

Forkhead as a cross-regulator of cell death and the innate immune response in the Malpighian tubules of *Drosophila melanogaster*

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Forkhead as a cross-regulator of cell death and the innate immune response in the Malpighian tubules of *Drosophila melanogaster*

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Abstract:

Forkhead (*fkh*) transcription factor derives its name from the distinctive appearance of the DNA-binding motifs when attached to DNA. In mammals, they are grouped into seven families from FOXA to FOXS. FoxA subfamily participates in several essential functions, including the development of various organs, the regulation of senescence, proliferation, and metabolic homeostasis. In this study, we provide evidence that the *Drosophila* orthologue of FOXA, known as *fkh*, is instrumental in preventing programmed cell death in *Drosophila* Malpighian tubules (MTs) during metamorphosis. This characteristic is unique to Malpighian tubules when compared to several other larval tissues. We also demonstrate its role in the immune function of the MTs, as its depletion in MTs suppresses the expression of Anti-Microbial Peptides (AMPs). Furthermore, reduced expression of NF-κB transcription factor, Relish and Dorsal, could contribute to the reduced synthesis of AMPs. *Fkh* also regulates the fluid secretion of the MTs and its absence disrupts the ion transporters thus affecting fluid homeostasis. Our results demonstrate that *fkh* plays an essential role in the survival of MTs, without that the MTs cannot survive and fail to mount an effective immune response.

Keywords- *Forkhead, FOXA, Drosophila, Malpighian tubules, Apoptosis, Immunity*

(1) Introduction :

Forkhead box (FOX), is an evolutionarily conserved gene family, and the name is derived from the *Drosophila melanogaster forkhead* gene (*fkh*), whose mutants exhibit head fold involution defects resulting in a spiked head appearance in adult flies [1]. The distinctive feature of this family of transcription factors is the Forkhead domain, a sequence of 110 amino acids, forming a helix-turn-helix motif or winged helix [2]. Different members of the *FOX* family have been allocated subclasses *FOXA* to *FOXN*, which is based on conserved Forkhead domain amino acid sequence phylogenetic analysis. These transcription factors participate in different biological processes ranging from embryonic development to the regulation of metabolism, cell death, growth, and organismal lifespan [3]. In *Drosophila*, *fkh* gene is an ortholog of the *FOXA* subfamily, and is closely related to *FOXA1* in humans and *FOXA3* in rats [4]. Similar to *fkh*, the *FOXA* proteins of the mammals are necessary for early embryonic development, the formation of specific organs, and they modulate specialized functions of differentiated tissues [5]. *FOXA2* is also necessary for the formation of gut [6], and both *FOXA1* and *FOXA2* are required for the development of endoderm-derived tissues, such as the pancreas, liver, prostate and lungs [5]. In *Drosophila*, *fkh* is necessary for normal development of the ectoderm and endoderm-derived parts of the gut, the larval salivary glands and the Malpighian tubules [7,8]. Furthermore, it plays an important role in the expression of *salivary gland secretion* protein genes (*sgs*), which are also known as glue proteins [9]. *Fkh* has a crucial role in regulating programmed cell death in a tissue-specific manner. It inhibits steroid-mediated cell death by repressing genes associated with steroid-triggered cell death genes and by up-regulating Akt activator PDK1 [10].

Fkh is negatively regulated by the pre-pupal ecdysone pulse, mediated through the early ecdysone-induced gene, *Broad-Complex (BR-C)* [10,11]. In addition to regulating the essential apoptotic gene expression, *fkh* also regulates the entire cassette of autophagic genes [12]. It has also been reported that *fkh* is involved in the regulation of innate immunity, independent of canonical immune signaling pathways in *Drosophila melanogaster* [13] and *Manduca sexta* [14].

Malpighian tubules consist of two pairs of single-layered epithelial tubes that develop at the midgut–hindgut junction during embryogenesis. These two tubules are divided into six functionally and structurally different segments: the initial, transitional, main, lower tubule, upper ureter, and lower ureter. The fluid secretion is modulated through the coordinated activity of two major cell types, ectodermally derived Type I or principal cells (PCs) and mesodermally derived Type II or stellate cells (SCs) [15]. PCs are enriched with V-ATPase (vacuolar H-ATPase), as well as K^+/H^+ exchangers [16,17] and K^+ channels [18] in the apical microvilli,

while Na⁺/K⁺-ATPase [19] and the Na⁺/K⁺/2Cl⁻ cotransporters [20] are present on the basolateral membrane, collectively, they ensure effective potassium transport through PCs. The ensuing imbalance of potassium is offset by the chloride ion movement through chloride channels in the SCs, leading to a net movement of water from the hemolymph through the aquaporin (Drip) in SCs, as well as through paracellular routes [21]. MTs are unique organs because unlike most larval tissues, they escape ecdysone-induced apoptosis during metamorphosis and the tubules formed during embryogenesis persist into the adult stage [22]. MTs of *Drosophila* primarily function in detoxification and osmoregulation and in addition to playing an important role in immune response [23,24]. The immune response in MTs is developmentally regulated and they exhibit a constitutive immune response independent of infection [24].

The results presented in this paper demonstrate a substantial increase in *fkh* expression in MTs as development proceeds from larval to adult stage. The loss of *fkh* leads to cell death within the tubules, resulting in lethality. Additionally, the fluid secretion is adversely disrupted in the absence of *fkh*. In addition, we observed that *fkh* positively regulates the production of AMPs. The identification of *fkh* binding sites in the promoter regions of genes responsible for immune response and ion transport clearly indicates an interconnected link between these two processes orchestrated by *fkh*.

(2) Material Methods:

2(a) Fly Stocks: The fly stocks used for the study were obtained from Bloomington Stock Centre except where mentioned. *c42Gal4* was used as a control. Principal Cell-specific Gal4 driver, *c42*; stellate cell-specific Gal4 driver, *c649* (a kind gift from Dr. J. A. T Dow, Institute of Biomedical Sciences, University of Glasgow, UK), *UAS-Fkh RNAi* (a kind gift from Dr. Margret H. Bulow, University of Bonn, Bonn, Germany), *UAS-Fkh RNAi* (BDSC no. 33760). All the stocks in this study were maintained at 24±1° C and on standard food media.

