Journal of Medical Entomology, XX(XX), 2023, 1–8
https://doi.org/10.1093/jme/tjad059
Research

Neurobiology, Physiology, Biochemistry

Suppression of the gene encoding PDZ domain-containing protein decreases cold tolerance and overwintering survival of the mosquito, *Culex pipiens* (Culicidae: Diptera)

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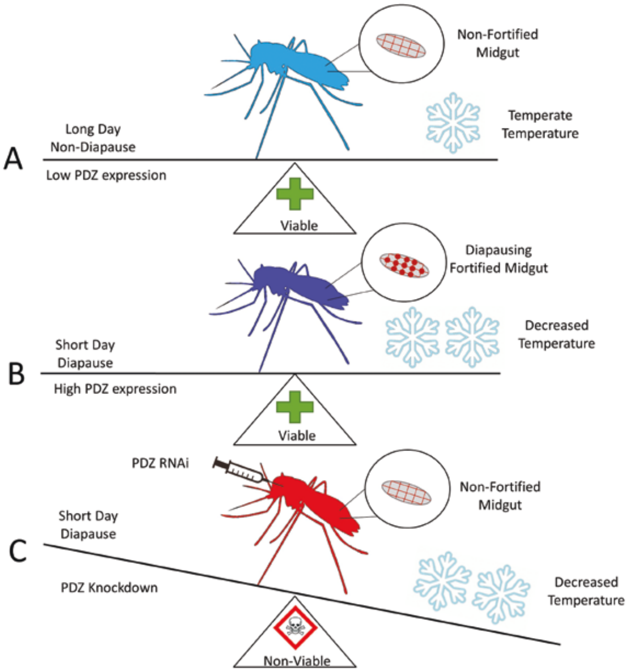
Subject Editor: Julian Hillyer

Received on 4 October 2022; revised on 14 April 2023; accepted on 9 May 2023

In diapausing mosquitoes, cold tolerance and prolonged lifespan are important features that are crucial for overwintering success. In the mosquito *Culex pipiens*, we suggest that PDZ domain-containing protein (PDZ) (post synaptic density protein [PSD95], drosophila disc large tumor suppressor [Dlg1], and zonula occludens-1 protein [zo-1]) domain-containing protein is involved with these diapause features for overwintering survival in *Culex* mosquitoes. The expression level of *pdz* was significantly higher in diapausing adult females in the early stage in comparison to their nondiapausing counterparts. Suppression of the gene that encodes PDZ by RNA interference significantly decreased actin accumulation in the midgut of early-stage adult diapausing females. Inhibition of *pdz* also significantly reduced the survivability of diapausing females which indicates that this protein could play a key role in preserving the midgut tissues during early diapause.

Key words: Culex pipiens, diapause, pdz, cytoskeleton, RNAi

Graphical Abstract



Introduction

Diapause allows mosquitos to survive the harsh winter season. In preparation for diapause, insects often increase cold tolerance and reduce the water content of digestive organs, which are prone to tissue breakdown by freezing, or strengthen the structure of the cytoskeleton (Denlinger 1991). The midgut is a major digestive organ and a vulnerable tissue in cold temperatures, and preserving it is critical for overwintering survival as well as blood-feeding, food intake, and digestion once normal development resumes in the spring. Thus, the preservation of midgut tissue mostly through actin accumulation is an important factor in cold tolerance and overwintering survival. Currently, genetic manipulation through the rapidly advanced CRISPR/Cas9 technology is actively being studied in *Culex* mosquitoes (Feng et al. 2021). If we understand which genes play integral roles in the winter survival of the mosquito *Cx. pipiens*, it is expected that this method can be applied to population control that stops the overwintering diapause program.

Recent studies on the molecular regulation of diapause in *Cx. pipiens* also show the importance of insulin and forkhead transcription factor (FOXO) as crucial elements in the signaling pathway that leads to the diapause phenotype. In short, the insulin signaling pathway is not activated in diapausing adult females, therefore lifting the suppression of FOXO. Upon this expression of FOXO, the induction of target genes associated with diapause, including enhanced stress tolerance, accumulation of fat reserves, and extended lifespan are then observed (Sim et al. 2009, 2011, 2015). Among these characteristics of diapause, increased resistance to cold stress is essential for survival in the cold winter season (Denlinger 2002), and several genes encoding stress-related proteins were revealed in the ChIP-seq analysis as being under the control of FOXO. A candidate gene encoding PDZ and LIM domain protein 2 was identified from our ChIP-seq analysis; this protein is an adapter protein that is located on the actin cytoskeleton, where it promotes cell attachment and controls muscle development (Sim et al. 2015).

