A Tissue Communication Network Coordinating Innate Immune Response During Muscle Stress
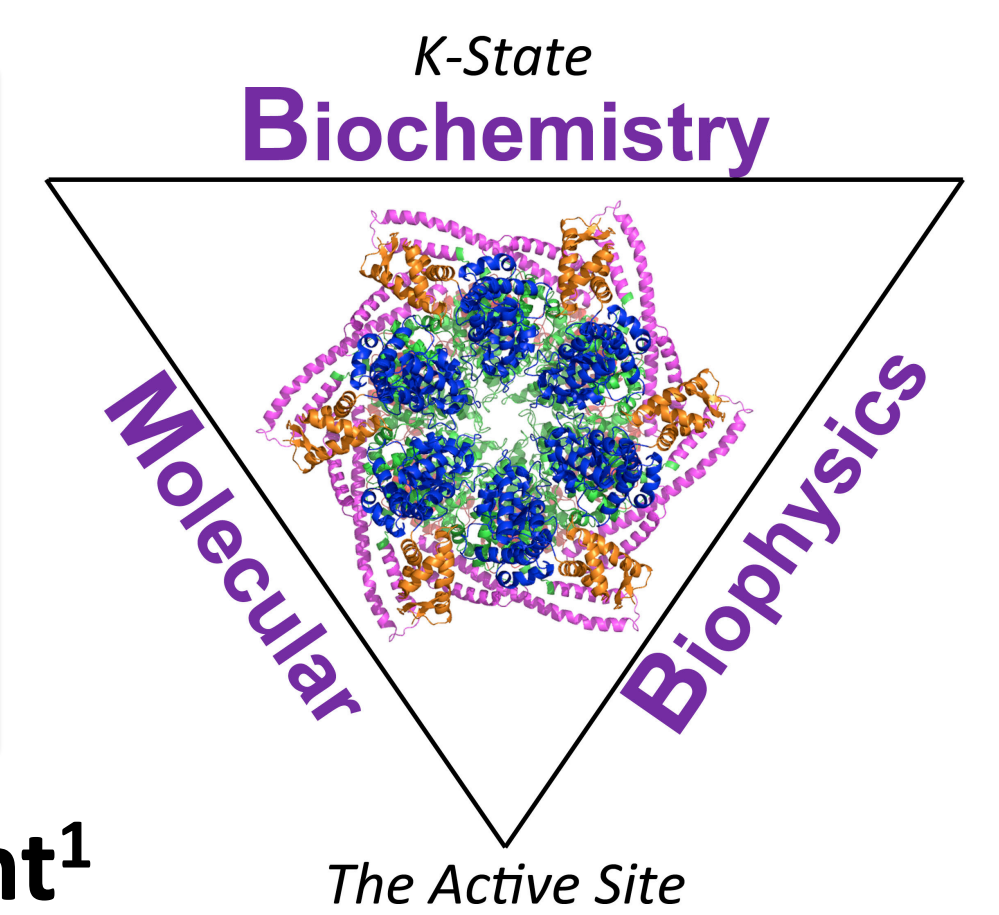
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