2(b) Antibodies and Immunostaining: MTs from desired stage and genotype were dissected in 1X PBS, fixed in 4% PFA for 20 minutes at Room temperature, washed in 0.1% PBST (1X PBS, 0.1% Triton X-100), blocked in blocking solution (0.1% BSA, 0.1% Triton X-100, 10% FCS, 0.02% thiomersal, 0.1% deoxycholate) for 2 hours at Room temperature. Tissues were incubated overnight in primary antibody at 4° C. After three washes with 0.1% PBST (20 minutes each), again tissues were blocked for 2 hours at Room temperature and incubated in secondary antibody for 2 hours. Then tissues were washed in 0.1% PBST and counterstained with DAPI (1 mg/ml, Molecular Probe) for 30 minutes. Again, washing was done in 0.1%

PBST and mounted in antifadant, DABCO (Sigma). The primary antibodies used were Relish (1:50, catalog number: AB_1553772, Developmental Studies Hybridoma Bank {DSHB}), Broad-core (1:50, catalog number: AB_528104, DSHB), EcR-B1 (1:20, catalog number: AB_2154902, DSHB), Dorsal (1:20, catalog number: AB_528204, DSHB), Vacuolar H⁺-ATPase (1:50, catalog number : AB_10805887 , DSHB), Drip (1:1000, a Kind gift from Dr. J.A.T Dow and Na⁺/K⁺ ATPase (1:20, catalog number, AB_2166869, DSHB). All the pictures were taken from Zeiss LSM 510 Meta confocal microscope and images were labelled and arranged using Adobe Photoshop 7.

2(c) Microscopy and Documentation: To take the image of whole larvae and pupae, a Sony Digital Camera (DSC-75) attached to a Zeiss Stemi SV6 stereo binocular microscope was used. For fluorescence and light microscopy, a Nikon E800 microscope was used. The images were taken with a Nikon DXM 1200 digital camera. Different objectives used were 10X, 20X etc. Confocal imaging was carried out LSM510 Meta Zeiss laser scanning confocal microscope, using appropriate filters and dichroics. All the images were assembled using Adobe Photoshop 7.0.

2(d) Quantitative Reverse transcription –PCR: For semi-quantitative RT-PCR and qRT-PCR analysis, total RNA was isolated from desired tissues (Malpighian tubules and salivary glands) from third instar larvae or 12-14 hours APF pupae of the desired genotypes using TRIzol followings the recommended protocol of the manufacturer (Sigma- Aldrich, India). RNA pellets were resuspended in nuclease-free water and quantified by Nano- drop. The RNA samples (1 µg) were incubated with 2U of RNase-free DNaseI (MBI Fermentas, USA) for 30 minutes at 37°C to remove any residual DNA. First-strand cDNA was synthesized using 1-2 µg of total RNA. The prepared cDNAs were subjected to semi-quantitative RT-PCR or real-time PCR using forward and reverse primer pairs as listed below. Real-time qPCR was performed using 5µL qPCR Master Mix (SYBR Green, Thermo Scientific), 2 picomol/µL of each primer per reaction in 10 µL of final volume in ABI 7500 Real-time PCR machine.

(50 ng), 25 pM each of the two specified primers, 200 µM of each dNTP (Sigma Aldrich, USA) and 1.5U of *Taq* DNA polymerase (Geneaid, Bangalore).

Primer sequences:

Drosocin (F): TTTTCCTGCTGCTTGCTTGC

(R): GGCAGCTTGAGTCAGGTGAT

mtk (F): GCTCAATCAATTCCCGCCAC

(R): CGAAAATGGGTCCCTGGTGA

dip (F): AGTTCACCATTACCGTCGCC

(R): GTAGGTGTAGGTGCTTCCCA
G3PDH (F): CCACTGCCGAGGAGGTCAACTA
 (R): GCTCAGGGTGATTGCGTATGCA
att (F): GATGGACGTGCTAATCTCTG
 (R): GGCTTAGCCGAAATGATGAG
cec (F): CGGGATGCAACCATTC AAGG
 (R): CCACATTGGCGGCCTGTT

2(e) Lethality Assay: Freshly hatched synchronized 1st instar larvae of both control and experimental genotype were collected within an hour interval and transferred to food vials **gently** and reared at 23±1°C. The total numbers of larvae that pupated and subsequently emerged as flies were counted in each case. At least eight replicates of 25 larvae each were examined in each case.

2(f) Next Generation RNA sequencing: Total RNA was isolated from 40 pairs of Malpighian tubules and salivary glands of the desired stage, using TRIzol (Invitrogen, USA) reagent. 1µg of the isolated RNA was subjected to DNase treatment using 2U of Turbo TM DNase (Ambion, Applied Biosystem) enzyme for 30 minutes at 37°C. The reaction was stopped using 15mM EDTA followed by incubation at 65°C for 5-7 minutes and purification on RNAeasy column (Qiagen). The purified RNA samples were processed for preparation of cDNA libraries following the standard protocol of the TruSeq Stranded Total RNA RiboZero H/M/R (Illumina) and were sequenced on HiSeq-2500 platform (Illumina) using 50bp pair-end reads, 12 samples per lane and each sample run across 2 lanes. This resulted in a sequencing depth of ~20 million reads. Biological duplicate or triplicate samples were sequenced in each case. The resulting sequencing FastQ files were mapped to the *Drosophila genome* (dm6) using **Tophat** with **Bowtie**. The aligned SAM/BAM file for each was processed for guided transcript assembly using Cufflink 2.1.1 and gene counts were determined.

Genes differentially expressed between experimental and control genotypes were categorised into various GO terms using **DAVID bioinformatics Resources 6.8** and/or **GORILLA, Gene Ontology enRIchment analysis and visualization tool** for gene ontology search. To find out the distribution of differentially expressed genes into various groups, Venn diagrams were prepared using the **Venny2.1** software.

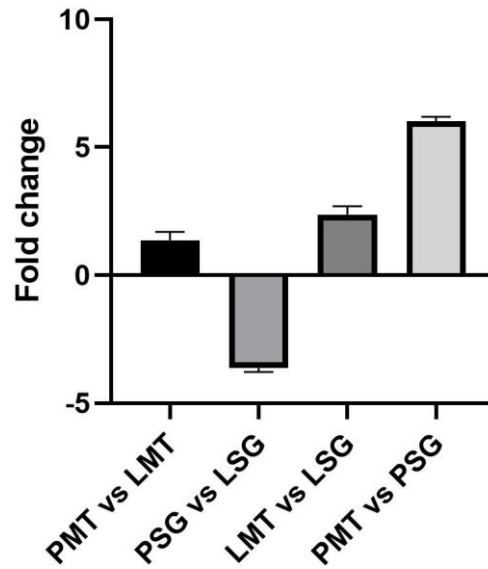
2(g) Statistical analysis: All statistical significance tests were calculated with Graph pad software using two-tailed Student's t-test. Data presented as mean $\pm$ standard deviation as values from at least three independent experiments. P values <0.05 (*) were considered as statistically significant.

