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Functional Characterization of the *Frost* Gene in *Drosophila melanogaster*: Importance for Recovery from Chill Coma

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Abstract

Background: Almost all animals, including insects, need to adapt to temperature fluctuations. The molecular basis of thermal adaptation is not well understood, although a number of candidate genes have been proposed. However, a functional link between candidate genes and thermal tolerance has rarely been established. The gene *Frost* (*Fst*) was first discovered when *Drosophila* flies were exposed to cold stress, but the biological function(s) of *Fst* has so far not been characterized. Because *Fst* is up-regulated after a cold stress, we tested whether it was essential for chill-coma recovery.

Methodology/Principal Findings: A marked increase in *Fst* expression was detected (by RT-PCR) during recovery from cold stress, peaking at 42-fold after 2 h. The GAL4/UAS system was used to knock down expression of *Fst* and recovery ability was assessed in transgenic adults following 12 h of chill coma at 0°C. The ability to recover from cold stress (short-, medium- and long-term) was significantly altered in the transgenic adults that had *Fst* silenced. These findings show that *Fst* plays an essential role in the recovery from chill coma in both males and females.

Conclusions/Significance: The *Frost* gene is essential for cold tolerance in *Drosophila melanogaster* and may play an important role in thermal adaptation.

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Introduction

Insects subjected to seasonally low temperatures have evolved a range of physiological and molecular adaptations to survive [1]. The molecular mechanisms behind cold stress and associated chilling injuries are complex and still poorly understood [2,3]. *Drosophila melanogaster* has adapted successfully to diverse thermal environments and provides a useful model system for understanding the molecular basis of thermal adaptation.

While some studies have considered genes that might be involved in cold tolerance in *Drosophila* [4], the molecular basis of cold stress resistance is poorly understood in comparison to heat resistance. It appears that more genes/proteins are activated during recovery phases following cold stress compared to the actual stress period [2,5] and these phases need to be differentiated in experimental studies [6]. Recovery from chill-coma is a trait widely studied by evolutionary geneticists (e.g. ref [4,7]) because it is adaptively significant [4,8] but its underlying molecular basis is not well-understood.

Frost (*Fst*) is one of the few candidate genes that have been implicated in cold tolerance in *D. melanogaster*. This gene was first discovered and characterized by Goto [9] in flies exposed to cold stress. Recent studies have also suggested that *Fst* might be a good candidate for thermal adaptation [11,12]. *Fst* was up-regulated during recovery from cold stress but, unlike heat-shock genes [7], *Fst* expression was not altered after heat stress [10]. However, a functional relationship between *Fst* and cold tolerance remains to be established. *Fst* has also been reported to respond weakly to a range of abiotic stressors, such as dietary shifts, desiccation, chemical toxicity, insecticide exposure and hypoxia [10,13–16]. *Fst* may also be involved in immune response against virus, bacteria and fungi [17–20].

In the present study we showed that the mRNA level of *Fst* was markedly increased in adults recovering from cold stress. We demonstrated that silencing *Fst* by transgenic RNA inference impaired the recovery process from chill coma in both sexes. Expression of *Fst* thus seems to be crucial for developing cold tolerance in *D. melanogaster* adults.

Methods

Drosophila stocks and breeding conditions

The wild type *D. melanogaster* strain was derived from about 50 females collected in Innisfail (Australian east coast) in May 2008 (see ref [7] for more details). RNAi-mediated *Fst* knockdown was achieved using the GAL4/UAS system [21]. The *UAS-Fst* line was

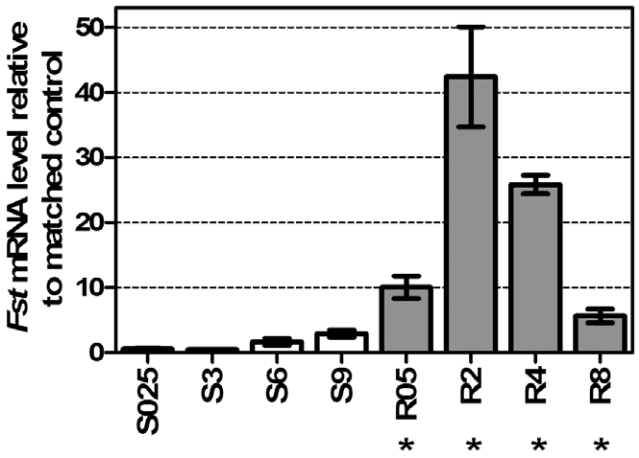

Figure 1. Upregulation of *Fst* during cold stress and recovery. White bars represent cold stressed treatment (S) at 0°C for 0.25 to 9 h and grey bars denote recovery (R) at 25°C for 0.5 to 8 h. Relative expressions are calculated using the $2^{-\Delta\Delta Ct}$ method. Expression levels of *Fst* are normalized against the housekeeping reference *RpS20* and values are expressed as fold change relative to control (mean±SE; $n=4$). The symbol (*) indicates when a value is significantly different from untreated controls (*t*-test).
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obtained from the Vienna Drosophila RNAi Center (transformant ID: KK102049) [22]. The *tubulin*-GAL4 (genotype: *w*; tubP-GAL4/TM3, Act-GFP JMR2, Ser*^{1}, provided by Phil Batterham, University of Melbourne) and the *actin5C*-GAL4 (Bloomington Drosophila Stock Center, #4414) lines were used separately to drive the expression of the UAS-*Fst*, both resulted in ubiquitous *Fst* mRNA knockdown. Progeny were tested in cold recovery assays. To control for genetic background effects, the same GAL4 driver lines were crossed to the w^{1118} line (from BDRC) and their progeny assayed alongside with their GAL4/UAS-*Fst* counterparts. Fly stocks were maintained in 250 ml bottles in uncrowded conditions. Bottles were kept at 25°C, 70% relative humidity, and continuous light on a standard fly medium as previously described [23].

