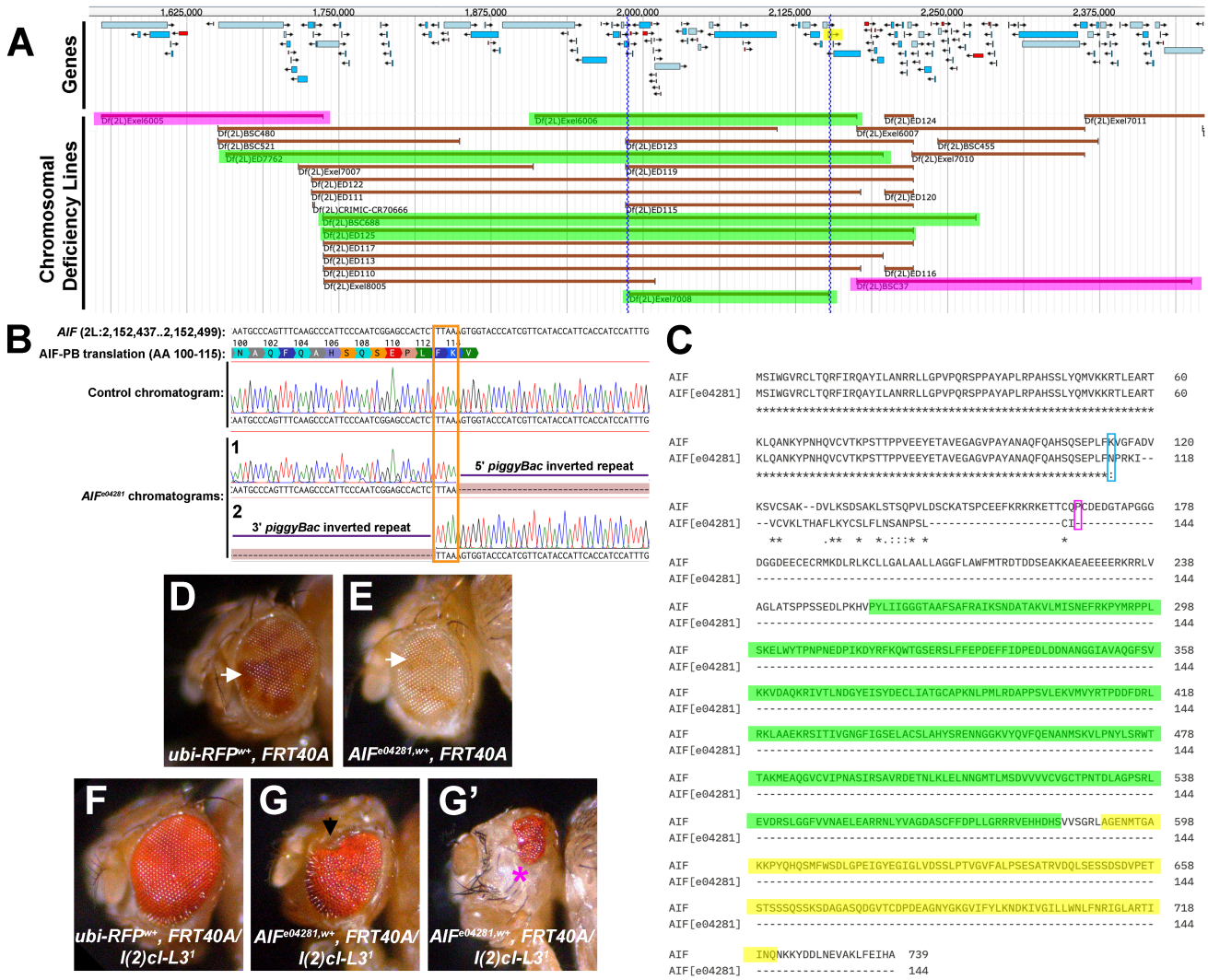

Figure 1. Molecular and phenotypic characterization of the *AIF*^{e04281} mutation in *Drosophila melanogaster*:

(A) Deficiency mapping localized the *AIF*^{e04281} mutation to a ~163 kb interval on chromosome arm 2L (2L:1,989,057..2,152,458, area between blue vertical lines), defined by overlapping deficiencies that failed to complement the mutant allele (green). Flanking complementing deficiencies (magenta) and *AIF* (yellow) are indicated. Adapted from FlyBase's JBrowse (*D. melanogaster* r6.62) (Öztürk-Çolak et al. 2024). (B) *ubi-RFP*$^{w+}$, *FRT40A* (control) and *AIF*^{e04281}, *FRT40A* (mutant) Sanger sequencing chromatograms aligned to the *AIF* genomic region (2L:2,152,437..2,152,499) and translation, corresponding to amino acids (AA) 100-115 of exon 2 of isoform AIF-PB. Two independent *AIF*^{e04281} chromatograms are shown, each using a different sequencing primer on opposing sides of the insertion. *AIF*^{e04281} sequence 1, generated from a primer 5' of the insertion, includes the 5' *piggyBac* inverted repeat, while *AIF*^{e04281} sequence 2, generated from a primer 3' of the insertion, includes the 3' *piggyBac* inverted repeat (purple lines). The TTAA insertion sequence is indicated (orange box). Adapted from Benchling DNA alignment results (Benchling [Biology Software]). (C) Clustal Omega Multiple Sequence Alignment (MSA) of wildtype and mutant AIF protein sequences revealed the location of the insertion after amino acid 114 (blue box), which introduces a frameshift and truncates the AIF protein (magenta box) (Sievers et al. 2011). The resulting mutant protein lacks conserved FAD/NAD(P)H-binding (green) and AIF_C (yellow) domains. (D-G) Mitotic recombination was induced in the developing eye using the FLP-FRT system to generate genetically mosaic tissue and assess the *AIF*^{e04281} phenotype relative to controls. Eyes are oriented with anterior to the left and dorsal at the top. (D) Control eyes (genotype: *w-, ey>FLP/Y; ubi-RFP*$^{w+}$, *FRT40A/FRT40A*) exhibited a higher proportion of red-pigmented tissue (white arrow; mean of 70.7% red to 29.3% white, n=40), indicating survival of homozygous *ubi-RFP*$^{w+}$ clones. (E) Eyes from *AIF*^{e04281} mutants (genotype: *w-, ey>FLP/Y; AIF*$^{e04281,w+}$, *FRT40A/FRT40A*) showed a marked reduction in red-pigmented mutant tissue (white arrow; mean of 14.7% red to 85.3% white, n=40), consistent with loss or underrepresentation of mutant clones. (F-G) A homozygous cell lethal allele (*l(2)cl-L3*^{1}) was used to eliminate homozygous wildtype clones. (F) Representative control eye (genotype: *w-, ey>FLP/Y; ubi-RFP*$^{w+}$, *FRT40A/l(2)cl-L3*^{1}, *FRT40A*) exhibiting normal morphology. (G-G') Representative *AIF*^{e04281} mutants in the

l(2)cl-L3^{1} background (genotype: *w-*, *ey>FLP/Y;* *AIF*$^{e04281,w^{+}}$, *FRT40A/l(2)cl-L3*^{1}, *FRT40A*) displaying smaller, irregularly shaped eyes with visible tissue defects. (G) *AIF*^{e04281} eye exhibiting ommatidial disorganization and dorsal tissue loss (black arrow) in the absence of homozygous wildtype tissue. (G') Small *AIF*^{e04281} eye displaying strong morphological defects, including tissue overgrowth and ectopic bristles in the interocular space (magenta asterisk) when homozygous wildtype tissue is eliminated.

Description

The Fly-CURE is a multi-institutional Course-Based Undergraduate Research Experience (CURE) that provides undergraduate students with hands-on experience mapping and characterizing novel mutants in *Drosophila melanogaster* (Merkle et al. 2023). The Fly-CURE aims to identify and study genes involved in cell growth regulation to better understand human disorders associated with abnormal cell proliferation (Neufeld and Hariharan 2002; Merkle et al. 2023; Chammout et al. 2024; Gruber et al. 2025; Patterson et al. 2025). In a previous forward genetic screen in *Drosophila*, mutant lines exhibiting defects in cell growth were identified in a background containing a mutation in *Death-associated APAF1-related killer* (*Dark*), a gene required for canonical apoptosis (Rodriguez et al. 1999; Mills et al. 2006; Kagey et al. 2012). When *Dark* is disrupted, cells defective in proper cell growth regulation evade apoptosis, allowing aberrant growth or proliferation phenotypes to be visualized in the developing *Drosophila* eye (Kagey et al. 2012). Following EMS mutagenesis in the *Dark* mutant background (allele *Dark*^{82}), mitotic recombination was induced on the right arm of chromosome 2 (2R) using the FLP-FRT system to identify mutant clones with aberrant growth phenotypes (Akdemir et al. 2006; Kagey et al. 2012; Weasner et al. 2017). Students in the Fly-CURE have characterized and mapped these mutants, resulting in 17 publications (Cosenza and Kagey 2016; Bieser et al. 2018; Bieser et al. 2019; Stamm et al. 2019; Siders et al. 2021; Talley et al. 2021; Vrailas-Mortimer et al. 2021; Evans et al. 2022; Mast et al. 2022; Moore et al. 2022; Cordes et al. 2023; Nowaskie et al. 2023; Chammout et al. 2024; Johnson et al. 2024; Thomson et al. 2024; Gruber et al. 2025; Patterson et al. 2025). To extend this approach to the left arm of chromosome 2 (2L), a mutant allele of *Apoptosis inducing factor* (*AIF*; allele *AIF*^{e04281}) was selected as the starting point for a new forward genetic screen (Bellen et al. 2004; Thibault et al. 2004). Because future modifier screens will rely on the *AIF*^{e04281} background to uncover mutant lines that alter eye development and cell growth, thorough molecular and phenotypic characterization of the *AIF*^{e04281} allele is essential. This study establishes that foundation and provides a critical reference point for identifying genetic enhancers and suppressors of *AIF*-dependent phenotypes.