3. Results:

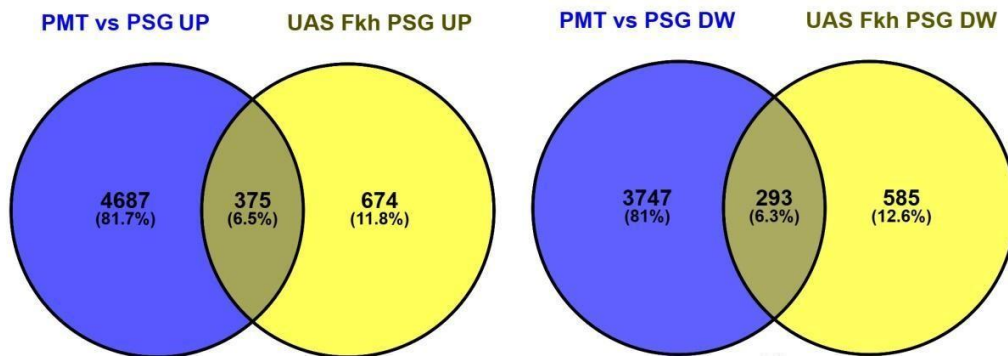
3.1. Comparative analysis of *fkh* expression in MTs and salivary glands during different stages of development

In our previous report, we delineated the differential gene expression in the Malpighian tubules of *Drosophila melanogaster*, by comparing them with *Drosophila* salivary glands [25]. This was done because salivary glands undergo ecdysone-induced cell death during metamorphosis, while Malpighian tubules escape cell death and continue in the adults. The rationale for this comparison was to decipher the probable reasons for the differing fates of the two larval tissues. Transcriptome analysis revealed consistently enhanced expression of *fkh* in MTs during larval and pupal stages [25].

Before further analysis of *fkh*, we confirmed *fkh* expression by q-PCR in MTs and salivary glands (SGs). The analysis was done at 110-112 hrs After Egg Laying (AEL), larval Malpighian tubules (LMT) and larval salivary glands (LSG), and 12-14 hrs pupal Malpighian tubules (PMT) and pupal salivary glands (PSG). Corroborating with the transcriptome data, a consistently high expression of *fkh* in LMT and PMT was observed, and there was a significant drop in expression in PSG compared to LSG, following late larval ecdysone pulse (-4 hrs APF) (Fig.1.A). This comparison shows an increase in the expression of *fkh* in Malpighian tubules at the pupal stage compared to the larval stage. On the other hand, in salivary glands, *fkh* was downregulated at the pupal stage compared to the larval stage. The increased expression of *fkh* in MTs, both at larval and pupal stages, in comparison to SGs, suggests that the elevation of *fkh* in MTs is necessary to prevent it from undergoing apoptosis during metamorphosis. These findings were consistent with earlier observations that *fkh* prevents apoptosis and promotes cell shape change during formation of the salivary glands [8].



(A)



(B)

(C)

Fig.1.A- C. Expression profile of Fkh in Malpighian tubules and salivary glands. qRT-PCR of Fkh in control MTs and SGs at 110- 112hour AEL larval 12-14hour APF pupal stages (A). Venn diagram showing common Fkh responsive genes in MTs. The number of commonly altered genes between the two comparisons is shown by the Venn diagram. PMT vs PSG represents the number of differentially expressed genes of PMT in comparison to PSG, while UAS Fkh PSG, represents the number Fkh responsive genes in PSG, as reported earlier by Liu and Lehmann, 2008. There are 375 genes commonly upregulated in PMT vs PMT UP (Blue), and UAS Fkh PSG UP (Yellow) (B); while, 293 genes are commonly downregulated in PMT vs LSG DW (Blue) and UAS Fkh PSG DW (Yellow) (C).

3.2. In silico analysis of Forkhead responsive genes in Malpighian tubules:

Fkh is a crucial suppressor of apoptosis, and we aimed to gain a comprehensive understanding of the genes expressed in response to *fkh*. We utilized a study wherein the authors over-expressed *fkh* in salivary glands and identified genes that were expressed in response to *fkh*, and designated them as '*fkh*-responsive genes'[12]. *Fkh* is a known pro-survival gene [26], and functions as a transcription factor. Our reasoning is based on the understanding that this comparison will allow us to identify genes that are transcribed in response to *fkh* in the MTs. At the pupal stage, the *fkh* levels are high in pupae in comparison to larvae, suggesting an increase in *fkh*-responsive genes. The rationale behind our analysis was to identify genes that may contribute to the survival of Malpighian tubules during pupation. As it is known that at this stage, physiological activity in MTs is minimal, it allowed us to hypothesize that the genes being expressed are necessary for both survival as well as immune response. In the salivary glands, the ectopic expression of *fkh*, will cause expression of the *fkh*-responsive genes. Since comparison was between similar stages, we did not expect context-dependent differences. Therefore, we compared the transcriptome of the Malpighian tubules and salivary glands from the pupa [25] with the data obtained by Liu and Lehmann, 2008.

A comparison between pupal MTs and larval MTs (PMT vs. LMT) showed an increase in *fkh* expression in pupal MTs relative to larval MTs, suggesting that the gene expression in pupal Malpighian tubules truly represents *fkh*-responsive genes. A total of 5062 genes were found to be upregulated in PMT vs PSG, and this group was compared with 1022 genes upregulated in response to *fkh* overexpression in SGs (Fig.1.B). Out of these, 375 genes were found to be commonly upregulated in both the groups. On the other hand, 3747 genes were downregulated in PMT vs PSG, while 585 genes were downregulated in response to *fkh* in SGs, (Fig.1.C). Among these, 293 genes were commonly downregulated. These genes, which are regulated in both comparisons are likely to represent the *fkh*-responsive genes in MTs.

3.3. Functional annotation of Forkhead responsive genes in Malpighian tubules

Using the David Bioinformatics Resources 6.8 annotation tool, we analyzed the commonly identified genes to categorize the major pathways affected by *fkh* in MTs based on their functions (Table 1). This comparison (PMTs vs. PSGs) is particularly informative because it allows us to highlight differentially expressed genes (DEGs) that may be associated with survival mechanisms in MTs, in contrast to the apoptotic response observed in SGs. It is important to note that while PCD (Programmed cell death), autophagy signaling, apoptotic pathway, and steroid hormone-mediated signaling, were found to be downregulated; genes related to innate immune response and protein glycosylation pathways were upregulated. The two pathways that could be of relevance to the Malpighian tubules are components of the immune system and cell death

pathways, which are unique to MTs [23,24]. These findings suggest that *fkh* plays multiple roles in the pathways involved in the function and survival of MTs.