Cold stress and recovery conditions

All tests were performed using synchronized 4-day old flies, sexed without CO_2 anaesthesia. To establish the *Fst* mRNA expression during the cold stress and during the recovery period, we used the same method as described in Colinet et al. [7]. Briefly, wild flies were cold stressed at 0°C to induce chill coma, and sampled after 0.25, 3, 6 and 9 h of cold stress (denoted as S025, S3, S6 and S9 respectively). After 9 h of cold stress, flies were allowed to recover at 25°C and *Fst* mRNA expression was measured after 0.5, 2, 4, and 8 h of recovery (denoted as R05, R2, R4 and R8 respectively). For every sampling time there was a

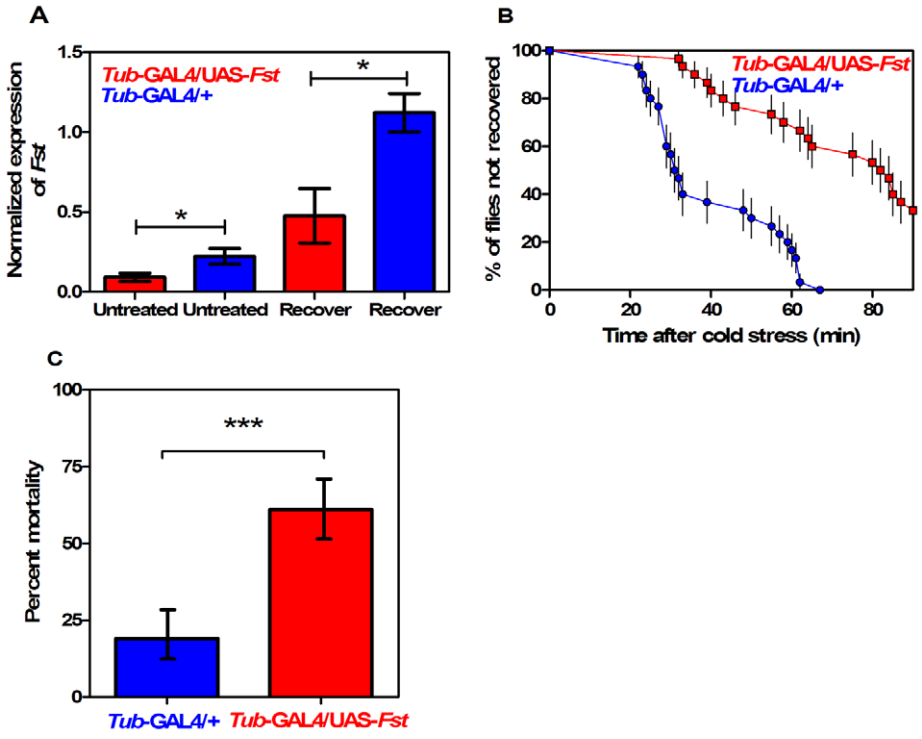

Figure 2. Silencing the cold-inducible *Fst* expression impairs chill coma recovery in *tub*-GAL4-driven females. (A) Expression of *Fst* mRNA in untreated (kept at 25°C) and recovering (2 h at 25°C after 12 h at 0°C) females. Expression levels of *Fst* are normalized against the housekeeping reference *RpS20* and values are $\sqrt{1/x}$ transformed (mean±CI; $n=3$). The symbol (*) indicates when the level is significantly different in *tub*-GAL4/UAS-*Fst* versus *tub*-GAL4/+ females (*t*-test). (B) Comparison of temporal recovery curves in *tub*-GAL4/UAS-*Fst* (squares) versus *tub*-GAL4/+ (circles) females. Time to recover from chill coma was monitored in females recovering at 25°C after 12 h of cold stress at 0°C. Each dot represents the mean percentage (±SE); 45 females were tested per line. (C) Mortality rate in *tub*-GAL4/UAS-*Fst versus tub*-GAL4/+ females. Mortality was assessed in flies recovering for 24 h at 25°C after 12 h of cold stress at 0°C. Bars represents the percentage (±CI) derived from 150 females in each line. The symbol (*) indicates a significant difference between lines (Chi square test).
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corresponding control, consisted of flies kept at 25°C for the same duration ($n = 4 \times 20$ flies).

