Non-apoptotic function of apoptotic proteins in the development of Malpighian tubules of *Drosophila melanogaster*

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Drosophila metamorphosis is characterized by the histolysis of larval structures by programmed cell death, which paves the way for the establishment of adult-specific structures under the influence of the steroid hormone ecdysone. Malpighian tubules function as an excretory system and are one of the larval structures that are not destroyed during metamorphosis and are carried over to adulthood. The pupal Malpighian tubules evade destruction in spite of expressing apoptotic proteins, Reaper, Hid, Grim, Dronc and Drice. Here we show that in the Malpighian tubules expression of apoptotic proteins commences right from embryonic development and continues throughout the larval stages. Overexpression of these proteins in the Malpighian tubules causes larval lethality resulting in malformed tubules. The number and regular organization of principal and stellate cells of Malpighian tubules is disturbed, in turn disrupting the physiological functioning of the tubules as well. Strikingly, the localization of β-tubulin, F-actin and Disclarge (Dlg) is also disrupted. These results suggest that the apoptotic proteins could be having non-apoptotic function in the development of Malpighian tubules.

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1. Introduction

Apoptosis is a highly conserved phenomenon and plays a major role during development, differentiation and tissue homeostasis by eliminating unwanted and harmful cells (Bright and Khar 1997). Apoptosis is identified by morphological changes like condensation of cytoplasm and chromatin, breakdown of nuclear membrane, membrane blebbing and, in epithelial cells, loss of membrane polarity and disruption of cell junction proteins (Jänicke *et al.* 1998; Atencia *et al.* 2000; Ianella *et al.* 2008). The canonical apoptotic pathway is evolutionarily conserved in invertebrates as well as in vertebrates (Aravind *et al.* 2001). In *Drosophila* this cascade is initiated by the activation of three pro-apoptotic genes, *reaper* (*rpr*), *head involution defective* (*hid*) and *grim,* which converge to activate the key players of cell death, which are a family of proteases known as caspases. Caspases (cysteinyl aspartate-specific proteinases) are so named because they cleave their target protein after an aspartate residue using cysteine in their active sites (Shi 2004; Hay and Guo 2006). Caspases exist as inactive zymogens in normal cells and become activated upon receiving the death signal through a proteolytic cleavage (Jacobson *et al.* 1997; Shi 2004). Counteracting the effect of caspases are a family of caspase inhibitors known as the inhibitor of apoptotic proteins (IAPs). *Drosophila IAP* (*diap1*) functions as E3-ubiquitin ligase to target Dronc for proteasomal degradation as well as for its own auto-regulation (Kaiser *et al.* 1998; Meier *et al.* 2000; Yin and Thummel 2004). Activation of these genes irreversibly set the cells on the path of destruction. Besides inducing apoptosis, many apoptotic proteins also have found to be important in non-apoptotic roles such as immune function (Stoven *et al.* 2003; Tanji and Ip 2005), cell proliferation (Chun *et al.* 2002; Salmena and Hakem 2005), cell differentiation (Kang *et al.* 2004; Lamkanfi *et al.* 2006) spermatid individualization (Arama *et al.* 2003; Huh *et al.*

Keywords. Apoptosis; *Drosophila*; Malpighian tubules

Abbreviations used: IAP, inhibitor of apoptotic protein; MT, Malpighian tubule; PC, principal cell; SC, stellate cells

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2004), cell migration (Geisbrecht and Montell 2004; Helfer *et al.* 2006) and cell shaping (Cullen and McCall 2004).

The *Drosophila* Malpighian tubules (MTs) perform excretory and osmo-regulatory roles analogous to vertebrate renal organs. MTs contain two main cell types, the type I or principal cells (PCs) and type II or stellate cells (SCs) and four different minor cell types (Sozen *et al.* 1997; Singh *et al.* 2007). Recent studies have shown that the PCs and SCs of MTs originate from two different lineages, with PCs originating from ectodermal epithelial buds in the hindgut and the SCs from the caudal mesoderm cells that lie above the hindgut in the region from where MTs bud (Denholm *et al.* 2003). Ecdysone regulates the proper intercalation and integration of these cell types as well as fluid secretion (Gautam and Tapadia 2010). During metamorphosis, extensive remodelling of the entire organism takes place wherein most of the larval tissues undergo apoptosis and adult-specific structures are made. These processes are triggered by ecdysone release from the pro-thoracic glands. Unlike most of larval tissues, which are histolysed during metamorphosis, MTs escape this fate and continue in the adult fly. Recent studies have shown that the different genes involved in apoptosis are expressed in the MTs but the proteins remain sequestered in the nucleus and are inhibited from being activated, thus allowing MTs to escape destruction (Shukla and Tapadia 2011).