Table 1. Major pathways affected from the list of common (upregulated and down regulated) Fkh responsive genes in Malpighian tubules (based on the DAVID bioinformatics Resources 6.8 functional annotation gene ontology search tool).

GO groups	Genes	Status
Humoral immune response	EIP75B, RAB11, MTK, HOP, JRA, PVR, KEY	Upregulated
Regulation of immune system process	WUN, SPN27A, CAT, CG1572, HOP, JRA, BRM, SOD2, PVR, KEY, CG32795, SMT3	Upregulated
Protein glycosylation	GALNAC-T1, CG8668, CG9536, PGANT3, PGANT6, CG30463, PGANT5	Upregulated
Apoptotic signaling pathway	CORP, JAFRAC2, RPR, HPO, EIP93F, CG12384, MIRO	Downregulated
Programmed cell death	CP1, CG3829, MMP1, HPO, MOD(MDG4), EIP93F, EIP74EF, AKAP200, LARP, OBP99D, CG9769, OBP99B, JAFRAC2, RPR, CG8149, CORP, AGO2, DOA, CG12384, BUN, IP3K2, ECT3, ATG7, MIRO	Downregulated
Autophagic cell death	CP1, CG3829, MMP1, EIP93F, EIP74EF, AKAP200, LARP, OBP99D, CG9769, OBP99B, RPR, CG8149, AGO2, DOA, BUN, IP3K2, ECT3, Atg7, Atg5	Downregulated
Steroid hormone mediated signaling pathway	EIP75B, HR39, HR38, EIP78C	Downregulated

3.4. *Fkh* is necessary for blocking cell death in MTs

Forkhead is essential for the survival of cells during programmed cell death, and its downregulation during metamorphosis is necessary in larval tissues undergoing apoptosis. To investigate the role of *fkh* in preventing cell death in MTs, its expression was reduced using the UAS-GAL4 system [27]. MTs-specific Gal4 driver was used to drive *UAS-Fkh RNAi*. Statistical analysis indicated 77% died at the pharate adult stage and only 23% of them eclosed as adults (Fig.2.A). The loss of *fkh* is also associated with cell death in salivary glands [10,28].

(A)

Genotype	No.of 1 st instar larvae transferred	No. of pupae formed	No. of flies hatched	Total lethality (%)
<i>c42Gal4</i>	311	308	308	0.96%
<i>c42Gal4>UAS Fkh RNAi</i>	509	506	116	77.27%



(B)

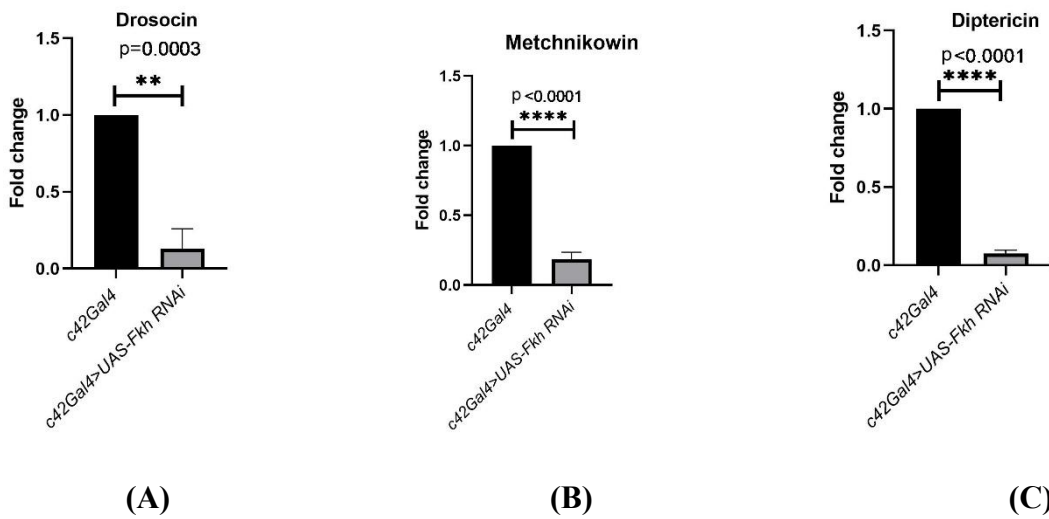
Fig.2. Fkh has a vital role in Malpighian tubule's survival. (A) Table showing percentage of lethality in control and *c42Gal4>UAS-Fkh RNAi*. (B) Image of day 10 pupa of control (left) and *c42Gal4>UAS-Fkh RNAi* (right). Control pupa emerges into fly while *c42Gal4>UAS-Fkh RNAi* pupa dies, where arrow indicates shrunken pupae inside the pupal case, showing the sign of desiccation.

3.5. Fkh-mediated activation of Toll and IMD pathways in the MTs

Fkh is known to regulate a subset of anti-microbial peptides (AMPs) in the major immune organs of *Drosophila*, the fat bodies and gut [13]. Since MTs are also immune organs [29], we examined the expression of AMPs mediated by the Toll and IMD pathways using RT-PCR analysis. A substantial decrease in the expression of *drosocin*, *metchnikowin* and *diptericin*, was observed in *c42Gal4>UAS-Fkh RNAi* larval MTs (Fig.3. A, B, C), whereas expression of other AMP remained unchanged (Data not shown). These observations are consistent with the previous finding highlighting the differential expression of AMPs in MTs [30].

We next investigated the expression of Relish, a NFkB transcription factor, regulating the IMD pathway. Under uninfected conditions, Relish is predominantly localized in the cytoplasm which, upon infection is activated and translocated to the nucleus [31]. In MTs there is a continuous synthesis of basal level of AMPs,

therefore in some cells the expression of Relish is observed in the nucleus as well, which increases following pathogenic challenge [30,31]. Immunostaining with an anti-Relish antibody indicates that there is a decrease in the expression of Relish in *c42Gal4>UAS-Fkh RNAi* larvae (Fig.3.E), in comparison to *c42Gal4* MTs (Fig.3.D). We also observed that its localization is restricted to cytoplasm in most of the cells in the absence of *fkh* compared to normal condition. After assessing the expression of Relish, we proceeded to examine the expression of Dorsal by immunostaining using anti-Dorsal antibody, which is another NFkB transcription factor, regulating the Toll pathway. We observed that the localization remained unchanged and it was present in the nucleus as well as cytoplasm. However, we observed a reduction upon *fkh* depletion in the 3rd instar larval MTs as compared to *c42Gal4* larval MTs (Fig.3.J'). Together these results provide strong indication that the diminished expression of AMPs can be a direct consequence of the decreased levels of Relish and Dorsal following *fkh* depletion. Since, *fkh* also regulates the immune response, we subjected *c42Gal4 >UAS Fkh RNAi* flies to pathogenic challenge to check the survival. Infection was done by continuous feeding on *E. coli* rather than septic injury. A significant reduction in survival of *c42Gal4>UAS Fkh RNAi* flies was observed after they were continuously fed a high dose of live *E. coli*-contaminated food (supplementary figure 1).