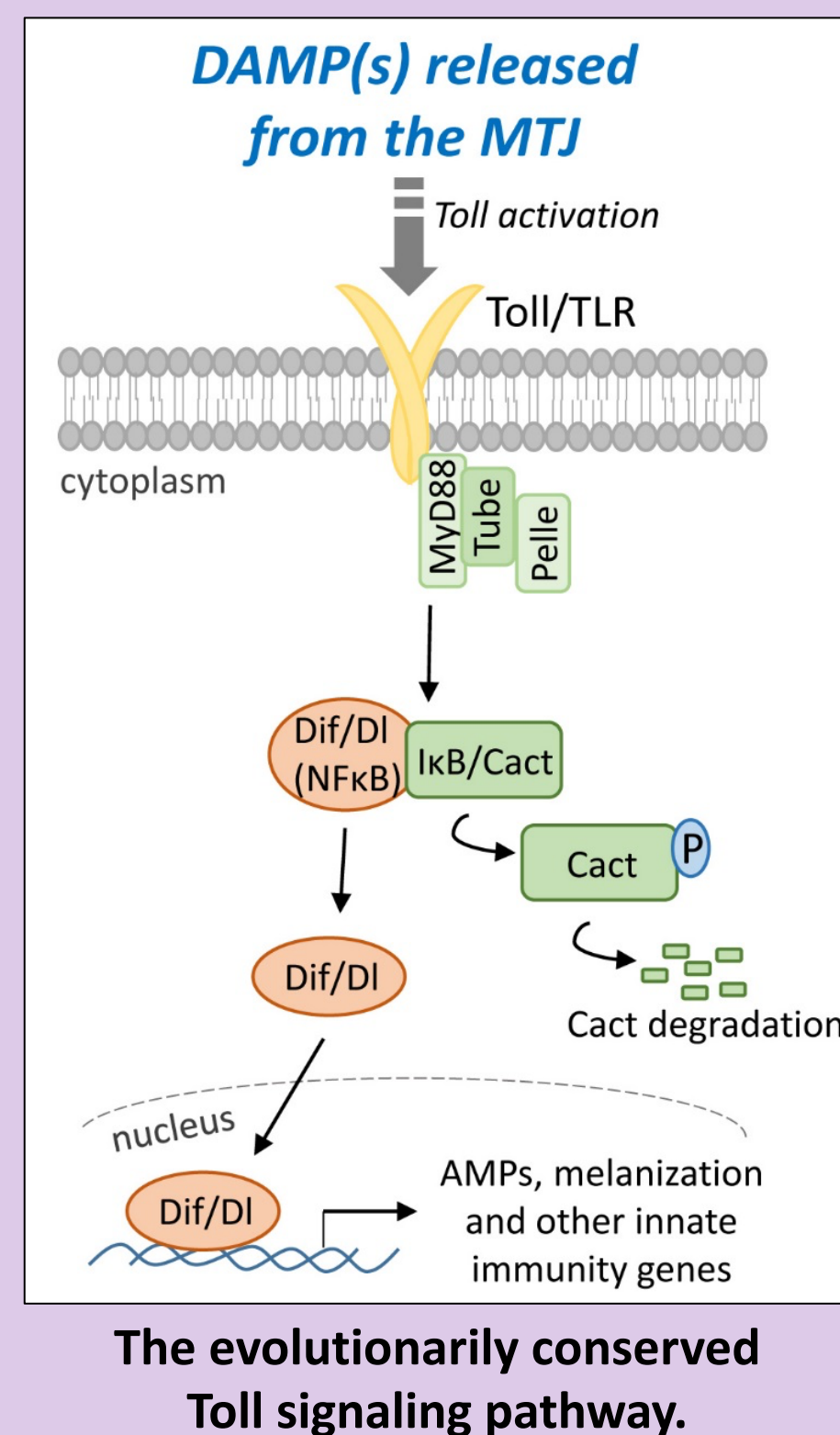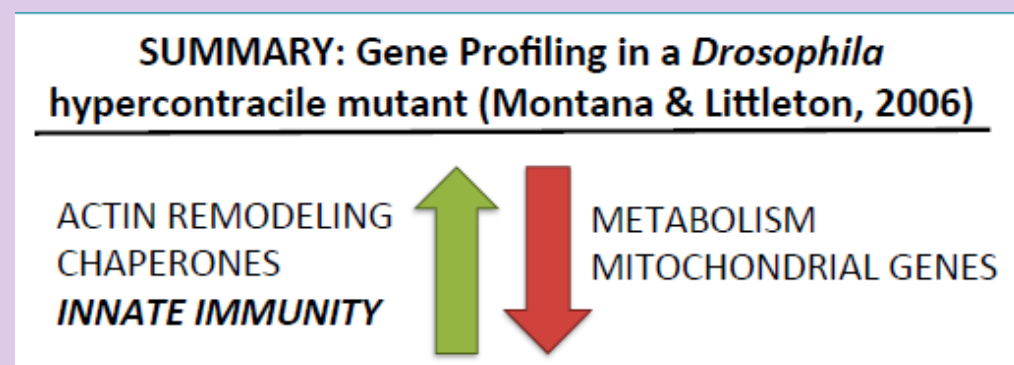*K-State* **Biochemistry** — **Molecular** **Biophysics** — *The Active Site*