PDZ domain-containing protein (PDZ) (post synaptic density protein [PSD95], drosophila disc large tumor suppressor [Dlg1], and zonula occludens-1 protein [zo-1]) domains are structurally conserved 80- to 100-aa modules present in a wide range of proteins either singly or tandemly (Nadelstadh et al. 2009). PDZ-LIM proteins act as adapters recruiting signaling molecules to the actin cytoskeleton. This is based on the ability of some PDZ-LIM proteins to associate with the actin cytoskeleton via their PDZ domain (Vallenius et al. 2000, 2004). Zasp52 biochemically interacts with α-actinin via an extended PDZ domain, and the PDZ domain is required for myofibril assembly (Liao et al. 2020). In most cases, the PDZ domains recognize very short C-terminal sequence motifs by specific side chain interactions between the amino acids of the ligand and those of the hydrophobic pocket formed between a beta B strand and an alpha B helix at the surface of the PDZ domain (Nadelstadh et al. 2009). Molecular studies in Drosophila have shown that a number of PDZ domain proteins are localized to adheren junctions. PDZ domains are protein–protein association domains that can interact with short carboxyl-terminal peptide motifs or with other PDZ domains (Liu and Lengyel 2000, Short et al. 1998). Studies done in *Drosophila* with RNA interference (RNAi) to knock down PDZ function show that when PDZ is knocked down, boarder cells did not migrate, follicle cell layers were thinner than controls, and nuclei were misaligned (Aranjuez et al. 2012). Another study with *Drosophila* shows when PDZ is removed from *Zasp67*, which is a Z-band PDZ motif protein, flies show a flightless and jumpless phenotype (González-Morales et al. 2019). In *Caenorhabditis elegans*, PTEN tumor suppressor is a gene that regulates dauer stage and has several PDZ-binding motifs. When PDZ was deleted from PTEN, larval dauer stage was significantly decreased and lifespan was significantly reduced, signifying that PDZ significantly impairs these functions and that PDZ containing proteins play a strong role in dauer form and increased lifespan (Solari et al. 2005). In diapausing insects, the ability to make acclimatory changes to cell structures such as membranes, cytoskeleton, and organelles to be able to handle cold stress for overwintering survival is crucial (Košťál et al. 2009).

In sum, PDZ proteins play a central role in maintaining tissue integrity, suggesting that their disruption would have a profound effect on signaling pathways or on the cytoarchitecture (Nourry et al. 2003). The *pdz* gene in *Cx. pipiens* is homologous to PDZ domain-contacting proteins in other model organisms, and in previous studies, PDZ shows a multitude of functions, such as cytoskeletal binding, maintaining tissue integrity, and cell signaling assembly (Ponting et al. 1997, Bezprozvanny et al. 2001, Velthuis et al. 2007), and PDZ domain proteins are known to bind α-actinin and play a role in muscle assembly and maintenance (Liao et al. 2016).

Actin is increased in the midgut of female mosquitoes, polymerizing and becoming more apparent during the diapause period (Kim et al. 2006, MacRae et al. 2010). In the fall field cricket, *Gryllus pennsylvanicus*, actin-associated proteins were similarly enhanced, including genes producing FRG and α-actinin. This altered actin-membrane anchoring may reduce tension or shearing damage in response to cold exposure (Martenaux et al. 2017). In addition, actin levels during early diapause in *Nasonia vitripennis* were significantly elevated (Wolschin et al. 2009). *Aphidius rhopalosiphi*, a parasitoid, showed a similar pattern (Colinet et al. 2012). This suggests that PDZ could help actin accumulation in tissues during stress events. If suppressed, inhibition of this stress response occurs, decreasing the odds of survival. In this study, we report that this actin accumulation in midgut tissue by activation of PDZ significantly contributes to increased cold stress tolerance in diapause mosquitoes, *Cx. pipiens*. This gene was cloned from *Cx. pipiens* and RNA interference was used to evaluate the function compared between diapausing females and their nondiapausing counterparts.

Materials and Methods

Mosquito Rearing

The *Culex pipiens* colony was established in September 2000 from larvae collected in Columbus, OH, and additional field-collected mosquitoes were added to the laboratory colony in 2009. The colony was reared at 25 °C and 75% relative humidity (RH) under a 12:12 light–dark (LD) photoperiod, as previously described Robich et al. (2007). When larvae reach their first or second instar stage, rearing containers were placed under 1 of 2 environmental conditions: nondiapausing adults were reared at 18 °C, 75% RH, and 15L:9D h (ND), and to induce diapause, mosquitoes were reared at 18 °C, 75% RH, and 9L:15D h (D). To confirm diapause status, primary follicle, and germarium lengths were measured, and the stage of ovarian development was determined according to methods previously described (Christophers 1911). Larvae were reared in dechlorinated tap water and fed Tetramin fish food (Tetra Holding Inc., Blacksburg, VA). Adults were provided with cotton wicks soaked in a 10–20% honey solution and kept in 33.5 cm × 30 cm × 30 cm screened cages.

Identification and Bioinformatics Analysis of Mosquito PDZ Proteins

Cx. pipiens gene encoding PDZ was utilized in discontinuous MEGA–BLAST searches on trace archives of genome data from the NCBI database (http://www.ncbi.nlm.nih.gov/blast/racemb.shtml), and identification of the retrieved other mosquito PDZs were confirmed by blasting against the VectorBase (https://vectorbase.org/vectorbase/app/workspace/blast/new). Multiple sequence alignments were performed by using ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic trees were constructed by the Neighbor-Joining (NJ) and maximum parsimony (MP) methods, both included in MEGA11 (Tamura et al. 2021). The accuracy of reconstructed trees was examined by the bootstrap test with 5,000 replications (Fig. 1). The percentage of replicate trees in which the associated PDZ proteins clustered together in the bootstrap test is shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Synthetic Dicer-Substrate siRNA Injection Into Adult Female Mosquitos

Targeting of the gene encoding PDZ (*pdz*, CPIJ001709) was performed as described previously (Olademehin et al. 2020). Briefly, the DsiRNAs were used in silencing experiments against the *pdz* gene. The siRNA duplexes were purchased from Integrated DNA Technology (IDT, Coralville, IA). The sequences of chosen siRNA duplexes are established through BLAST searches and have no significant homology to *Cx. pipiens* genes other than for *Culex pdz*, which are as follows: dicer-substrate RNAi of *Cx. pipiens pdz* (dsi-*pdz*): 5'-rCrUrGrUrUrCrCrUrCrCrUrGrArArGrUrCrGrCrUrCrCr UrCrGrArU-3'/5'-rCrGrArGrGrArGrCrGrArCrUrUrCrArGrGrArGrGrArArCAG-3'. A scrambled negative control dsiRNA, a dicer-substrate RNAi of control (dsi-control) duplexes lacking significant sequence homology to any genes in the *Cx. pipiens* genome, is used for control experiments, dsi-control: 5'rGrArArGrArGrCrArCrUrGrArUrArGrArUrGr UrUrArGrCGT-3'/5'rArCrGrCrUrArArCrArUrCrUrArUrCrArGrUrGrCrUrCrUrUrCrCrG-3'. The dsi-control injected females were not significantly different from wild type females for any of the phenotypes assessed in this study. Each mosquito received 300–500 nl of dsiRNA via a microinjector system.