RNA extraction and quantitative real time PCRs

RNA extractions were performed using the RNeasy RNA extraction kit and the RNase-Free DNase Set (Qiagen, Australia) as described in Colinet et al. [7]. cDNA was synthesized using the Superscript III First-Strand Synthesis System (Invitrogen, Australia), according to manufacturer's instructions. *Fst* primers were designed with the Primer3 module (http://www.angis.org.au) (forward: 5′-GGAACAGAGGTGGAATAGCCAAAATC-3′ and reverse: 5′-GCCTTGATTGTTTCCGTGAGATTG-3′). The qRT-PCRs were performed on the LightCycler® 480 system (Roche Diagnostics, Australia) following the method previously described [7]. Relative expression ratios (i.e., fold change) were calculated using the $2^{-\Delta\Delta Ct}$ method [24]. *RpS20* was used as a housekeeping reference gene (see ref [7]). To verify the extent of gene knockdown, *Fst* mRNA levels were compared between the untreated flies, kept at 25°C (i.e. basal expression) and the treated flies, recovering for 2 h after cold stress (i.e. during *Fst* up-regulation). Such a comparison in *Fst* expression was conducted separately in males and females ($n = 3 \times 20$ flies per line).

Chill-coma recovery assays

Three types of assays were used to measure recovery abilities after 12 h of chill-coma at 0°C. Firstly, '*short-term recovery*' was assessed by comparing recovery times of both GAL4/UAS-*Fst* and GAL4/+ lines at 25°C. Flies were considered recovered when they stood up [25]. Recovery curves were compared between lines using Mantel-Cox analysis with a censoring factor for individuals that did not recover at the end of the experiment. Forty-five flies were monitored for each line. To test for '*long-term recovery*', the mortality of flies after cold stress was assessed when they had been held in food vials at 25°C for 24 h. Chi square contingency tests were used to compare mortality rates between GAL4/UAS-*Fst* and GAL4/+ lines. Mortality rates were based on 150 flies for each line. Finally, an additional '*medium-term recovery*' test was performed with flies derived from *act*-GAL4 crosses. This test was designed to monitor mobility status during 8 h following the cold stress, and represents a modified version of a climbing activity test described elsewhere [26]. Briefly, flies were individually transferred to a 9.5 cm plastic vial. The height flies reached within 7 sec after a mechanical stimulation was noted. Flies were divided into three categories: (a) *injured*, no climbing; (b) *recovering*, slow climbing without reaching the top of the vial within 7 sec; (c) *fit*, fast climbing and reaching the top of the vial within 7 sec. The 7 sec observation time was chosen because preliminary assays showed that all unstressed flies reach the top of a vial within 6 sec (5.1 ± 1.3 sec, $n = 50$). This test was performed repeatedly on the same individuals after 2, 4, 6 and 8 h of recovery (25°C). Flies were maintained on food during this period. Chi square contingency tests were carried out to compare numbers of flies in the three categories for the *act*-GAL4/UAS-*Fst* and *act*-GAL4/+ lines. Seventy flies were tested for each line. This test was not