The *AIF*^{e04281} mutation provides a foundation for studying the genetic regulation of apoptosis and tissue growth. This homozygous lethal allele results from a *piggyBac* (*PBac*) transposon insertion that disrupts the *AIF* gene (Häcker et al. 2003; Bellen et al. 2004; Thibault et al. 2004), the predicted *Drosophila* ortholog of mouse *AIF* and human *AIFM1*, a conserved mitochondrial flavoprotein critical for energy metabolism and induction of caspase-independent apoptosis (Susin et al. 1999; Joza et al. 2001; Joza et al. 2008; Joza et al. 2009). Studying *AIF* function in *Drosophila* can provide valuable insights into the cellular mechanisms underlying human disorders associated with *AIFM1* mutations, including Cowchock syndrome and other mitochondrial dysfunction syndromes (Rinaldi et al. 2012; Bano and Prehn 2018; Heimer et al. 2018; Nguyen et al. 2025).

To validate the genomic location of the *piggyBac* insertion in *AIF* and to establish a baseline for mapping future alleles generated in the *AIF*^{e04281} mutant background, complementation testing was conducted. Virgin females heterozygous for *AIF*^{e04281} were crossed with heterozygous males from a collection of overlapping chromosomal deficiency lines spanning chromosome 2L (Ryder et al. 2007; Cook et al. 2012). Since the *AIF* mutation and deficiency chromosomes are homozygous lethal and maintained with balancer chromosomes containing a dominant phenotypic marker that causes curly wings in adults, failure to complement results were evidenced by the absence of straight-winged progeny. Since some 2L deficiency lines did not exhibit the expected curly-wing phenotype attributed to the balancer chromosome, the collection requires re-balancing before mutants from a forward genetic modifier screen can be mapped.

Initial mapping showed that *Df(2L)BSC688* failed to complement *AIF*^{e04281}, while flanking lines *Df(2L)Exel6005* and *Df(2L)BSC37* complemented *AIF*^{e04281}, narrowing the mutation to nucleotides 1,737,249 to 2,175,620 on chromosome 2L (Table 1 and Figure 1A). Additional deficiencies within this interval, including *Df(2L)ED125*, *Df(2L)ED7762*, and *Df(2L)Exel6006*, also failed to complement *AIF*^{e04281} (Table 1 and Figure 1A). The smallest non-complementing deficiency, *Df(2L)Exel7008* (nucleotides 1,989,057 to 2,152,458), defined an interval containing part of the *AIF* gene (nucleotides 2,151,754 to 2,155,389), confirming the location of the mutation that causes homozygous lethality of the *AIF*^{e04281} allele (Table 1 and Figure 1A). Complementation testing with an independent allele, *AIF*^{GE14994}, also failed to complement *AIF*^{e04281}, reinforcing that the lethal phenotype results from disruption of *AIF* and not a nearby locus (Table 1).

To validate the precise location of the *piggyBac* insertion associated with *AIF*^{e04281}, genomic DNA from heterozygous *AIF*^{e04281} mutant and *ubi-RFP*$^{w+}$ control flies was extracted and subjected to PCR and Sanger sequencing. The insertion was reported to start at genomic position 2L:2,152,458 in exon 2 of mRNA transcripts *AIF-RB* and *AIF-RC* (Thibault et al. 2004; Öztürk-Çolak et al. 2024). Three primers were designed per student group: two to amplify the native genomic region flanking the insertion, and another targeting within the *piggyBac* insertion sequence and extending to the native *AIF* gene.