Abstract

Tissue communication is required for maintaining organismal homeostasis during development. The coordination of metabolism, immune activation, and circadian rhythms typify the complex tissue networks necessary for organismal health. Our lab is using the *Drosophila* muscle attachment site (MAS) as a model to understand the connection between innate immune activation and muscle maintenance. A pupal lethal screen for abnormal pupal morphology revealed a previously unknown role for the extracellular matrix (ECM) protein Fondue (Fon), in muscle development. Previously characterized for its role in clot integrity, loss of *fon* caused a reduction in larval locomotion due to the detachment of body wall muscles. More interestingly, a sensitized background screen revealed a subset of coagulation proteins, Fon, Tiggrin (Tig), and Larval Serum Protein 1 γ (Lsp1γ), that are secreted from the fat body and incorporated into MASs for stabilization. In *fon* mutants with muscle detachment, we also observed abnormal melanin accumulation along the MAS, pathogen-independent translocation of Dorsal (Dl) in the fat body, constitutive expression of the antimicrobial peptide (AMP) *drosomycin*, and recruitment of hemocytes to damaged muscle. In a *fon*-sensitized background assay, we identified genetic interactions between *fon* and Toll pathway genes, including loss of the NFκB inhibitor/IκB, cactus, and overactivation of SPE which enhance muscle detachment. We analyzed Toll activation in the absence of hemocytes, and our data suggests that hemocytes are not necessary for Toll activation upon muscle detachment. Understanding the mechanisms by which muscle detachment or stress activate the innate immune system will advance our knowledge of how tissue stresses can be sensed and the molecular mechanisms eliciting multi-tissue responses.

Introduction

Figure 1. Activation of the Toll Pathway.

Our lab recently found that the extracellular matrix protein Fondue (Fon) accumulates at muscle-tendon attachment sites and is required for stable muscle attachment (Green, et al., 2016). In the data on this poster, we find that loss of Fon also promotes activation of the innate immune Toll pathway. Interestingly, innate immune genes are upregulated in a *Drosophila* model of hypercontraction (Montana et al., 2006), suggesting a further link between muscle damage and immunity.

SUMMARY: Gene Profiling in a *Drosophila* hypercontractile mutant (Montana & Littleton, 2006)
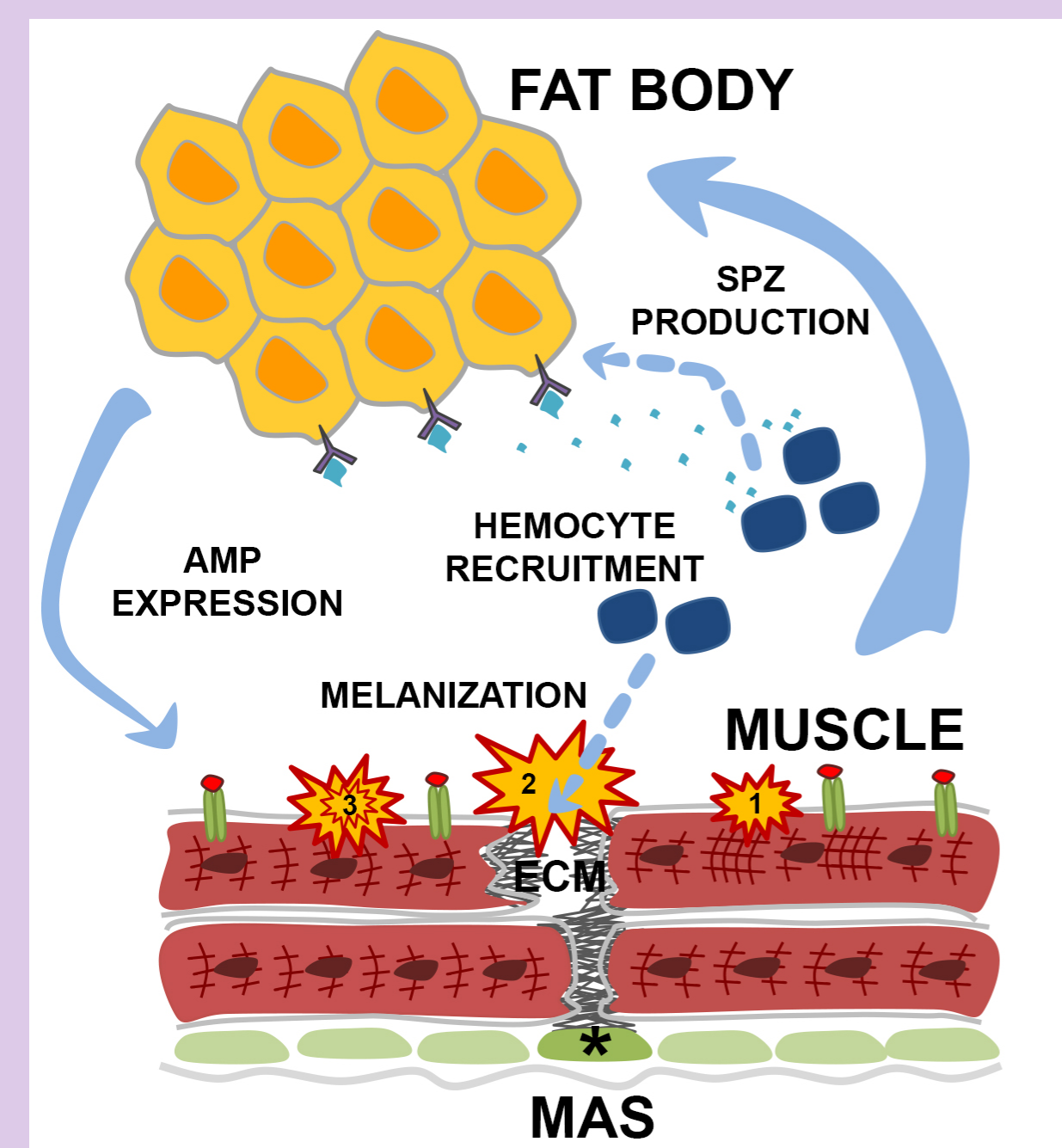ACTIN REMODELING CHAPERONES INNATE IMMUNITY ↑ — METABOLISM MITOCHONDRIAL GENES ↓