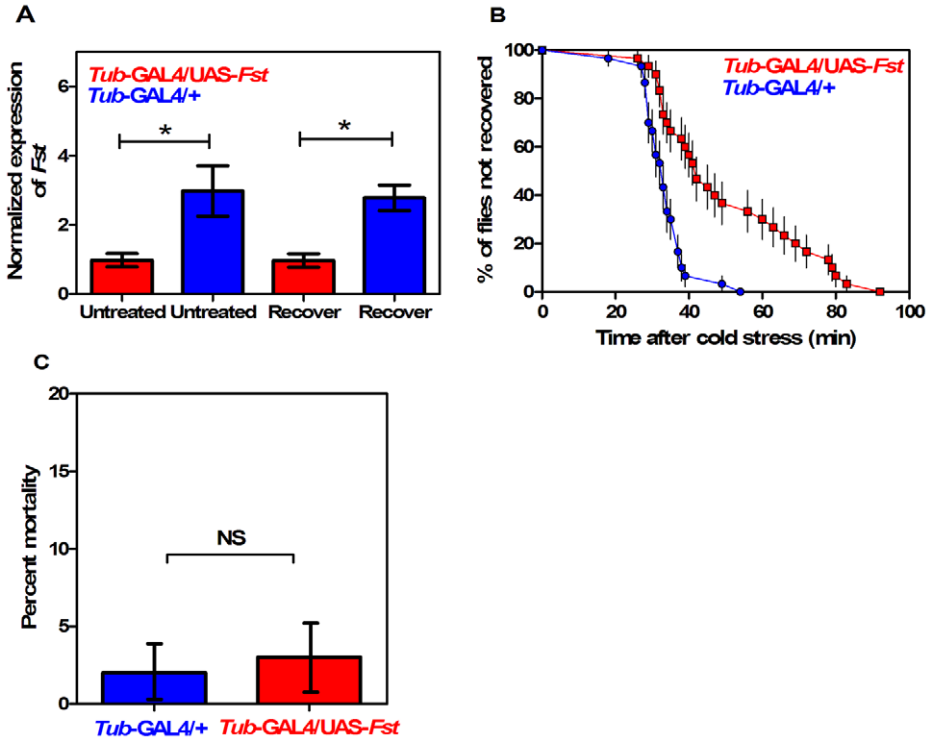

Figure 3. Silencing the cold-inducible *Fst* expression impairs chill coma recovery in *tub*-GAL4-driven males. (A) Expression of *Fst* mRNA in untreated (kept at 25°C) and recovering (2 h at 25°C after 12 h at 0°C) males. Expression levels of *Fst* are normalized against the housekeeping reference *RpS20* and values are $\sqrt{1/x}$ transformed (mean±CI; $n = 3$). The symbol (*) indicates when the level is significantly different in *tub*-GAL4/UAS-*Fst* versus *tub*-GAL4/+ males (*t*-test). (B) Comparison of temporal recovery curves in *tub*-GAL4/UAS-*Fst* (squares) versus *tub*-GAL4/+ (circles) males. Time to recover from chill coma was monitored in males recovering at 25°C after 12 h of cold stress at 0°C. Each dot represents the mean percentage (±SE); 45 males were tested per line. (C) Mortality rate in *tub*-GAL4/UAS-*Fst* versus *tub*-GAL4/+ males. Mortality was assessed in flies recovering for 24 h at 25°C after 12 h of cold stress at 0°C. Bars represents the percentage (±CI) derived from 150 males in each line. The symbol (*) indicates a significant difference between lines (Chi square test).
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performed on flies derived from *tub*-GAL4 crosses which were less vigorous even when they were unstressed (34% did not reach the top of the vial within 10 sec, $n = 50$). All statistical tests were performed using Prism V 5.01 (GraphPad software, Inc. 2007).

Results

Expression of *Fst* was not altered during the cold stress period, but *Fst* was significantly up-regulated during the recovery phase at 25°C. Expression peaked after 2 h of recovery, when there was a maximal 42-fold change relative to controls (Fig. 1). Because of this significant up-regulation during recovery from cold stress, we suspected that *Fst* may have an essential role in chill-coma recovery.

Lines derived from *tubulin*-GAL4 driver

Fst mRNA expression was significantly reduced in *tub-GAL4/UAS-Fst* females compared to *tub-GAL4/+* females, both when flies were untreated ($t = 10.11$, $P<0.001$, IC: 0.166−0.094, $r^2 = 0.962$) and when they were recovering from the cold stress ($t = 13.36$, $P<0.001$, IC: 0.779−0.511, $r^2 = 0.978$) (Fig. 2A). *Fst* expression was also significantly repressed in *tub-GAL4/UAS-Fst* males compared to *tub-GAL4/+* males, both when flies were untreated ($t = 11.43$, $P<0.001$, IC: 2.490−1.517, $r^2 = 0.970$) and recovering from cold stress ($t = 18.78$, $P<0.001$, IC: 2.080 to 1.544, $r^2 = 0.988$) (Fig. 3A). *Fst* knockdown had a significant effect on short-term recovery in both sexes but particularly in females (Fig. 2B, 3B), resulting in significantly different recovery curves (Mantel-Cox: $\chi^2 = 34.33$; $df = 1$; $P<0.001$ for females and $\chi^2 = 20.50$; $df = 1$; $P<0.001$ for males). In females (Fig. 2B) all the *tub*-GAL4/+ flies recovered within 62 min, while 33% of flies still had not recovered in the *tub*-GAL4/UAS-*Fst* group after 90 min. In males (Fig. 3B) all flies recovered within 90 min but recovery time was longer in the *tub*-GAL4/UAS-*Fst* group. Nevertheless all flies did eventually recover. For the long-term assay, there was a significant difference in mortality between females from the two lines (Fig. 2C) ($\chi^2 = 37.41$; $df = 1$; $P<0.001$), with mortality reaching 61% in the *tub*-GAL4/UAS-*Fst* flies compared to 19% in the *tub*-GAL4/+ controls. In males, mortality in the two groups did not differ significantly ($\chi^2 = 0.16$, $df = 1$; $P = 0.68$) (Fig. 3C).

Lines derived from *actin*-GAL4 driver

Fst mRNA expression was significantly reduced in *act-GAL4/UAS-Fst* females compared to *act-GAL4/+* females, both when flies were untreated ($t = 5.47$, $P = 0.005$, IC: 0.273−0.089, $r^2 = 0.882$) and when they were recovering from the cold stress ($t = 6.19$, $P = 0.003$, IC: 1.615−0.615, $r^2 = 0.905$) (Fig. 4A). *Fst* expression was also significantly suppressed in *act-GAL4/UAS-Fst* males compared to *act-GAL4/+* males, both when flies were untreated ($t = 37.60$, $P<0.001$, IC: 0.833−0.719, $r^2 = 0.997$) and recovering from the cold stress ($t = 15.78$, $P<0.001$, IC: 2.913−2.041, $r^2 = 0.984$) (Fig. 5A). Short-term recovery was significantly different between lines for both sexes (Fig. 4B, 5B) (Mantel-Cox: $\chi^2 = 12.50$; $df = 1$; $P<0.001$ for females; $\chi^2 = 9.63$; $df = 1$; $P = 0.002$ for males). For females (Fig. 4B), all the *act*-GAL4/+ control flies