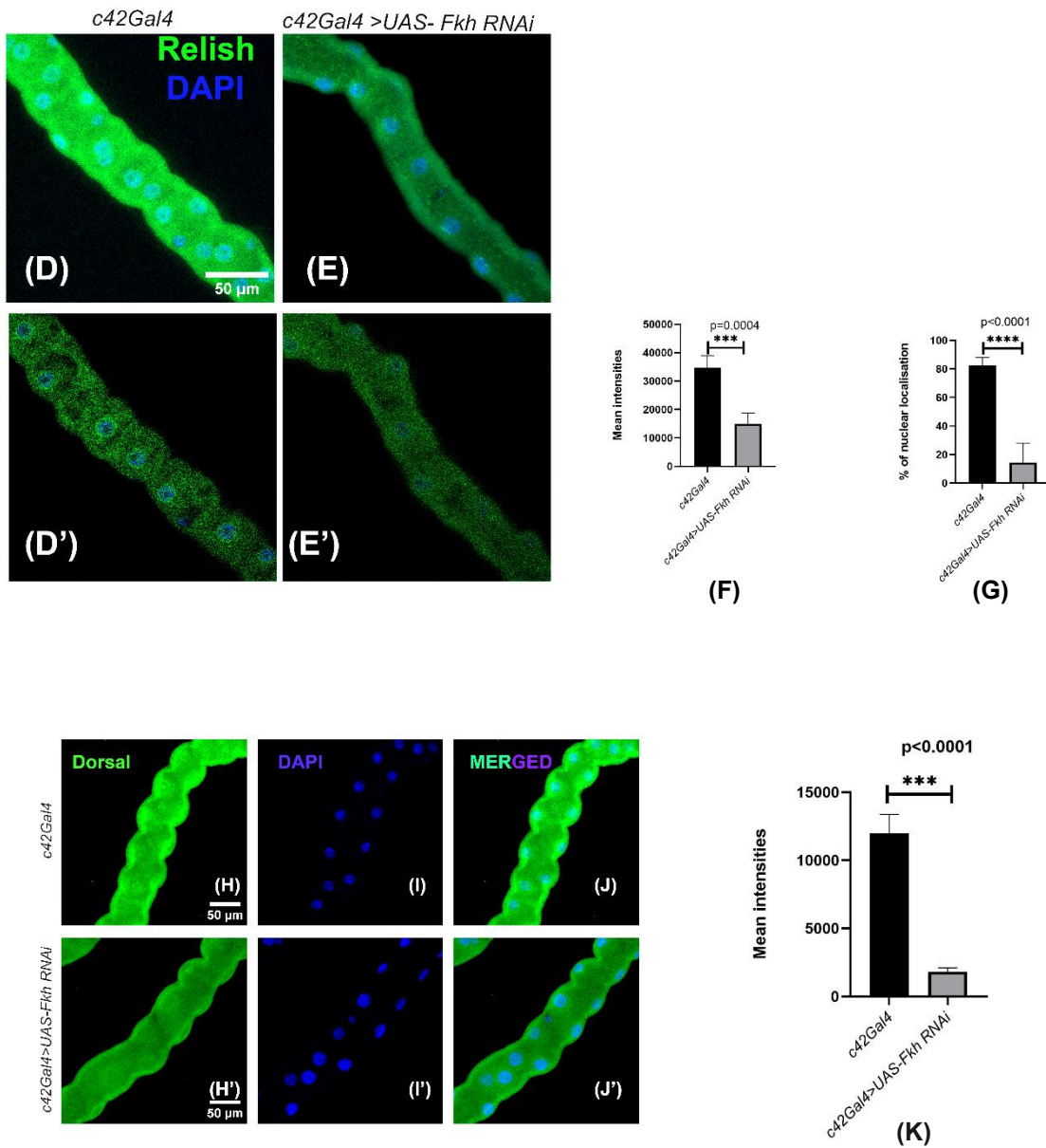


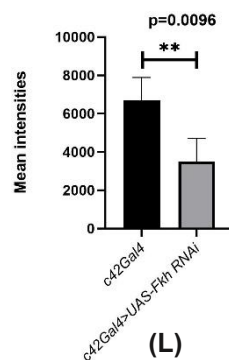
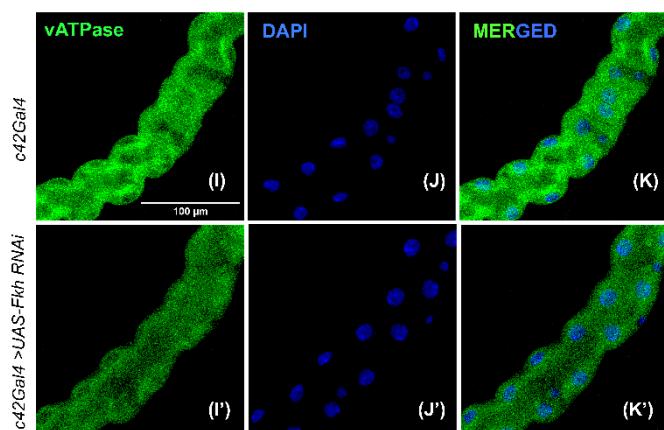
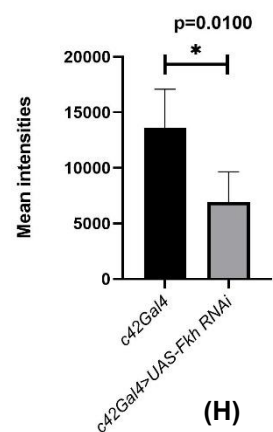
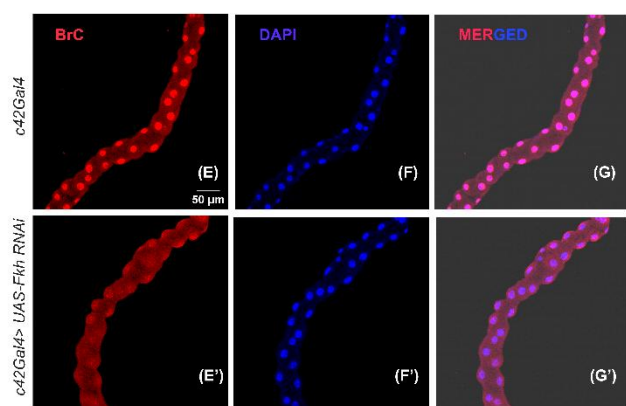
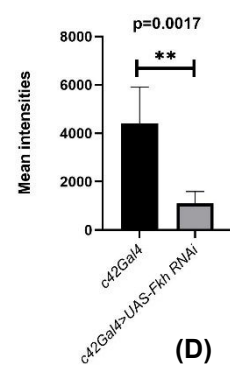
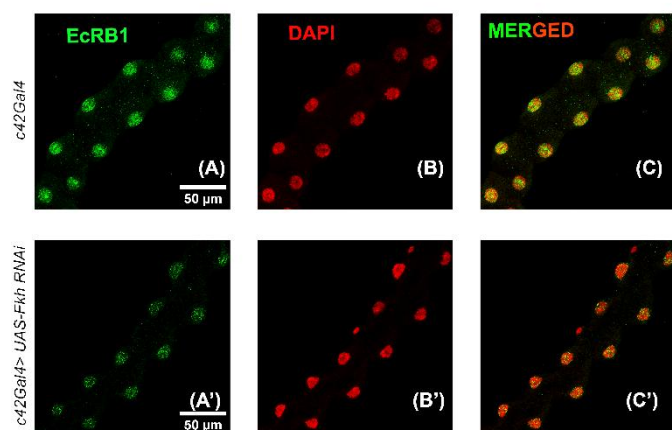
Fig.3. Reduced expression of AMPs, Relish, and Dorsal in MTs on Fkh depletion. RT-PCR analysis of transcript level of AMPs in MTs from third instar larvae of *c42Gal4* and *c42Gal4 >UAS-Fkh RNAi*. *drosocin*, *metchnikowin* and *diptericin* showed reduced expression in *c42Gal4 >UAS-Fkh RNAi*, to that of *c42 Gal4* (A, B, C, respectively). ‘*’ indicates $p=0.0003$, and $p<0.0001$.