Binding of extracellular molecules to Toll/Toll-like receptors (TLR) results in activation of an intracellular cascade that targets Cactus (Cact) for protein degradation. Cact is an inhibitor of the transcription factors Dif and Dorsal (Dl). Thus, the destruction of Cact allows Dif/Dl to translocate to the nucleus and initiate the transcription of antimicrobial peptides (AMPs), such as *Drosomycin (Drs)* or *Drosocin*. These AMPs are crucial for immune defense against invading pathogens.

DAMP(s) released from the MTJ — Toll activation — Toll/TLR — cytoplasm — MyD88 Tube Pelle — Dif/Dl (NFκB) — IκB/Cact — Cact P — Cact degradation — Dif/Dl — nucleus — Dif/Dl — AMPs, melanization and other innate immunity genes

The evolutionarily conserved Toll signaling pathway.

Figure 2. Model: Tissue communication during muscle stress

Toll signaling (Fig. 1) can be activated in response to two different types of extracellular signals:
- *P*athogen-*a*ssociated *m*olecular *p*atterns (PAMPs) are typically foreign pathogens.
- *D*amage-*a*ssociated *m*olecular *p*atterns (DAMPs) are normally intracellular components that are released into the extracellular environment upon cell or tissue damage.

Our current data suggests a model (right panel) whereby the absence of Fon activates a Toll-mediated immune response through yet undefined DAMPs. DAMP(s) released in response to either muscle hypercontraction and/or from the damaged myotendinous junction (MTJ) triggers release of the Toll ligand Spätzle (Spz) from hemocytes. Spz activates the Toll innate immune pathway through binding to fat body Toll receptors to mount a systemic immune response.

FAT BODY — SPZ PRODUCTION — AMP EXPRESSION — HEMOCYTE RECRUITMENT — MELANIZATION — MUSCLE — ECM — MAS

Results

Figure 3. Loss of *fon* activates innate immune processes.

Previous experiments with Fon showed that loss of *fon* results in a **loss of clot integrity**. Mutations in *fon* also cause **diffuse melanization** and the formation of **melanotic tumors.** Consistent with melanotic phenotypes, *fon* is also a Toll responsive gene and *fon* mutants constitutively express the AMP, *Drosomycin (Drs)* (Scherfer et al., 2006).