Gel electrophoresis results validated the general reported position of the insertion, and DNA sequencing from four independent sets of PCRs validated the position of the insertion at nucleotide 2,152,458 in *AIF*^{e04281} mutant DNA at the predicted *PBac{RB}* TTAA insertional target sequence (Figure 1B, orange box) (Cary et al. 1989; Häcker et al. 2003; Thibault et al. 2004). The genomic sequence flanking this position aligned between the control and *AIF*^{e04281} sequence reads, while the remainder of the sequence aligned with *piggyBac* inverted repeats (Figure 1B, purple lines). This insertion introduces a frameshift in exon 2 starting at amino acid 114, leading to a premature stop codon that truncates over 80% of the protein sequence (Figure 1C). The mutant AIF protein lacks conserved domains required for mitochondrial redox function and apoptosis, such as a mitochondrial Apoptosis-inducing factor C-terminal (AIF_C) dimerization domain and an FAD/NAD(P)H-binding (NirB) domain (Figure 1C) (Maté et al. 2002; Joza et al. 2008; Blum et al. 2025; Nguyen et al. 2025).

Mitotic recombination, mediated by the FLP-FRT genetic system, was used to assess the *AIF*^{e04281} phenotype in the adult *Drosophila* eye. In this system, flippase (FLP) drives mitotic recombination at flippase recognition target (FRT) sites near the centromere on chromosome 2L (*FRT40A*). Additionally, FLP activity is driven by enhancers of the eye-specific gene *eyeless* (*ey>FLP*) during development. Since the *AIF*^{e04281} *piggyBac* carries a *mini-white* cassette (w^{+mC}), clones produced by mitotic recombination are genetically distinguished by differences in eye pigmentation: the insertion yields red pigment in *AIF* homozygous mutant and heterozygous clones, while homozygous wildtype cells lacking w^+ appear white. In control mosaic eyes, the average eye composition was 70.7% red tissue (homozygous or heterozygous for *ubi-RFP*$^{w+}$) and 29.3% white tissue (Figure 1D, n=40). In *AIF* mosaic eyes, however, red *AIF*^{e04281} mutant tissue was significantly reduced (mean=14.7%), resulting in an overabundance of homozygous wildtype tissue (mean 85.3%) (Figure 1E, n=40). These results indicate a strong growth disadvantage or loss of *AIF* mutant cells in *AIF*^{e04281} mosaic eyes, suggesting a requirement for *AIF* in autonomous cell survival.

To investigate the role of *AIF* in cell proliferation and tissue organization, a cell lethal allele (*l(2)cl-L3*^{1}) was introduced on the non-mutant chromosome to eliminate homozygous wild-type clones. After mitotic recombination, adult eyes consisted of only homozygous mutant and heterozygous cells. In *ubi-RFP*$^{w+}$ controls, adult eyes exhibited a normal morphology (Figure 1F). However, *AIF*^{e04281} mosaic eyes often appeared misshapen and reduced in size, with variability in the presence and severity of morphological defects (Figure 1G,G'). These results indicate that the *AIF*^{e04281} mutation leads to loss or underproliferation of mutant cells, even in the absence of wild-type competition, and that *AIF* function is critical for eye development and tissue integrity. Morphological defects included misaligned ommatidia, irregular bristle placement, and uneven interocular spacing, further supporting *AIF*'s developmental role (Figure 1G,G').

In eukaryotes, loss of *AIF* function impairs mitochondrial apoptosis and may activate compensatory nuclear apoptosis pathways (Joza et al. 2008; Joza et al. 2009; Bano and Prehn 2018; Nguyen et al. 2025). The reduced presence of *AIF*^{e04281} mutant clones, along with their morphological abnormalities, reflects a cell-autonomous requirement for *AIF* in maintaining tissue viability. Interestingly, *AIF*^{e04281} mutant eyes often retained size and shape when wildtype clones were present, suggesting a compensatory proliferative response by adjacent wildtype cells. This supports a non-cell-autonomous mechanism of tissue homeostasis during eye development, wherein surrounding cells respond to clone loss (Bergmann 2025).

Because the *AIF* gene shares homology with human *AIFM1*, which regulates caspase-independent apoptosis, also called parthanatos, the *Drosophila AIF*^{e04281} allele provides a tractable model to study conserved mitochondrial and nuclear apoptotic pathways and their relevance to human disease (Susin et al. 1999; Fatokun et al. 2014). Disorders linked to *AIFM1* mutations include Cowchock syndrome, X-linked deafness-5, spondylometaphyseal dysplasia, and early-onset sensorimotor neuropathies (Rinaldi et al. 2012; Bano and Prehn 2018; Heimer et al. 2018; Nguyen et al. 2025). These pathologies involve impaired mitochondrial function and cell death regulation, consistent with phenotypes observed in *AIF*^{e04281} mutant flies (Figure 1D,G,G') (Joza et al. 2008).