Relish gets localized in cytoplasm in *c42Gal4 >UAS-Fkh RNAi* larval MTs (E’), whereas it localized in cytoplasm and nucleus in control MTs (D’). The expression of Relish is also decreased after Fkh depletion (E). Chromatin was counterstained with DAPI. (D) and (E) images are projections of optical sections whereas (D’) and (E’) are the single optical section images obtained by Zeiss LSM 510 Meta Confocal microscope. Scale bar is 50 μ m. Intensity profile shows decreased expression of Relish (F) and graph (G) represents % of nuclear localization of Relish. ‘*’ indicates $p=0.0004$ and $p<0.0001$.

Dorsal localized both in the cytoplasm and nucleus in 3rd instar larval MTs. There is no change in the localization of Dorsal upon Fkh depletion, however in *c42Gal4>UAS-Fkh RNAi* its expression gets reduced in 3rd instar larval MTs (H') as compared to *c42Gal4* larval MTs (H). All images are the projection images of optical sections obtained by Zeiss LSM 510 meta confocal microscope. Scale bar is 50µm. Graph is showing decreased level of Dorsal (K). '**' indicates $p < 0.0001$.

3.6. Reduced expression of Ecdysone Receptor and Broad-core receptor and ion channels in the absence of *fkh* in Malpighian tubules-

In MTs, ecdysone signaling regulates immune response pathways. Ecdysone binds to EcR-B1 and B2 receptors, which then activates Broad-complex (Br-C). Br-C regulates the IMD pathway by activating the Relish and physically interacting with it to activate AMPs expression [30]. We checked the expression of EcR-B1 by immunostaining using Anti-EcR-B1 antibody and a reduction in expression was observed in *c42Gal4>UAS-Fkh RNAi* larval MTs (Fig.4.A') as compared to *c42Gal4* (Fig.4.A). We next checked the expression of Br-C by immunostaining using anti-Br-C antibody and observed its expression to be greatly reduced in *c42Gal4>UAS-Fkh RNAi* larval MTs as compared to *c42 Gal4* (Fig.4.E'). These results together show that the *fkh* could be the downstream target of the ecdysone pathway to regulate AMP. We next looked into the expression of ion transporters such as V-ATPase, Drip and Na⁺/K⁺ATPase, as they are crucial for fluid secretion [19,20,21,32,], and have been reported to regulate the immune response [30]. Depletion in the level of V-ATPase (Fig.4.I'), Drip (Fig.4.M') and Na⁺/K⁺ATPase (Fig.4.Q') in the *c42Gal4>UAS-Fkh RNAi* larval MTs, respectively indicated that *fkh* plays an important role in fluid secretion as well as immunity regulation of MTs.