(A-C) *WT* larvae are free from melanization upon normal conditions and dissection in the presence of the melanin-precursor, DOPA (low mag, B; high mag, C). (D) *fon* mutants spontaneously accumulate melanin at muscle attachment sites (MASs, black arrows). Similarly, we observe melanin at MASs when *fon* mutant fillets are dissected with DOPA (E-F, yellow boxes and black arrows). (G-I) Hemocytes normally circulate throughout the hemolymph or reside in sessile populations underneath the body wall muscles, largely absent from the MAS (G, arrows). Detached muscles in *WT* larvae caused during dissection (H) or caused by loss of *fon* (I) recruit hemocytes to sites of damage.
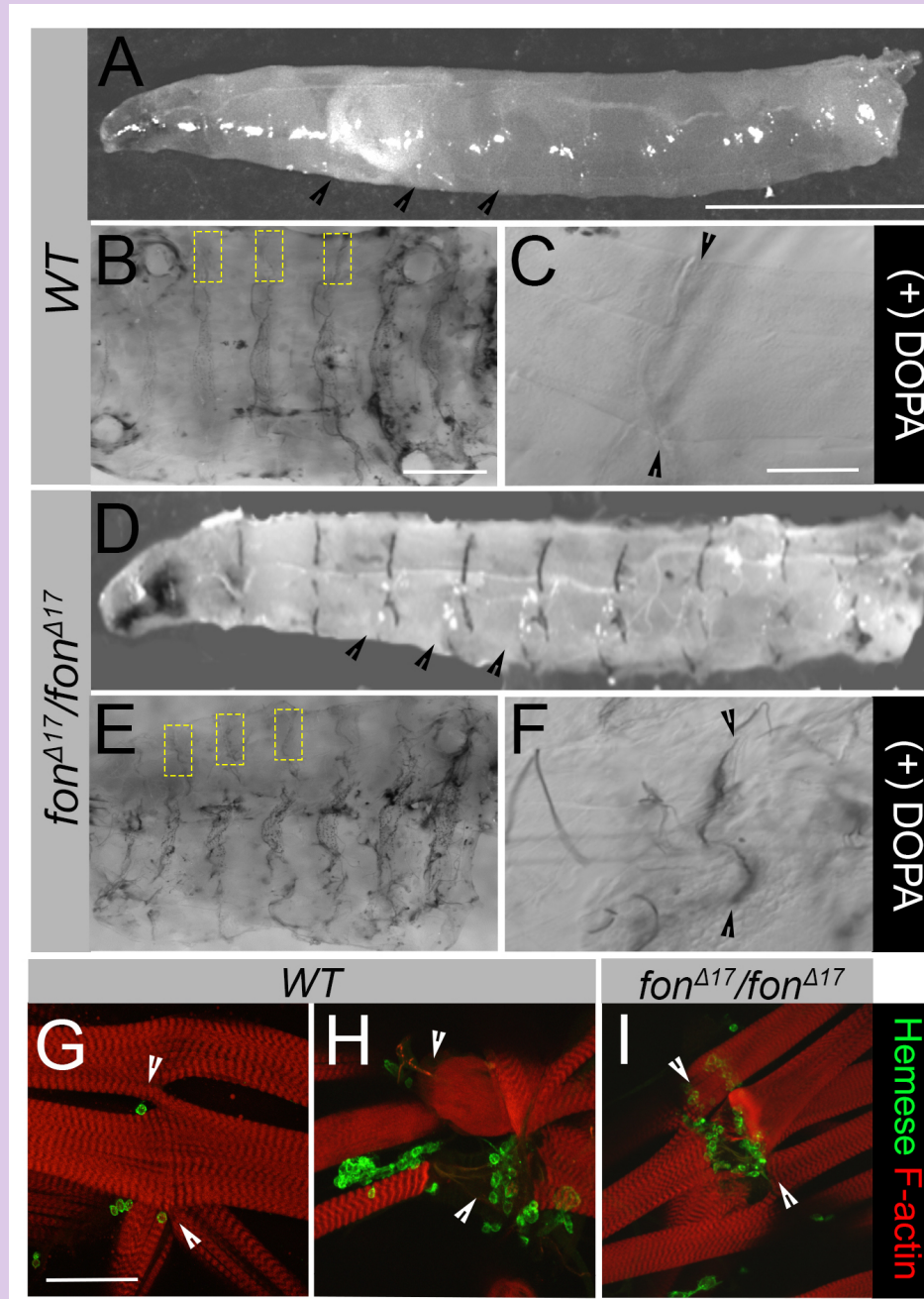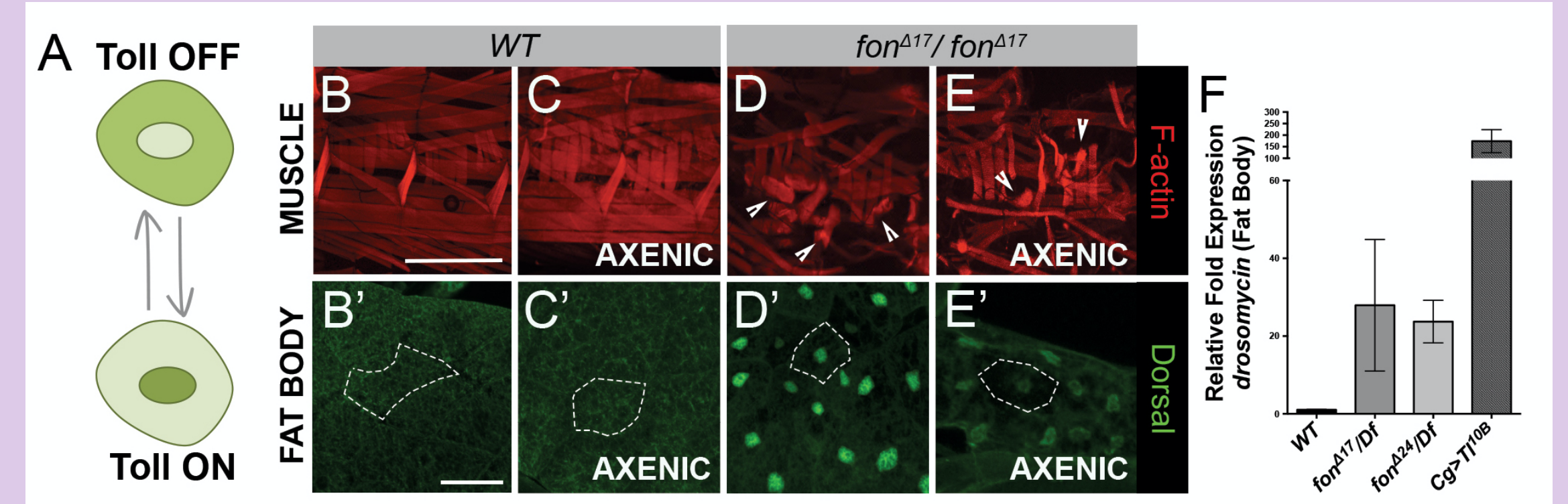