RNAi Efficiency Evaluation Using Quantitative Real-time PCR (qRT-PCR)

All qRT-PCR reactions of the dsi-RNA-injected mosquitoes were performed as previously described (King et al. 2019, 2021). Briefly, total RNA samples were extracted with TRIzol (Invitrogen) from 3 batches of 20 adult female mosquitoes on various days after dsi-RNA injection. To remove genomic DNA contamination, RNA samples were treated with DNase I following the manufacturer's instructions (50–375 units/µl; Invitrogen). For reverse transcription, 5 µg of total RNA was reverse-transcribed with SuperScript III RNase H-reverse transcriptase (Invitrogen). All reactions were performed in triplicate in a total volume of 20 µl containing 10 µl SYBR Green PCR Master Mix (Bio-Rad,

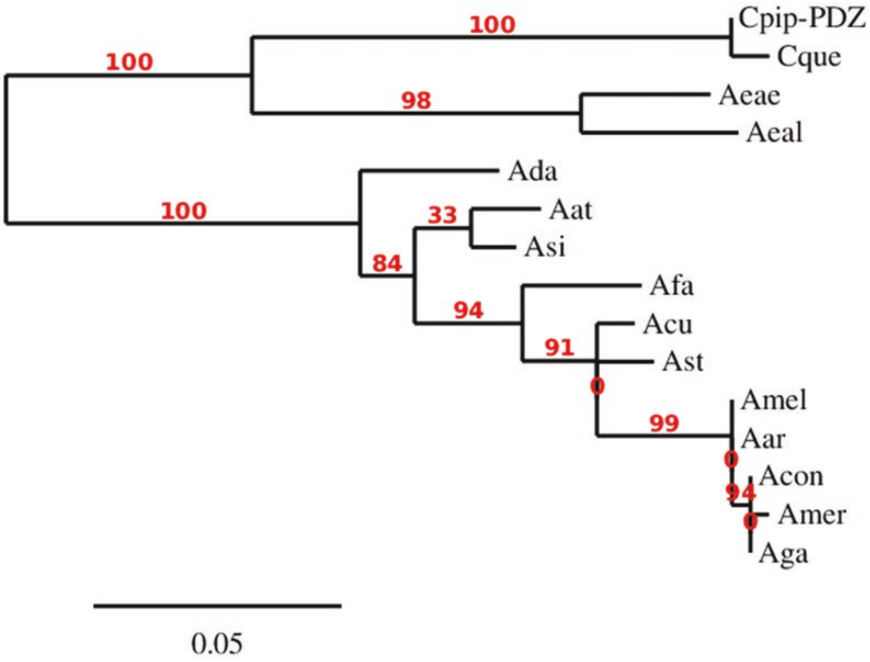


Fig. 1. (A) Phylogenetic tree of mosquito PDZ domain-containing protein (PDZ) generated with the maximum likelihood method and bootstrap analysis. Bootstrap values are shown at the base of the branches and represent the percentage of times that grouping was supported. Cpip-PDZ, *Culex pipiens* PDZ, CPIJ001709. Cque, *Culex quinquefasciatus* PDZ, CQUJHB011912 Aeae, *Aedes aegypti* PDZ, AAEL009904. Aeal, *Aedes albopictus* PDZ, AALF016890. Afa, *Anopheles farauti* PDZ, AFAF020364. Acu, *Anopheles culicifacies* PDZ, ACUA004266. Ast, *Anopheles stephensi* PDZ, ASTEI00950. Ada, *Anopheles darlingi* PDZ, ADAC010163. Aat, *Anopheles atroparvus* PDZ, AATE005752. Asi, *Anopheles sinensis* PDZ, ASIS019004. Acon, *Anopheles coluzzii* PDZ, ACON003938. Aar, *Anopheles arabiensis* PDZ, AARA017649. Amer-, *Anopheles merus* PDZ, AMEM002570. Amel, *Anopheles melas* PDZ, AMEC002140. Aga, *Anopheles gambiae* PDZ, AGAP003938. The scale bar indicates an evolutionary distance of 0.05 amino acid substitutions per position. (B) Alignment of the mosquito PDZ domain-containing proteins from Cpi-PDZ, Cque-PDZ, Aeae-PDZ, Aeal-PDZ, Afa-PDZ, Acu-PDZ, Ast-PDZ, Ada-PDZ, Aat- PDZ, Asi-PDZ, Acon-PDZ, Aar-PDZ, Amer-PDZ, Amel-PDZ, and Aga-PDZ. PDZ domain is located in 47-114 a.a. of the Cpi-PDZ protein (black box). Red is for a.a. K and R, blue is for a.a. A, F, I, L, M,V and W, green is for a.a. N, Q, S, and T, teal is for a.a. H and Y, pink is for a.a. C, purple is for a.a. D and E, yellow is for a.a P and orange is for a.a. G, respectively.