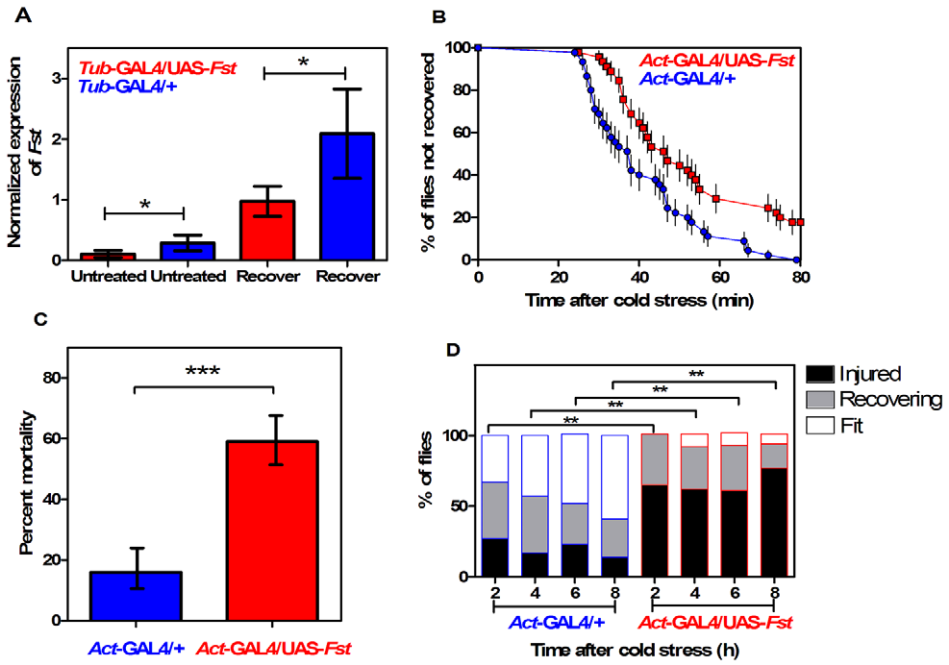

Figure 4. Silencing the cold-inducible *Fst* expression impairs chill coma recovery in *act*-GAL4-driven females. (A) Expression of *Fst* mRNA in untreated (kept at 25°C) and recovering (2 h at 25°C after 12 h at 0°C) females. Expression levels of *Fst* are normalized against the housekeeping reference *RpS20* and values are $\sqrt{1/x}$ transformed (mean±CI; $n = 3$). The symbol (*) indicates when the level is significantly different in *act*-GAL4/UAS-*Fst* versus *act*-GAL4/+ females (*t*-test). (B) Comparison of temporal recovery curves in *act*-GAL4/UAS-*Fst* (squares) versus *act*-GAL4/+ (circles) females. Time to recover from chill coma was monitored in females recovering at 25°C after 12 h of cold stress at 0°C. Each dot represents the mean percentage (±SE); 45 females were tested per line. (C) Mortality rate in *act*-GAL4/UAS-*Fst* versus *act*-GAL4/+ females. Mortality was assessed in flies recovering for 24 h at 25°C after 12 h of cold stress at 0°C. Bars represents the percentage (±CI) derived from 150 females in each line. The symbol (*) indicates a significant difference between lines (Chi square test). (D) Climbing activity monitored in *act*-GAL4/UAS-*Fst* versus *act*-GAL4/+ females. Measurements were taken in recovering females after 2, 4, 6 and 8 h at 25°C following 12 h at 0°C. Flies were categorized as fit (fast climbing) or recovering (slow climbing) or injured (no climbing). The symbol (*) indicate significant differences between lines (Chi square test, $n = 70$).
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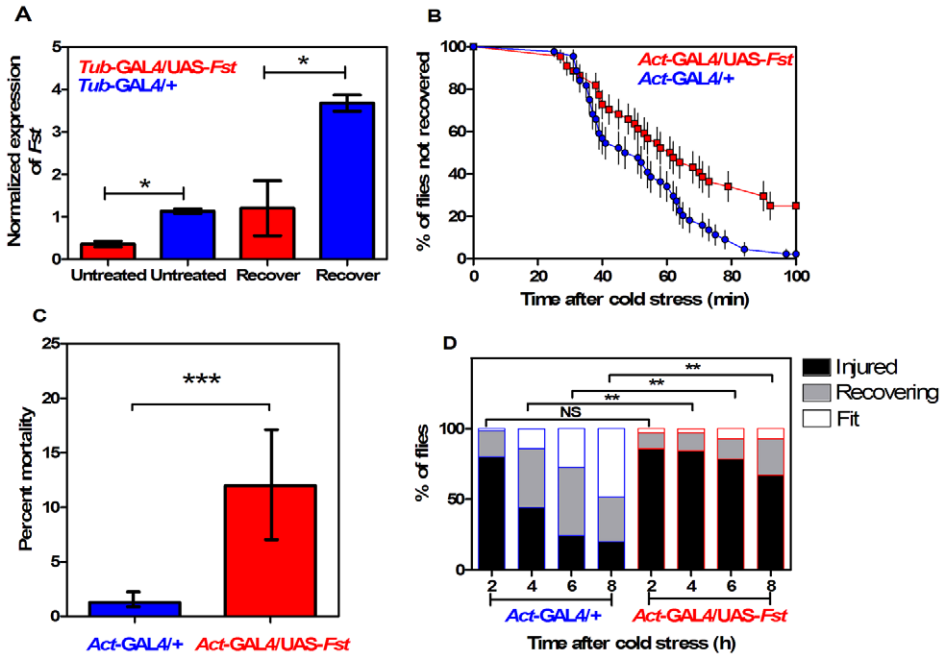