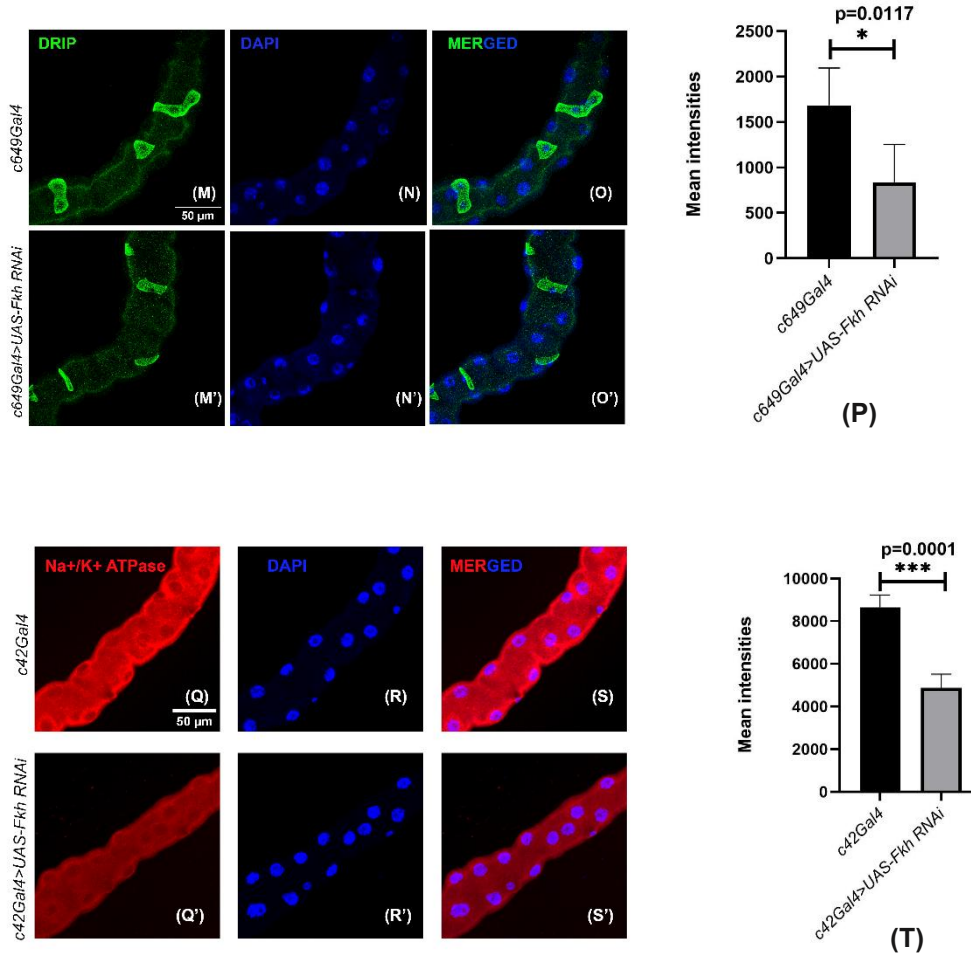


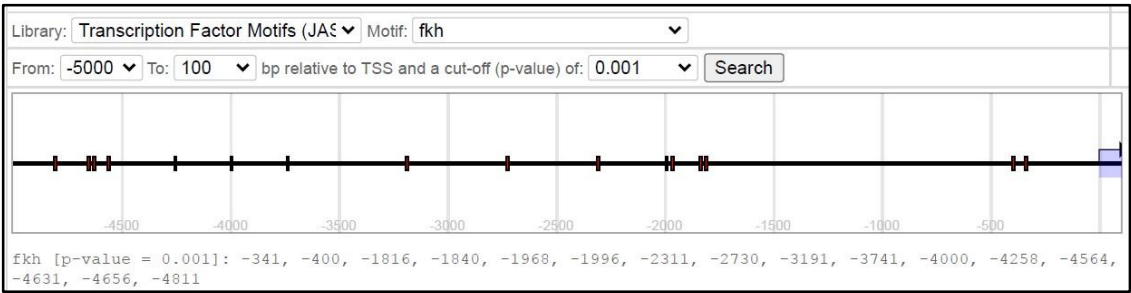
Fig.4. Depletion of EcR, Br-C, V-ATPase, Drip and Na⁺/K⁺-ATPase after decrease in Fkh. EcR level gets reduced in *c42Gal4>UAS-Fkh RNAi* larval MTs (A') as compared to *c42Gal4* (A). Nuclei are counterstained by DAPI (pseudo-red) (B&B'). All images are the projection images of optical sections obtained by Zeiss LSM 510 meta confocal microscope. Scale bar is 50μm. Intensity profile shows reduced expression of EcR (D) '**' indicates p=0.0017. The expression of Br-C gets reduced in *c42Gal4>UAS-Fkh RNAi* larval MTs (E') as compared to *c42Gal4* larval MTs (E), depicts that localization of Relish in nucleus is restricted because of low level of Br-C in Fkh depleted condition. Nuclei are counterstained by DAPI (F and F'). All images are the projections of optical sections obtained by Zeiss LSM 510 Meta Confocal microscope. Scale bar is 50μm. Graph is showing decreased level of Br-C (H). '**' indicates p=0.0100.

Downregulating Fkh causes depletion of V-ATPase (I'), Drip (M') and Na⁺/K⁺-ATPase (Q') respectively, in *c42Gal4>UAS-Fkh RNAi* larval MTs. Nuclei are counterstained by DAPI. Scale bar is 100μm (image I to K) and 50μm (image M to O), and 50μm (image Q to S), respectively. Graph is showing decreased expression of V-ATPase, Drip, and Na⁺/K⁺-ATPase (L, P and T). '**' indicates p=0.0096, p=0.0117, and p=0.0001 respectively.

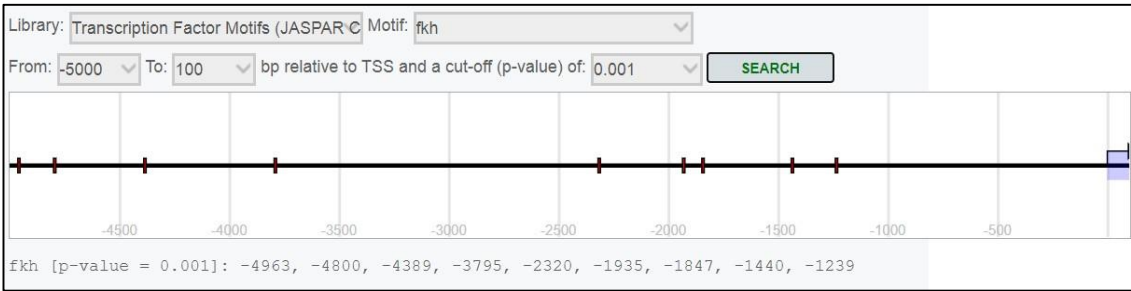
3.7. Fkh binding sites are present in genes regulating fluid secretion and immune response

Fluid secretion depends on the correct functioning of different ion channels. It is well established that V-ATPase, Na pump, and Drip are essential for the formation and regulation of fluid secretion [19,20,21,32] and on the other hand ion channels also play an important role in immune response [30], thus it was hypothesized that *fkh* could regulate ion channels expression. Hence, upstream regulatory regions of V-ATPase subunit genes, Na pump subunit genes, and Drip were scanned using Transcription factor motifs (JASPAR CORE 2018 DATABASE), to find out binding sites for *fkh*. The presence of many *fkh* binding sites in these genes ensured the involvement of *fkh* in the regulation of ion channels mediating urine secretion and immune response of MTs (Fig. 5.A-C). Next, we checked the promoter region of Relish (Fig.5.D) for *fkh* binding sites, which also exhibited multiple *fkh* binding sites, again suggesting a role of *fkh* in regulation of Malpighian tubules’s immune response. These results suggested an important role of *fkh* in fluid secretion and immune response in MTs.