Figure 4. Toll signaling is activated in *fon* mutants.

(A) Translocation of Dorsal (Dl; green) from the cytoplasm acts as a reporter for Toll activation. (B-B') In healthy conditions, larval muscles (red) remain attached and functional via stable junctions comprised of ECM proteins. Immune responses are not required and are inactive in the fat body (green). (D-D') However, in larvae homozygous for the *fon* null allele, *Δ17*, muscles are detached (B,C) and Dorsal translocates to the nucleus as a result of active Toll signaling in the fat body. These trends also hold true in axenic, or germ-free conditions (C-C', E-E') indicating that **muscle detachment and Toll activation caused by loss of fon is pathogen-independent and related to a damage-induced response.** (F) Loss of *fon* is sufficient to induce expression of the Toll-dependent antimicrobial peptide (AMP), *drosomycin*, in the fat body during the systemic immune response.

Figure 5. Genetic interactions between *fon* and Toll pathway components enhance muscle detachment.

Toll pathway members were screened using a *fon*-sensitized background assay. The extent of muscle detachment present upon ubiquitous knockdown of a candidate RNAi is compared to the RNAi knockdown of each transcript in a heterozygous mutant *fon* background. (A, E) Neither *WT* muscles or *fon* heterozygotes have detached muscles. (B-D) Knockdown of RNAi candidates appear to be *WT*. RNAi knockdown of the NFκB inhibitor *cact* (F) or overexpression of the NFκB protein Dorsal (Dl) (G) or Spatzle-processing enzyme (SPE) (H) in a *fon*-sensitized background leads to enhanced muscle detachment (arrows). (I) Quantification of muscle detachment. Overexpression of *Dif* and the constitutively active allele, *Tl[10B]* could not be analyzed due to lethality. (J) Validation of *cact* transcript knockdown. All other stocks have been previously published. **Therefore, activation of Toll signaling in fon-sensitized muscles leads to a breakdown of tissue maintenance processes.**
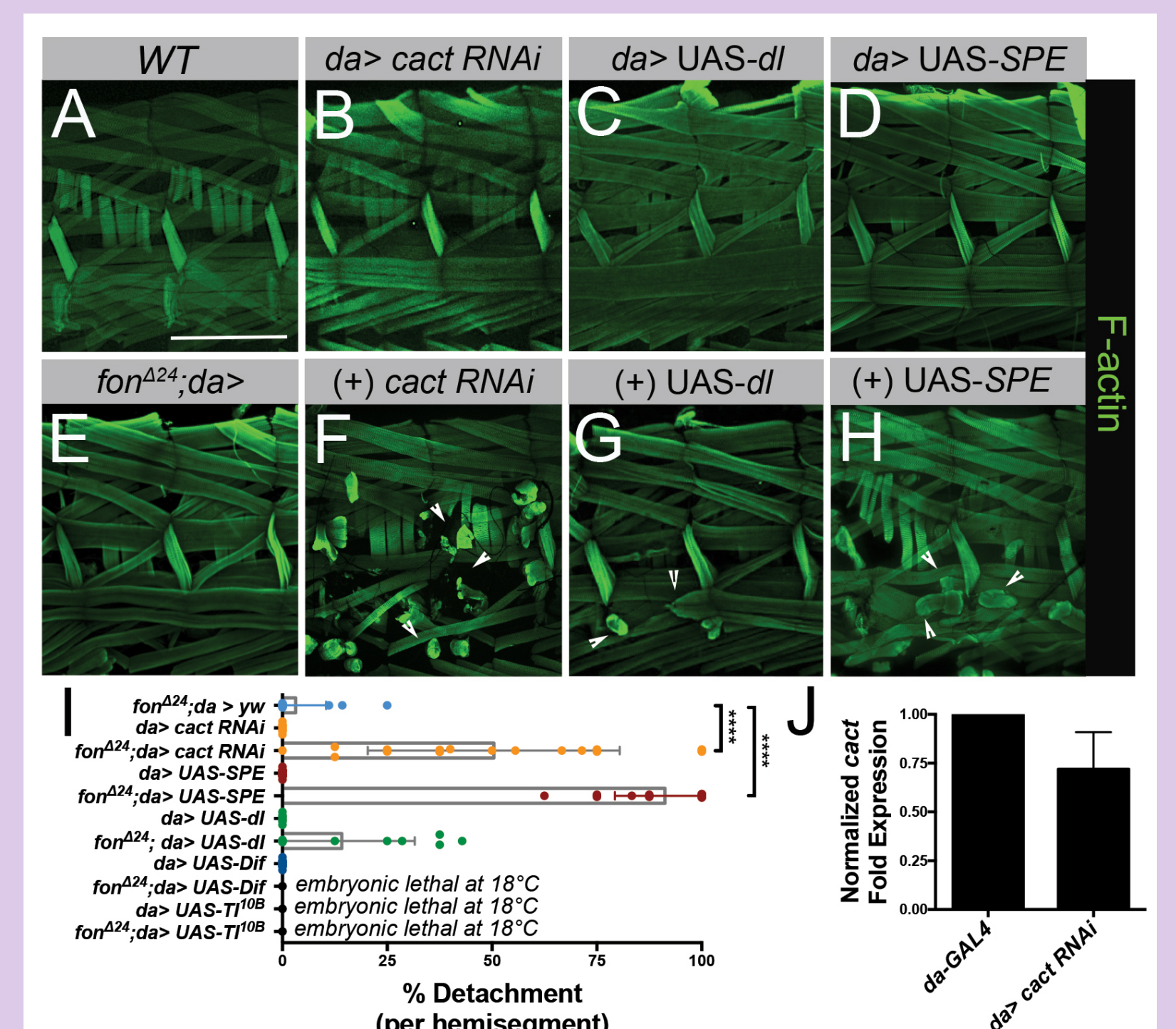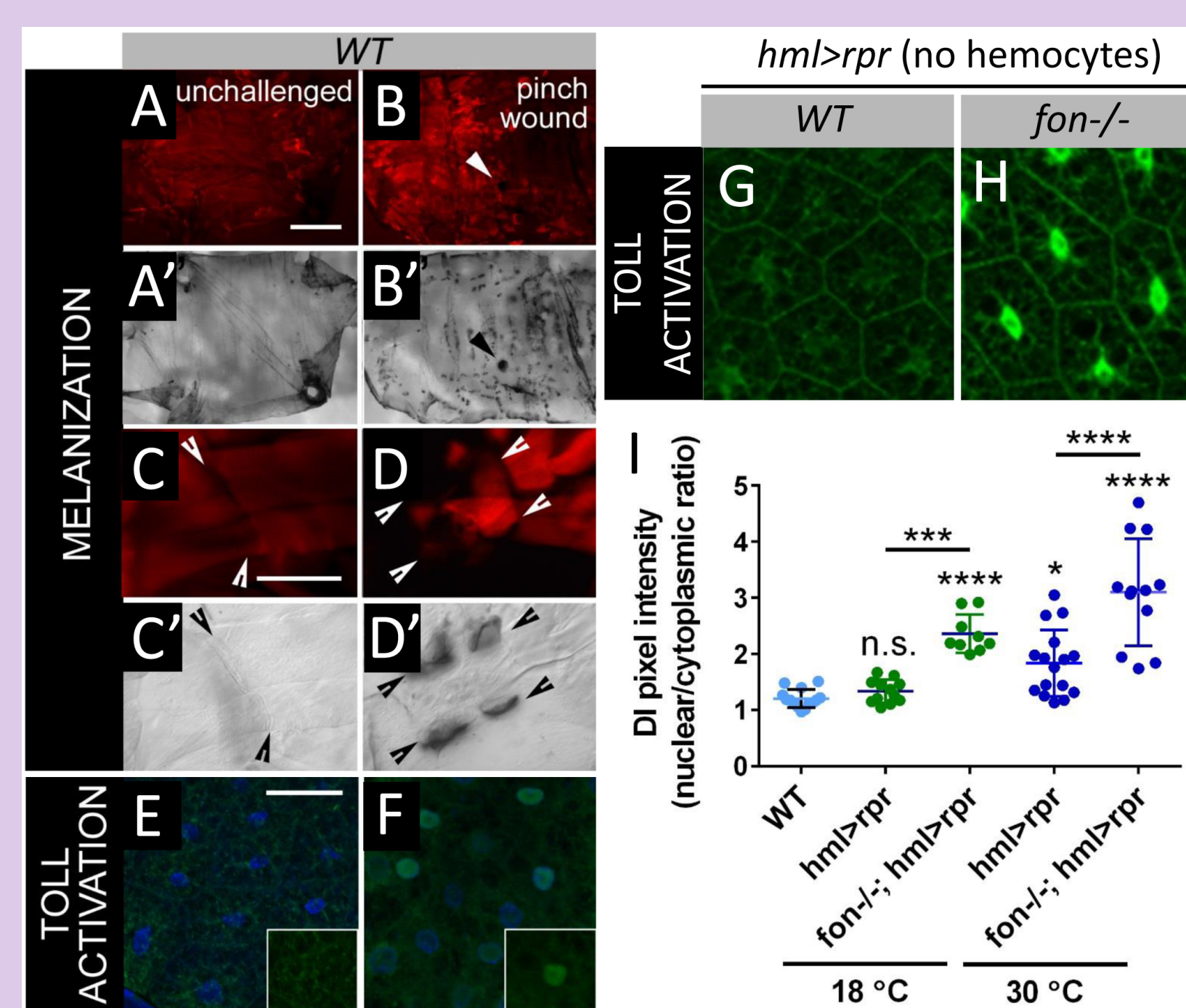