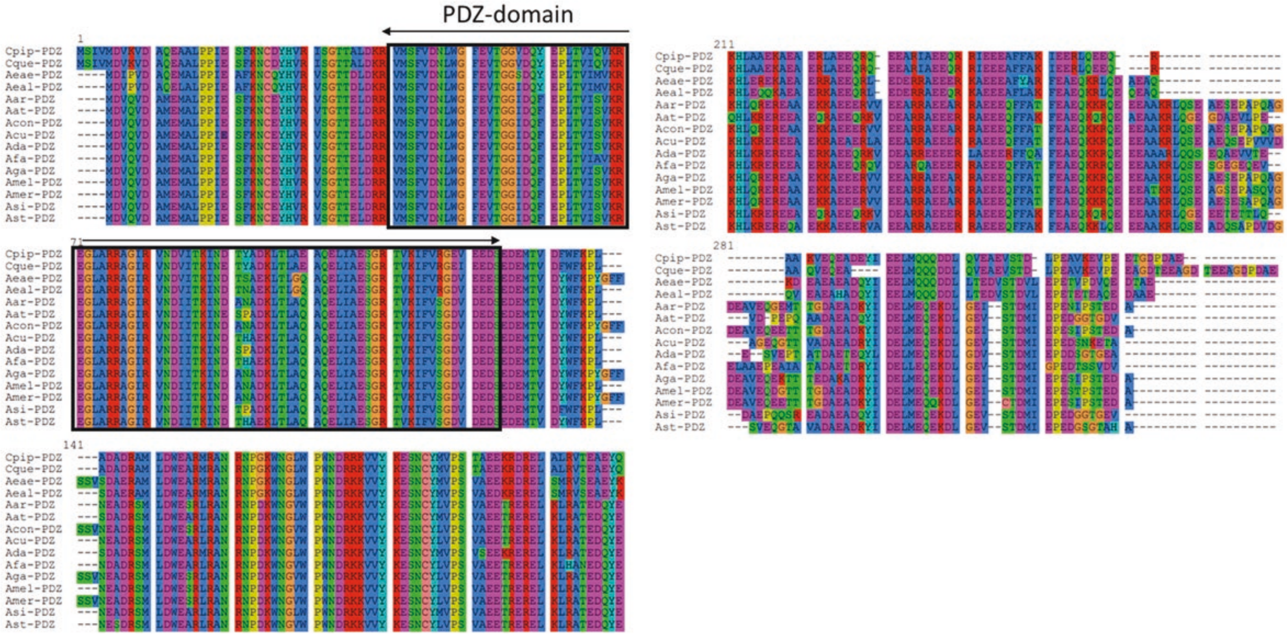


Fig. 1. Continued

Hercules, CA) and 300 nmol of each primer at the following conditions: 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The following qRT-PCR primers were used: q-pdz, 5′-TACCAACCACCTTGCATTGA-3′ and 5′-TTCGCTCG TACGTGAAGATG-3′; q-rpl19, 5′-CGCTTTGT TTGATCGTGTGT-3′ and 5′-CCAA TCCAGGAGTGCTTTTG-3′. The ribosomal protein large subunit 19 gene (rpl19) was used as a loading control (Sim et al. 2008). Expression data were normalized to the geometric mean of housekeeping gene rpl19 to control the variability in expression levels and were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Then, the statistical significance of differences in transcript levels was determined using a Student's *t*-test between the relative transcript values of dsi-*pdz* injected vs. control samples (dsi-control injected), using 3 biologically independent replicates for each gene. A *P*-value less than 0.05 was considered as a significant transcript-level change.

Low-Temperature Treatment and Detecting the Accumulated F-actin in the Midgut Muscle

Dsi-*pdz* and Dsi-*control* treated nondiapausing and diapausing females were exposed to −4 °C for 3 h by placing them in a −4 °C freezer. At least 29 females were used for each treatment as previously described (Kim et al. 2006); in short, treatments consisted of diapause (D) and nondiapause (ND) controls (no freezing temperatures of 18 °C), D and ND placed in −4 °C for 3 h, D and ND injected with control RNAi and placed in −4 °C for 3 h, D and ND injected with pdz RNAi and placed in −4 °C for 3 h. All samples were dissected after 3 h in −4 °C. Midgut tissues of cold-treated mosquitos were dissected in insect saline (Ringer's saline: 150 mM NaCl, 6.4 mM KCl, 1.0 mM CaCl, 1.0 mM $MgCl_2$, 25 mM HEPES, and 5 mM glucose, pH 7.0) under a Zeiss Axiskop fluorescent microscope and immediately placed on ice. Samples were fixed with paraformaldehyde for 30 min, moved to FBS (fetal bovine serum) for 1 h, then moved to Rhodamine phalloidin (1:1,000, Molecular Probes R-415), washed with insect saline solution, and then imaged, all at room temperature. ImageJ, software that is available through the National Institutes of Health website (https://imagej.nih.gov/ij/), was used for resizing images and calculating mean pixel intensity. Tracing of the tissues and analysis of the regions of interest were done to analyze between 29 and 43 individuals per group (Harig 2013). Measurements were taken and an average of at least 3 images per midgut sample. A χ^2 goodness of fit test was performed to determine whether the proportion of action-accumulation in RNAi-*pdz* treated group was equal to control groups (W.T and RNAi-*control* treated group).