Figure 5. Silencing the cold-inducible *Fst* expression impairs chill coma recovery in *act*-GAL4-driven males. (A) Expression of *Fst* mRNA in untreated (kept at 25°C) and recovering (2 h at 25°C after 12 h at 0°C) males. Expression levels of *Fst* are normalized against the housekeeping reference *RpS20* and values are $\sqrt{1/x}$ transformed (mean±CI; $n = 3$). The symbol (*) indicates when the level is significantly different in *act*-GAL4/UAS-*Fst* versus *act*-GAL4/+ males (*t*-test). (B) Comparison of temporal recovery curves in *act*-GAL4/UAS-*Fst* (squares) versus *act*-GAL4/+ (circles) males. Time to recover from chill coma was monitored in males recovering at 25°C after 12 h of cold stress at 0°C. Each dot represents the mean percentage (±SE); 45 males were tested per line. (C) Mortality rate in *act*-GAL4/UAS-*Fst* versus *act*-GAL4/+ males. Mortality was assessed in flies recovering for 24 h at 25°C after 12 h of cold stress at 0°C. Bars represents the percentage (±CI) derived from 150 males in each line. The symbol (*) indicates a significant difference between lines (Chi square test). (D) Climbing activity monitored in *act*-GAL4/UAS-*Fst* versus *act*-GAL4/+ males. Measurements were taken in recovering males after 2, 4, 6 and 8 h at 25°C following 12 h at 0°C. Flies were categorized as fit (fast climbing) or recovering (slow climbing) or injured (no climbing). The symbol (*) indicate significant differences between lines (Chi square test, $n = 70$).
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recovered within 80 min, while 18% of flies had not recovered in the *act*-GAL4/UAS-*Fst* group. A similar pattern was observed in males (Fig. 5B) with 25% of flies failing to recover in the *act*-GAL4/UAS-*Fst* group after 100 min. All flies eventually recovered. For the long-term recovery assay, a significant difference was observed in females ($\chi^2 = 60.23$; $df = 1$; $P<0.001$), mortality reached 59% in the *act*-GAL4/UAS-*Fst* flies compared to 16% in the *act*-GAL4/+ controls (Fig. 4C). Males also differed significantly for mortality ($\chi^2 = 0.13$; $df = 1$; $P = 0.002$), which reached 12% in the *act*-GAL4/UAS-*Fst* flies and 1.5% in the *act*-GAL4/+ flies (Fig. 5C). In addition, the medium-term recovery tests revealed significant differences in movement patterns between the *act*-GAL4/UAS-*Fst* and the *act*-GAL4/+ control flies (Fig. 4D, 5D) (χ^2 tests: $P<0.05$). A high proportion of females were initially injured in the *act*-GAL4/UAS-*Fst* group and this proportion remained high during the observation period (Fig. 4D). In contrast, females from the *act*-GAL4/+ group gradually recovered, with the proportion of designated as 'fit' increasing while 'injured' flies decreased in proportion (Fig. 4D). A similar pattern was observed in the males (Fig. 5D) where the *act*-GAL4/+ flies gradually recovered while the majority of the *act*-GAL4/UAS-*Fst* flies remained injured.

Discussion

D. melanogaster is a chill-susceptible species. At 0°C it falls almost instantly into deep chill-coma because of an inability to maintain muscle resting potentials [27]. In addition to this neuromuscular perturbation, chilling injuries accumulate at low temperatures as a result of various physiological dysfunctions (see ref [3] for review). The molecular mechanisms underlying cold stress and recovery from chill-coma are complex and not well understood. Genes involved in heat shock response are known to affect recovery from cold stress in insects [7,28,29]. In addition to heat shock genes, the regulation of other genes is presumably important for cold-tolerance. Indeed, multiple genes appear to be up-regulated during recovery from cold stress [30] and *Fst* is among the candidates suspected to play a role in cold tolerance.

However, the functional relationship between *Fst* and cold tolerance has not been established prior to this study. Using transgenic gene silencing techniques, the expression of *Fst* was knocked down. All recovery traits analyzed (i.e. short-, medium- and long-term) were significantly affected in flies where *Fst* expression was suppressed. Our findings thus show that *Fst* plays an important role in chill coma recovery in both sexes. This is the first time, to our knowledge, that a biological function has been demonstrated for *Fst*. QTL and microarrays studies have suggested that *Fst* might be a candidate for thermal adaptation [11,12] and our findings indicate that this gene is indeed important for cold recovery.