Altogether, the *AIF*^{e04281} allele constitutes a lethal loss-of-function mutation in *Drosophila* and reveals critical roles for *AIF* in apoptosis, tissue homeostasis, and eye development. Its effects on clone survival and tissue morphology make it a valuable background for genetic modifier screens on chromosome 2L. Identifying enhancers or suppressors of the *AIF*^{e04281} mutant phenotype may uncover new regulators of apoptosis and growth control pathways conserved across

species. These findings reinforce *Drosophila melanogaster* as a powerful model for studying the molecular mechanisms of cell growth control and their disruption in human disease.

Table 1. Complementation analysis between *AIF^{e04281}* and chromosome 2L deficiency lines or an independent *AIF* allele. Complementation testing was performed between *AIF^{e04281}* and overlapping chromosomal deficiencies on 2L. Initial results narrowed the candidate region to 2L:1,737,249..2,175,620. Additional deficiency lines within this interval also failed to complement, defining the smallest non-complementing region as 2L:1,989,057..2,152,458. A known mutant allele of *AIF* also failed to complement *AIF^{e04281}*, confirming disruption of the *AIF* gene.

Bloomington *Drosophila* Stock Center (BDSC) 2L Deficiency Kit			
Deficiency	**BDSC Stock #**	**Chromosomal Region**	**Complementation Result**
Df(2L)BSC688	26540	2L:1,736,964..2,273,572	Fail to complement
Df(2L)Exel6005	7492	2L:1,555,098..1,737,249	Complement
Df(2L)BSC37	7144	2L:2,175,620..2,450,829	Complement
Additional Deficiency Lines			
Deficiency	**BDSC Stock #**	**Chromosomal Region**	**Complementation Result**
Df(2L)Exel7008	7780	2L:1,989,057..2,152,458	Fail to complement
Df(2L)Exel6006	8000	2L:1,911,627..2,175,599	Fail to complement
Df(2L)ED7762	24119	2L:1,657,408..2,197,121	Fail to complement
Df(2L)ED125	24120	2L:1,737,465..2,222,091	Fail to complement
Single Gene Allele			
Genotype	**BDSC Stock #**	**Gene Affected**	**Complementation Result**
AIF^{GE14994}/CyO	26887	*AIF*	Fail to complement

Reagents

w-; PBac{w^{+mC}=RB}AIF^{e04281}, FRT40A/CyO (this study; generated from RRID:BDSC_18244)

Bloomington *Drosophila* Stock Center 2L Deficiency Kit (Cook et al. 2012)

w-, ey>Flp; FRT40A (RRID:BDSC_5615)

w-, ey>FLP; l(2)cl-L3^{1}, FRT40A/CyO (RRID:BDSC_5622)

w-; P{w^{+mC}=Ubi-mRFP.nls}2L, FRT40A/CyO (RRID:BDSC_34500)

w^{1118}; Df(2L)Exel7008/CyO (RRID:BDSC_7780)

w^{1118}; Df(2L)Exel6006, P{w^{+mC}=XP-U}Exel6006/CyO (RRID:BDSC_8000)

w^{1118}; Df(2L)ED7762, P{w$^{+mW.Scer\FRT.hs3}$=3'.RS5+3.3'}ED7762/SM6a (RRID:BDSC_24119)

w^{1118}; Df(2L)ED125, P{w$^{+mW.Scer\FRT.hs3}$=3'.RS5+3.3'}ED125/SM6a (RRID:BDSC_24120)

w-; P{w^{+mC}=EP}AIF^{GE14994}/CyO (RRID:BDSC_26887)

Forward primer 1 (*AIF*): 5’ GTC GAT TTC AGC TCC TCT TC 3’

Reverse primer 1 (*AIF*): 5’ TGT CCG ACT TTA ACA CAT CC 3’

Reverse primer 2 (*PBac*): 5’ GTA TCG CTC TGG ACG TCA TC 3’

Forward primer 2 (*PBac*): 5’ CCT CGA TAT ACA GAC CGA TAA AAC AC 3’

Reverse primer 3 (*AIF*): 5’ TAG TCG CTT TGC AGG AAT CCA 3’

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