Survival Analysis

By injecting 0.5 µl of dsi-*pdz* into the thorax of 150 mosquitoes, the knockdown impact of *pdz* on survival rate was investigated. The same dosage of dsi-*control* was injected into the thorax of 154 individuals as a control group. Mosquitoes were held at 18 °C, 75% relative humidity, and 9:15 L:D cycle, with access to sugar, and survival is assessed every 5 days. Survival curves were fitted and analyzed using Cox proportional hazards model (R version 4.0.4, "survival," "survminer" packages) (R core team 2014). Univariate Cox regression and Wald test were used for testing the statistical significance of the differences between the dsi-*pdz* and dsi-*control* injected mosquitoes (Cox 1972). Individuals who did not die by 35 days after dsi-*control* injections were censored, otherwise recorded as death event (0 = death event did not occur; 1 = death event occurred). The individual females with dsi-*pdz* injections were not censored, because the experiment finished with the death of all dsi-*pdz* injected females.

In addition, 51 and 57 mosquitoes were intrathoracically injected with ~0.5 µl of dsi-*pdz* or dsi-*control*, respectively, to test the cold shock impact of −4 °C for 3 h. These mosquitoes were given 1 h to recover from their injections before being exposed to −4 °C for 3 h. The survival of the mosquitoes is evaluated every 5 days at 18 °C, 75% relative humidity, and a 9:15 L:D cycle, with access to sugar. Survival curves were fitted as described previously.

Results

Phylogenetic Analysis of *Cx. pipiens* PDZ

We identified other mosquito genes encoding PDZ by performing BLAST searches on the nonredundant genomic database using sequences of *Cx. pipiens pdz* gene. The sequences of *Cx. pipiens pdz* shared the highest identities, 100% with *pdz* from a sister species, *Cx. quinquefasciatus*. The 1,470-bp mRNA fragment and 302 amino acid sequence of *Cx. pipiens pdz* shared 78% identity with *pdz* from *Aedes albopictus* and 63% identity to *pdz* sequences from *Anopheles gambiae*. The deduced amino acid sequences PDZ, based on a pfam search, belong to a PDZ family which is essential to bind either the carboxyl-terminal sequences of proteins or internal peptide sequences (Ponting et al. 1997). A phylogenetic analysis and protein alignment of known mosquito PDZ was performed to infer relationships of the *Culex* PDZ with those identified in *Cx quinquefasciatus, Ae. Aegypti*, and *An. gambiae* mosquitoes, showing strong conservation between *Cx. pipiens, Ae. aegypti*, and *Ae. albopictus*, but based on phylogenetic analysis, *Anopheles* mosquitoes are evolutionarily more distant (Fig. 1).

Transcript Levels of pdz From Diapausing and Nondiapausing Females of *Cx. pipiens* and RNAi Efficiency Evaluation Using qRT-PCR

The transcript levels of the *pdz* gene in nondiapausing and diapausing *Cx. pipiens* were obtained using qRT-PCR. Diapausing mosquitoes had much higher PDZ transcript levels than nondiapausing mosquitoes (Fig. 2A). In nondiapausing females, *pdz* levels were relatively low in comparison to diapausing females. Increased expression levels of the *pdz* gene in mosquitoes in response to short day length (destined to diapause) suggested the possibility that this gene is involved in regulating cold tolerance stress during early diapause period.

In addition, RNAi efficiency was assessed by qRT-PCR. In contrast to the relatively high induction of *pdz* in dsi-*control* injected mosquitoes, more than 75% reduction of *pdz* transcript was observed in dsi-*pdz* injected, diapausing mosquitoes, using qRT-PCR and primers corresponding to the *pdz* gene (Fig. 2B). This result shows that the injection of dsi-*pdz* successfully inhibited the expression of the *pdz* gene in diapausing females. It was also shown that the level of suppressed expression of this gene was similar to that of the nondiapausing counterparts. The expression of the *rpl19* gene used as the loading control did not show a significant difference among each cDNA sample. Thus, the low expression levels observed for the *pdz* gene were related to the knockdown effect of dsi-*pdz* injections rather than variation in sample loading. All cDNA samples were normalized before qRT-PCR was conducted.

Dicer-Substrate RNAi-*pdz* Decreases the Formation of Actin Accumulation in Diapausing Females

There was no change in the actin polymerization patterns in the midguts of ND females regardless of RNAi treatments (Figs. 3A–D and 4). However, in the midgut of diapause-destined females with the same temperature of 18 °C (Fig. 3E), actin polymerization was increased and midgut actin accumulation was observed even in the early stages of diapause (Figs. 3F, G, and 4), and an enhanced amount of actin was present at 21 days (Supplementary Fig. 1F and G). It was observed that the midgut actin accumulation under these diapause conditions was increased to a more significant level when the cold stress was introduced by exposure at −4 °C for 3 h (Figs. 3F, G and 4). In addition, a χ^2 goodness-of-fit test was conducted to compare the rate of actin accumulation in the midgut between the test group treated with dsi-*pdz* injection and the control group treated only with cold shock (−4 °C for 3 h). When the expression of *pdz* was inhibited with dsi-*pdz* injection, actin accumulation greatly decreased in the midgut 7–10 days after adult eclosion. (Fig. 3H and Supplementary Figs. 1 and 2) (χ^2 (1, n = 71) = 45.2, $P < 0.001$, Fig. 4) A similar actin accumulation pattern was also observed at 21 days post adult eclosion (χ^2 (1, n = 67) = 45.5, $P < 0.001$, Fig. 4) (Supplementary Figs. 1 and 2).