Figure 6. Mechanical stress-induced Toll activation may be hemocyte-independent

(A-D') Muscle morphology (F-actin; red) of dissected L3 fillets which have been dissected in the PO substrate, DOPA, and imaged by DIC for the presence of melanization (black). Muscle damage caused by pinch wounding (site of injury indicated by solid arrow B,B') show melanization near MASs (indented arrowheads) or the ends of damaged muscles as compared to uninjured *WT* muscle fillets which are free of melanin except for areas along the cuticle (A',C'). (E,F) Pinch wounding causes Dl (green) nuclear localization in fat body tissue. A single fat body cell is pictured in insets within each panel. (G-I) Analysis of Dl (green) as a proxy for Toll activation upon hemocyte ablation in a *WT* or *fon-/-* background. The Toll pathway is still activated upon loss of hemocytes and Fon, **suggesting that hemocytes are not necessary for Toll activation upon muscle detachment.** (I) Scatter plot quantitation. Scale bars 100 μm A-B', 500 μm C-D', 50 μm E,F.

Conclusions

- Fon function is integral in the maintenance of muscle attachment sites.
- Loss of Fon results in the activation of key innate immunity features, including melanization, hemocyte recruitment, and nuclear Dl accumulation.
- Fondue interacts with multiple Toll pathway components, specifically genes that result in Toll activation.
- Toll activation can cause muscle detachment in a manner similar to loss of *fon*.
- Systemic Toll activation from local tissue damage may be hemocyte-independent.

FUTURE DIRECTIONS
- Determine the level of hemocytes needed for sufficient Toll pathway activation.
- Determine if Fon binds to Spz to another upstream component to activate Toll signaling.

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