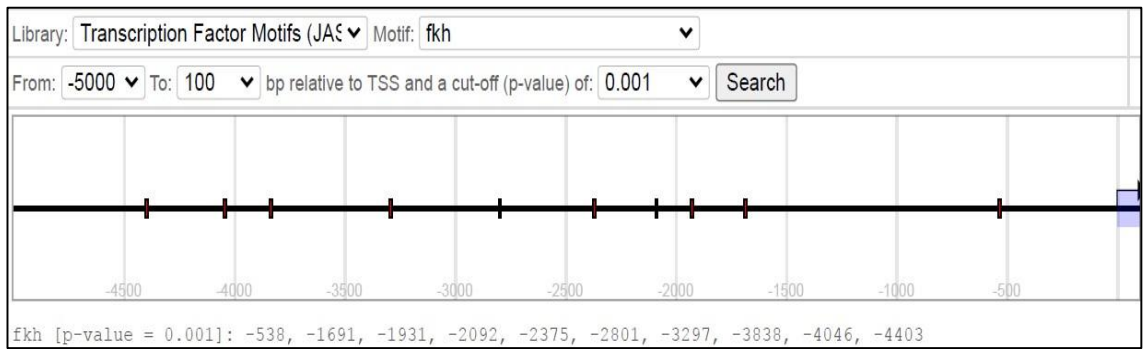
5. A Vacuolar H[+]-ATPase SFD subunit (VhaSFD):



5.B Na pumpalpha subunit (Atpalpha):



5.C Drip:



5.D Relish:

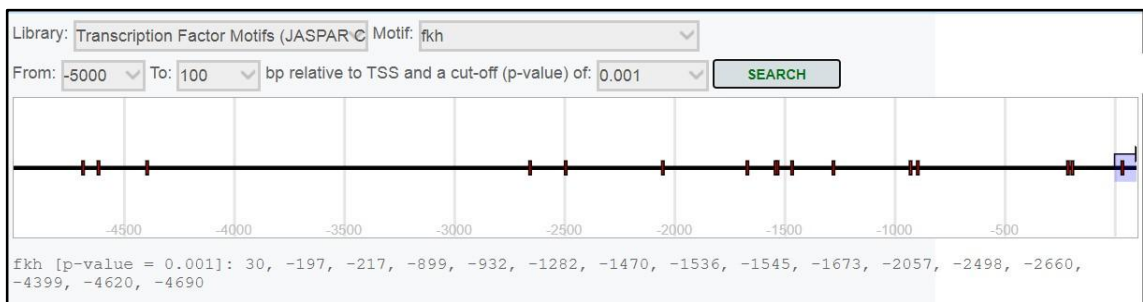


Fig.5. Images showing transcription factor binding sites of Fkh in (A)Vha; (B) Na pump; (C) Drip; (D) Relish. Search for transcription binding sites was done using Transcription factor motifs (JASPAR CORE 2018 DATABASE), with a p-value of 0.001.

4. Discussion

In the present study, we show that *fkh* is the unifying factor for two unique properties of Malpighian tubules. First, it is vital for inhibiting program cell death in Malpighian tubules of *Drosophila*, which allows them to escape ecdysone-mediated cell death during metamorphosis. Second, it regulates the AMP secretion by the Toll and IMD pathway. The results demonstrated in this article place *fkh* as a critical regulator of cellular properties that define the survival and immune response of MTs.

Fkh suppresses PCD in larval salivary glands by repressing the expression of apoptotic genes, *rpr* and *hid* [33], and its downregulation by ecdysone pulse during the prepupal-pupal stage, releases the brakes on *reaper* and *hid*, leading to the onset of apoptosis. Likewise, ectopic expression of *fkh* in mid-prepupal has

been shown to repress salivary gland cell death [10]. *Fkh* is highly expressed in MTs even at the adult stages [34], and as observed, expression in PMT is several-fold higher than PSG, despite the late larval ecdysone pulse. Thus, the hypothesis put forward was that *fkh* must be crucial for MTs survival. On the other hand, a decrease of *fkh* in pupal SGs is necessary so that it can undergo apoptosis. The similarity between the *fkh*-responsive genes in MTs, with those in salivary glands [12], confirmed that *fkh* is negatively regulating apoptotic and autophagic pathways in MTs. Consistent with this inference, cell death ensues in MTs of *c42Gal4>UAS-Fkh RNAi* confirming the belief that apoptosis/autophagy is kept in check by *fkh*. Another factor that could be involved in preventing PCD in MTs was the reduced expression of *E93* [25], which is also an *fkh*-responsive gene and is negatively regulated by *fkh*. Together these results suggest that MTs are susceptible to PCD, which is probably kept in check by maintaining a higher expression of *fkh*.

Fkh is downregulated in salivary glands by Br-C, following pre-pupal ecdysone pulse, but contrary to this well-existing paradigm, *fkh* levels remain the same in MTs, despite high levels of Br-C [30]. As Br-C is known to regulate secretion of AMPs [23,30], its expression remains high in MTs even at pupal stage, to regulate immune response [29]. Unlike SGs, BR-C does not seem to negatively regulate *fkh*. This paradoxical situation implies that gene regulation in MTs is not the same as in SGs. This orchestration is important for the second function of MTs: its role in immune response, which is governed by *fkh* as well as Br-C. It signifies that AMPs can be activated by *fkh* under non-infectious conditions, which may be important for protection of insects from microbial infection during molting and metamorphosis. This is confirmed by the observation of reduced immunity with reduced expression of AMPs in *fkh*-depleted conditions. NF- κ B transcription factors Relish and Dorsal/Dif regulate the expression of AMPs in the Imd and Toll pathways, respectively [35]. The localization and expression of Relish get affected after *fkh* depletion. However, there is no change in the localization of Dorsal whereas its expression gets decreased in MTs upon downregulation of *fkh*. The expression of EcR and Broad-core also gets reduced in *c42Gal4>UAS-Fkh RNAi* third instar LMTs.

Drosophila MTs are the simple epithelial tissues, which detoxifies and maintain fluid homeostasis with the help of several ion transporters. *Fkh* depletion causes disruption of ion channels, which is important, because *fkh* is considered as master regulator of V-ATPase Proton Pump Subunits in the Inner Ear, Kidney and Epididymis in mice [36]. Apart from excretion, ion channels also regulate immunity [30] and the presence of multiple *fkh* binding sites in promoter regions of V-ATPase subunit genes, Na pump subunit genes and Drip channels strengthens the probability that *fkh* is playing prime role in urine formation and immune regulation via ion channels in MTs. The expression of V-ATPase, Drip and Na⁺/K⁺ ATPase is reduced in *c42Gal4>UAS-Fkh RNAi* third instar LMTs. The significance of MTs not undergoing histolysis

during pupation could be for protection against infection when fat bodies and other AMP-producing tissues are histolyzed by programmed cell death. Thus, *fkh* appears to be involved in multiple functions in MTs, albeit, in a “context-dependent” manner.

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Data Availability statement:

Data will be available on request.

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