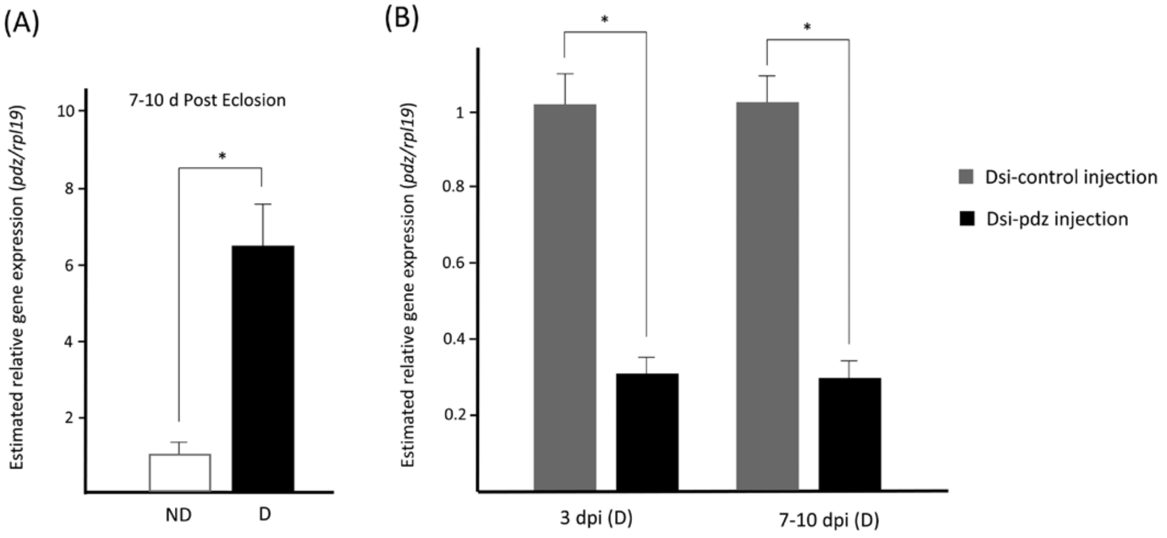


Fig. 2. (A) Quantitative Real-Time PCR showing relative expression levels of the gene encoding PDZ protein in nondiapausing (ND) and diapausing (D) females 7–10 days post adult eclosion (dpe). ND (gray bar) = programmed by long day length for nondiapause, D (black bar) = programmed by short day length for diapause. Both groups were maintained at 18 °C. (B) RNA interference efficiency targeting PDZ (dsi-*pdz*) in diapausing *Cx. pipiens.* Transcript levels of the gene encoding PDZ protein in females injected with dsi-*pdz* (white bar) were compared with the dsi-control (black bars). Expression levels were measured by qRT-PCR at 3 days and 7–10 days post dsi-RNA injection (pdi). qRT-PCR data were normalized using a ribosomal protein large subunit 19 (rpl19) as a loading control. Bars (mean ± s.e., n = 3 groups of 10 individuals each) with asterisks (*) indicate significant differences at $P < 0.05$, t-test.