Although the mechanistic details of how *Fst* functions as a protein have not been resolved, the primary sequence of *Fst* suggests that it resembles a mucin-like protein. Frost contains multiple tandem repeats rich in serine, threonine and proline [9], a typical feature of mucins [31]. Like secreted mucins, Frost contains an 18-amino acid signal peptide at the N-terminus [9]. A homology search in annotated protein database (http://www.geneontology.org/) identified two *D. melanogaster* mucins: Mur18B

and Muc11A. *Fst* mRNA is highly enriched in adult malpighian tubule and midgut [32,33]. Similarly, *Mur18B* and *Muc11A* transcripts are enriched in the tubule of adult flies [34]. The function of insects mucin-like proteins are currently poorly characterized [34] and the relationship between mucins and protection from abiotic stress has not been firmly established. A *Drosophila* mucin gene (*Muc68Ca*) was suggested to play an undefined role in heat shock response [35]. There is evidence that mucins protect from oxidative stress [36,37], which is a typical feature of chilling-injury [38]. Mucins also provide a physical barrier to cells against pathogens and allow homeostasis of local molecular environments with respect to hydration, ionic composition and concentration [31,39]. This mucin function may be critical because perturbation of ion homeostasis is directly linked to chilling injuries [40,41] and its reestablishment occurs during recovery [42]. Among the genes up-regulated during cold stress recovery, many encode membrane-related proteins [30]. This is not surprising since the cell membrane is a primary site of chilling or cold-shock injury, as a result of damage to intracellular organelles and the leakage of ions and other solutes across cell membranes [43,44]. The *Fst* gene product, presumably a mucin-like protein, may help protect membrane integrity and hence recovery from cold [1]. Silencing *Fst* might thus impair some protective functions against oxidative stress and/or alter aspects of osmoregulation across membranes in the tubule and midgut. Taken together, this study provides evidence that *Fst* is essential for chill-coma recovery in adult *D. melanogaster* and highlights the need to further examine this gene from evolutionary and mechanistic perspectives.

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Author Contributions

Conceived and designed the experiments: HC SFL. Performed the experiments: HC SFL. Analyzed the data: HC. Contributed reagents/materials/analysis tools: HC AAH. Wrote the paper: HC SFL AAH.