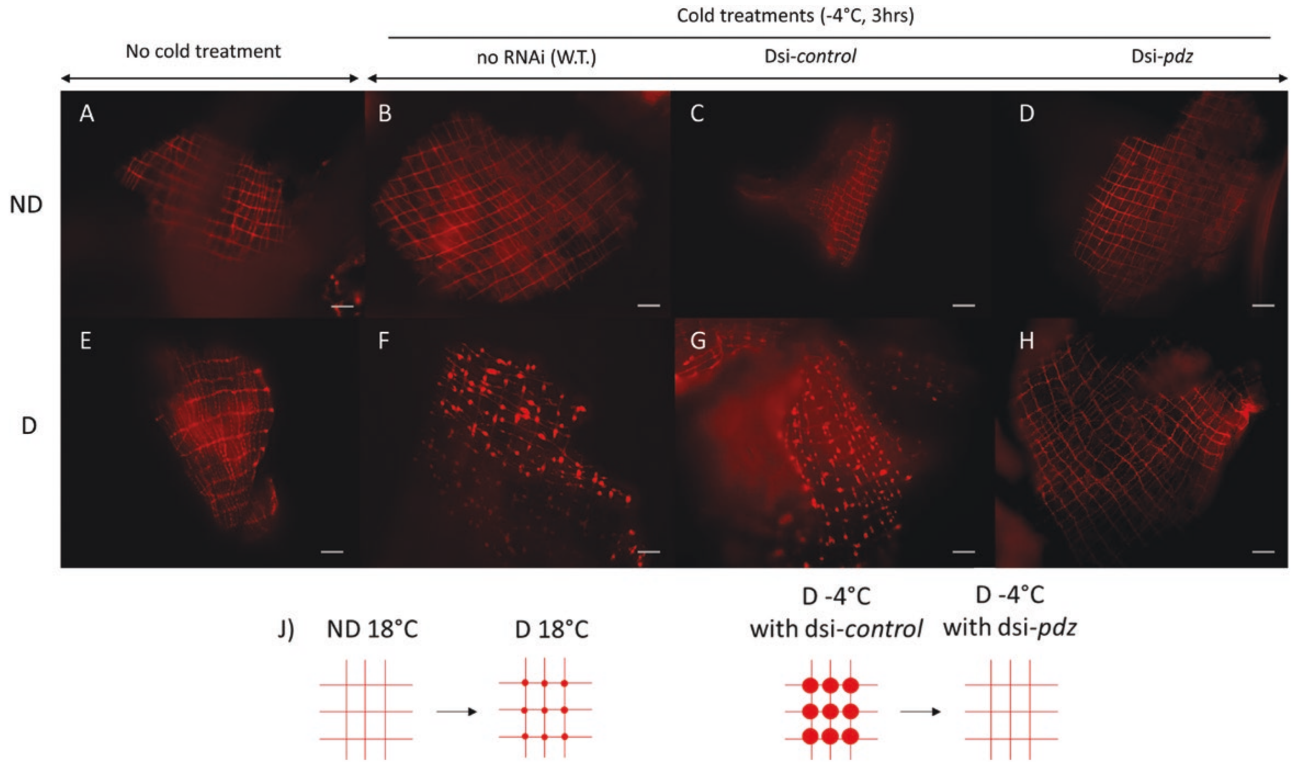


Fig. 3. Representative midguts from nondiapausing (ND) and diapausing (D) females, prepared using Rhodamine phalloidin (Molecular Probes R-415) post adult eclosion under FITC filter. (A) Midgut of 18 °C ND females 7–10 days post eclosion. (B) Midgut of ND females 7–10 days post eclosion in −4 °C for 3 h. (C) Midgut of ND females 7–10 days post eclosion with dsi-control in −4 °C for 3 h. (D) Midgut of ND females 7–10 days post eclosion with dsi-*pdz* in −4 °C for 3 h. (E) Midgut of 18 °C D females 7–10 days post eclosion. (F) Midgut of D females 7–10 days post eclosion −4 °C for 3 h. (G) Midgut of D females 7–10 days post eclosion with dsi-control in −4 °C for 3 h. (H) Midgut of D females 7–10 days post eclosion with dsi-*pdz* in −4 °C for 3 h. Bar for measurement is 50 μm. (J) Schematic representation of the changes in F-actin accumulation in the midgut between nondiapausing and diapausing adults in response to the low temperature (−4 °C) and dsi-*pdz* treatment.

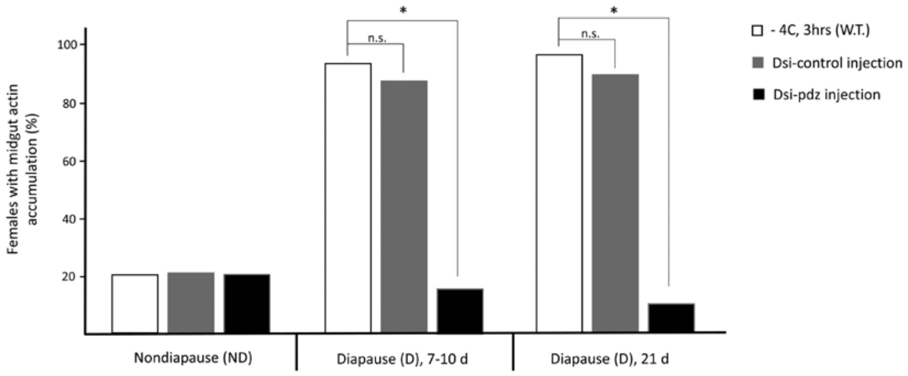


Figure 4. The proportion of female mosquitoes with midgut in a state of actin accumulation following an injection of dsi-*control* or dsi-*pdz*. Nondiapause (ND) = programmed by long day length for nondiapausing females. Diapause (D), 7–10 days = programmed by short day length for diapausing females 7–10 days post adult eclosion (dpe). D, 21 days = programmed by short day length for diapausing females 21 dpe. Female mosquitoes in response to the low temperature (−4 °C, 3 h, W.T.) (white bar). Female mosquitoes in response to the low temperature (−4 °C, 3 h) and dsi-*contorl* treatment (gray bar). Female mosquitoes in response to the low temperature (−4 °C, 3 h) and dsi-*pdz* treatment (black bar). *N*= 29–43 individuals per group. *Significant differences from untreated control (χ^2 goodness of fit test at $P < 0.001$ and df = 1).

Reduced Survival of Diapausing *Cx. pipiens* in Response to dsi-*pdz* Injection

The midgut tissue of the female treated with dsi-pdz was severely damaged (Supplementary Fig. 1B), and it was observed that the female fatality rate was increased by more than 50% (Fig. 5). The experimental results that inhibit the function of the *pdz* gene suggest that this gene product effectively enhances cold tolerance in early-stage diapause. In addition, survival analysis using the Cox's Proportional Hazards model showed a higher death risk (hazard ratio; HR) for dsi-*pdz* injected females (Fig. 5). Hazard ratio estimate of exp(coef) = 26.35 with 95% CI (15.59, 44.55). This result suggests that, at least with early diapause, the suppression of *pdz* expression significantly increases mortality of diapausing *Cx. pipiens*, due to possible consequences of reduced actin accumulation activity at 18 °C. In addition, when diapausing females are subjected to temperatures of −4 °C for 3 h, the effect of actin accumulation is intensified. As a result, the survival rates of mosquitoes whose *pdz* gene expression was suppressed were drastically reduced (Supplementary Fig. 3).

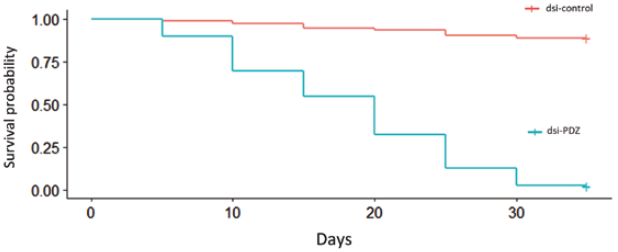


Fig. 5. The survival probability of female mosquitoes after injection of dsiRNAs targeting the gene encoding PDZ domain-containing protein (dsi-*pdz*) and control (dsi-control) at 18 °C. Survival curves were fitted and analyzed using Cox proportional hazards model. *N* = 150–154 individuals per group.

Discussion

The PDZ protein contains protein domains linked to the actin cytoskeleton that promote cell adhesion and muscle development (Bezprozvanny and Maximov 2001). A phylogenetic analysis and protein alignment of known mosquito PDZs were performed to determine how the PDZs of various mosquito species are evolutionarily related (Fig. 1). According to phylogenetic analysis, *Cx. pipiens* and other *Aedes* species are more closely related to *Anopheles* mosquitoes. Some mosquito species of the Aedes genus initiate a diapause program mostly during the egg or larva stage, which is interesting in light of research on the evolution of diapause in mosquitoes. Nevertheless, mosquitoes of the Culex and Anopheles genera typically undergo diapause once they have reached the adult stage (Denlinger and Armbruster 2014). Each stage of development is at risk when entering diapause during the winter when prevention of cold stress is essential. Yet, it is clear that the case of entering diapause as an adult is particularly precarious. Phylogenetic analysis of these PDZs, however, reveals that the *Culex* PDZ protein is more closely related to the Aedes genus than initially assumed. This stress response may have been utilized as a result of modifications in other evolutionary mechanisms that affect regulatory elements among the genes involved in cold hardiness. This specific gene regulation system may be altered and activates the genes related to cold stress signaling. The insulin/Foxo singling pathway is one proposed mechanism. Consequently, the development of cold stress response genes, such as *pdz*, appears not to be analogous to the evolution of mosquito diapause. As our knowledge of the molecular mechanisms regulating mosquito diapause at the egg, larval, pupal, and adult stages grows, we will be better able to draw inferences about the complex evolutionary process related to diapause and cold stress responses in mosquitoes as well.

There has been little study on diapause-induced changes in actin-filament-related genes in insects. The pharate first-instar larvae of *Lymantria dispar* express a brain-specific actin pre- and post-diapause period but not during diapause (Lee et al. 1998). In the solitary bee *Megachile rotundata*, actin is only high in post-diapausing pupae (Yocum et al. 2005). In diapausing mosquito species, actin is up-regulated in adult *Culex pipiens* during early diapause but downregulated during late diapause. *Cx. pipiens* is also diapausing species in which actin expression changes in response to diapause, but it is the first time that actins are specifically up-regulated during the early diapause. (Robich et al. 2007, Kim et al. 2006). According to this distinct actin activation in early diapause period, actin may be connected to the diapause-preparatory stage, including cold tolerance.

In this study results, first of all, actin in the midgut was observed in the musculature of the midgut overall at the moderate temperature of 18 °C, but more clearly in the intersections of muscle fibers. Moreover, at this moderate temperature, actin accumulation in midgut tissue in diapause started to show stronger signals than in nondiapause conditions. These results are the same as previous experiments and suggest that the start of the diapause program is related to the activation of genes involved in actin polymerization (Robich et al. 2007, Kim et al. 2006). Second, in the case of cold shock treatment at 4 °C for 3 h, these effects are further amplified, as can be shown (Fig. 3). No specific change was observed in the midgut tissue in the nondiapause state (Fig. 3B), but in the diapause state, specifically concentrated actin accumulation was observed at the intersection of muscle fibers (Fig. 3F). It seems that the diapause program activates actin accumulation in the midgut, and cold shock stress further enhances actin accumulation at the intersection of muscle fibers.

In the early diapause period, the expression of the *pdz* gene is more than 5 times higher than in the nondiapause state (Fig. 2A). Interestingly, when the expression of this gene was suppressed in the early diapause period through injection of dis-*pdz*, it was observed that actin accumulation in the midgut fiber intersection disappeared (Fig. 3H). These RNAi gene knockdown experiments targeting the *pdz* gene show that the *pdz* gene plays an important role in the enhancement of midgut fibers induced by diapause and cold shock. As can be seen from the potential role of the PDZ domain protein already listed in the introduction, the PDZ domain is known to interact with the actin cytoskeleton and several related proteins (Bezprozvanny and Maximov, 2001, Liao et al. 2016, 2020, Nandelstadh et al. 2009, Nourry et al. 2003, Short et al. 1998, Vallenius et al. 2004). However, the functions related to diapause and cold hardness are reported for the first time in this study. In fact, what mechanism activates actin polymerization in the strengthening of the actin cytoskeleton or specifically in the intersection of midgut fibers is a part that needs further clarification.

In subsequent experiments, this midgut tissue was analyzed 3 wk after the *pdz* gene was suppressed. In this sample, although actin accumulation was slightly increased, it was observed that the continuously connected muscle fibers were disconnected in many places and degeneration of midgut tissue occurred (Supplementary Fig. 1B). These results can be understood in the same context as in the survival rate assay experiment (Fig. 5). In the case of inhibiting the *pdz* gene, it is in line with the rapid increase in mortality rate from 1-wk post adult eclosion. This implies that the PDZ protein strengthens the tissues of the midgut against cold shock by reinforcing the structure of the actin cytoskeleton and eventually protects these tissues from cold stress during the winter diapause period. However, further studies are needed to determine whether the expression of the *pdz* gene protects tissues of different periods throughout or is limited to midgut tissues.

In conclusion, we have demonstrated that the gene encoding a PDZ offers a role in protecting diapausing mosquitoes and also plays a key role in protecting diapausing midgut tissue from enhanced cold stress. However, additional research is needed on how this gene plays a role in actin accumulation in the tissue outside of diapause, as well as in other forms of diapause in different insects.

Acknowledgments

This work was supported in part by the National Institutes of Health under grant number R15AI139861 and by the National Science Foundation under grant number IOS-1944214.

Author Contributions

C.S. and B.K. designed research; B.K., A.I., and M.L. performed research and analyzed data; and C.S. and B.K. wrote the paper.

Data Availability

The sequences reported in this paper have been deposited in the GenBank database (accession no. *Culex pipiens pdz*, KP057870).

Supplementary Material

Supplementary material is available at *Journal of Medical Entomology* online.

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