References

1. Doucet D, Walker VK, Qin W (2009) The bugs that came in from the cold: molecular adaptations to low temperatures in insects. Cell Mol Life Sci 66: 1404–1418.
2. Clark MS, Worland MR (2008) How insects survive the cold: molecular mechanisms-a review. J Comp Physiol B 178: 917–933.
3. Chown SL, Terblanche JS (2006) Physiological diversity in insects: ecological and evolutionary contexts. Adv in Insect Phys 33: 50–152.
4. Hoffmann AA, Sorensen JG, Loeschcke V (2003) Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. J Therm Biol 28: 175–216.
5. Colinet H, Nguyen TT, Cloutier C, Michaud D, Hance T (2007) Proteomic profiling of a parasitic wasp exposed to constant and fluctuating cold exposure. Insect Biochem Mol Biol 37: 1177–1188.
6. Colinet H, Lee SF, Hoffmann A (2010) Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult *Drosophila melanogaster*. FEBS J 277: 174–185.
7. Anderson AR, Hoffmann AA, McKechnie SW (2005) Response to selection for rapid chill-coma-recovery in *Drosophila melanogaster*: physiology and life history traits. Genet Res 85: 15–22.
8. Gibert P, Moreteau B, Petavy G, Karan D, David JR (2001) Chill-coma tolerance, a major climatic adaptation among *Drosophila* species. Evolution 55: 1063–1068.
9. Goto SG (2001) A novel gene that is up-regulated during recovery from cold shock in Drosophila melanogaster. Gene 270: 259–264.
10. Sinclair BJ, Gibbs AG, Roberts SP (2007) Gene transcription during exposure to, and recovery from, cold and desiccation stress in *Drosophila melanogaster*. Insect Mol Biol 16: 435–443.
11. Morgan TJ, Mackay TFC (2006) Quantitative trait loci for thermotolerance phenotypes in *Drosophila melanogaster*. Heredity 96: 232–242.
12. Laayouni H, García-Franco F, Chávez-Sandoval BE, Trotta V, Beltran S, et al. (2007) Thermal evolution of gene expression profiles in *Drosophila subobscura*. BMC Evol Biol 7: 42.
13. Carsten LD, Watts T, Markow TA (2005) Gene expression patterns accompanying a dietary shift in *Drosophila melanogaster*. Mol Ecol 14: 3203–3208.
14. Affleck JG, Neumann K, Wong L, Walker VK (2006) The effects of methotrexate on *Drosophila* development, female fecundity, and gene expression. Toxicol Sci 89: 495–503.
15. Jensen HR, Scott IM, Sims SR, Trudeau VL, Arnason JT (2006) The effect of a synergistic concentration of a *Piper nigrum* extract used in conjunction with pyrethrum upon gene expression in *Drosophila melanogaster*. Insect Mol Biol 15: 329–339.
16. Liu G, Roy J, Johnson EA (2006) Identification and function of hypoxia-response genes in *Drosophila melanogaster*. Physiol Genomics 25: 134–141.
17. De Gregorio E, Spellman PT, Rubin GM, Lemaitre B (2001) Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. Proc Natl Acad Sci USA 98: 12590–12595.
18. Apidianakis Y, Mindrinos MN, Xiao W, Lau GW, Baldini RL, et al. (2005) Profiling early infection responses: *Pseudomonas aeruginosa* eludes host defenses by suppressing antimicrobial peptide gene expression. Proc Natl Acad Sci USA 102: 2573–2578.
19. Chamilos G, Lewis RE, Hu J, Xiao L, Zal T, et al. (2008) *Drosophila melanogaster* as a model host to dissect the immunopathogenesis of zygomycosis. Proc Natl Acad Sci USA 105: 9367–9372.
20. Buchon N, Broderick NA, Poidevin M, Pradervand S, Lemaitre B (2009) *Drosophila* intestinal response to bacterial infection: activation of host defense and stem cell proliferation. Cell Host Microbe 5: 200–211.
21. Duffy JB (2002) GAL4 system in Drosophila: a fly geneticist's swiss army knife. Genesis 34: 1–15.
22. Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, et al. (2007) A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature 448: 151–156.
23. Hoffmann AA, Shirriffs J (2002) Geographic variation for wing shape in *Drosophila serrata*. Evolution 56: 1068–1073.
24. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta Ct)}$ method. Methods 25: 402–408.
25. Hoffmann AA, Anderson A, Hallas R (2002) Opposing clines for high and low temperature resistance in *Drosophila melanogaster*. Ecol Lett 5: 614–618.
26. Minois N, Le Bourg E (1999) Resistance to stress as a function of age in *Drosophila melanogaster* living in hypergravity. Mech Ageing Dev 109: 53–64.
27. Hosler JS, Burns JE, Esch HE (2000) Flight muscle resting potential and species-specific differences in chill coma. J Insect Physiol 46: 621–627.
28. Rinehart JP, Li A, Yocum GD, Robich RM, Hayward SA, et al. (2007) Up-regulation of heat shock proteins is essential for cold survival during insect diapause. Proc Natl Acad Sci USA 104: 11130–11137.
29. Koštál V, Tollarová-Borovanská M (2009) The 70 kDa heat shock protein assists during the repair of chilling injury in the insect, *Pyrrhocoris apterus*. PLoS ONE 4: e4546.
30. Qin W, Neal SJ, Robertson RM, Westwood JT, Walker VK (2005) Cold hardening and transcriptional change in *Drosophila melanogaster*. Insect Mol Biol 14: 607–613.
31. Hollingsworth MA, Swanson BJ (2004) Mucins in cancer: protection and control of the cell surface. Nature Rev Cancer 4: 45–60.
32. Wang J, Kean L, Yang J, Allan A, Davies S, et al. (2004) Function-informed transcriptome analysis of *Drosophila* renal tubule. Genome Biol 5: R69.
33. Chintapalli VR, Wang J, Dow JA (2007) Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. Nat Genet 39: 715–720.
34. Syed ZA, Härd T, Uv A, van Dijk-Härd IF (2008) A Potential Role for *Drosophila* Mucins in development and physiology. PLoS ONE 3: e3041.
35. Jensen LT, Nielsen MM, Loeschcke V (2008) New candidate genes for heat resistance in *Drosophila melanogaster* are regulated by HSF. Cell Stress Chap 13: 177–182.
36. Felton GW, Summers CD (1995) Antioxidant systems in insects. Arch Insect Biochem Physiol 29: 187–197.
37. Takeyama K, Dabbagh K, Jeong Shim J, Dao-Pick T, Ueki IF, et al. (2000) Oxidative stress causes mucin synthesis via transactivation of epidermal growth factor receptor: role of neutrophils. J Immunol 164: 1546–1552.
38. Rojas RR, Leopold RA (1996) Chilling injury in the housefly: Evidence for the role of oxidative stress between pupariation and emergence. Cryobiology 33: 447–458.
39. Hegedus D, Erlandson M, Gillott C, Toprak U (2009) New insights into peritrophic matrix synthesis, architecture, and function. Ann Rev Entomol 54: 285–302.
40. Kostal V, Vambera J, Bastl J (2004) On the nature of pre-freeze mortality in insects: water balance, ion homeostasis and energy charge in the adults of *Pyrrhocoris apterus*. J Exp Biol 207: 1509–1521.

41. Kostal V, Yanagimoto M, Bastl J (2006) Chilling-injury and disturbance of ion homeostasis in the coxal muscle of the tropical cockroach (*Nauphoeta cinerea*). Comp Biochem Physiol B Biochem Mol Biol 143: 171–179.
42. Kostal V, Renault D, Mehrabianova A, Bastl J (2007) Insect cold tolerance and repair of chill-injury at fluctuating thermal regimes: role of ion homeostasis. Comp Biochem Physiol A Mol Integr Physiol 147: 231–238.
43. Denlinger DL, Lee RE (1998) Physiology of cold sensitivity. In: Hallman GJ, Denlinger DL, eds. Temperature sensitivity in insects. Application for integrated pest management. Boulder: Westview Press. pp 55–95.
44. Lee JRE, Damodaran K, Yi S-X, Lorigan GA (2006) Rapid cold-hardening increases membrane fluidity and cold tolerance of insect cells. Cryobiology 52: 459–463.