In the present study, we have extended the observation on expression of apoptotic proteins in the MTs during development and examined their role in the development of MTs. We show that the pro-apoptotic proteins, Hid and Grim are expressed in the MTs during embryonic and early larval stages of development. Ectopic expression of *reaper, hid* and *grim* specifically in the MTs, using the UAS-Gal4 system (Brand and Perrimon 1993), causes lethality and hampers proper development of these structures. Reduction in the size of MTs with reduced number of cells and mis-organization of PCs and SCs is also associated with overexpression. The excretory process is also disrupted in these progeny. The localization of different cytoskeletal proteins and membrane-associated proteins are also disrupted, thus affecting the formation of a stabilized tubular structure. These results points to the non-apoptotic role of the apoptotic proteins primarily in the development of MTs.

2. Results

2.1 *Expression of pro-apoptotic proteins in MTs during early development*

The four MTs primordia, ectodermal in origin, are derived from the midgut and hindgut junction, their numbers increase by cell division. Later, by stage 13, the PCs are generated by cell rearrangement and elongation (Liu 1998; Denholm *et al.* 2003; Jung *et al.* 2005). The SCs, which are of mesodermal origin, undergo mesenchymal-to-epithelial transition and get intercalated in between the PCs at stage 15 (Liu 1998). The expression of pro-apoptotic genes, *rpr*, *hid* and *grim* and their ectopic expression leading to activation of caspases and apoptosis, is well correlated with cell death (Song *et al.* 2000). In our recent report (Shukla and Tapadia 2011) we have shown that these proteins also express and show nuclear localization in the 3rd instar larval and pupal MTs, which are tissues that do not undergo apoptosis during metamorphosis like other larval structures. To further investigate the expression of *rpr*, *hid* and *grim* in MTs at embryonic and 1st instar larval stages, MTs were immunostained with anti-Rpr, Hid and Grim antibodies and expression of different primary apoptotic proteins was examined at stage 13 and 15 of wild type embryos. Our results show that at stage 13 and 15 of embryonic development, Hid (figure 1J, J′, J′′) and Grim (figure 1R, R′, R′′) are expressed in MTs while Rpr is not (figure 1F, F′). To unequivocally identify the MTs in the developing embryos, we used anti-Cut, which expresses specifically in the principal cells of the MTs (figure 1A, A′, E, E′, I, I′, I′′, Q, Q′, Q′′). The merged images (figure 1D, D′, H, H′, L, L′, L′′, T, T′, T′′) show the co-localization of these proteins, demonstrating that the apoptotic genes do indeed express in MTs. We next wanted to know if the expression of apoptotic proteins continues in larval stages; so, MTs from wild type 1st instar larvae were immunostained. Similar to that in embryos, Hid (figure 2G) and Grim (figure 2J) expression was observed in the wild type 1st instar larval MTs, while the expression of Rpr was not observed (figure 2D). The images were scanned at the setting at which the negative control did not show any signal (figure 2A). The nuclei were counterstained with DAPI (figure 2B, E, H, K) and the merged images show that these proteins localize predominantly to the cytoplasm with little or no expression in the nucleus (figure 2C, F, I, L). This is in contrast to our earlier report that the localization of apoptotic protein in the 3rd larval and pupal MTs is predominantly nuclear (Shukla and Tapadia 2011). These data suggest that the expression of apoptotic genes *hid* and *grim*, but not *rpr*, commences from early development of the MTs during embryogenesis and continues into the larval stages. However, based on the difference in localization of these apoptotic proteins, we believe that initially in the 1st instar larval stage these proteins show cytoplasmic localization and they later move into the nucleus by 3rd instar larval stage.

2.2 *Ectopic expression of apoptotic proteins leads to lethality and abnormal tubules*

We next wanted to examine the effect of altering the levels of the apoptotic proteins in MTs during development. The UAS-Gal4 system (Brand and Perrimon 1993) was used to

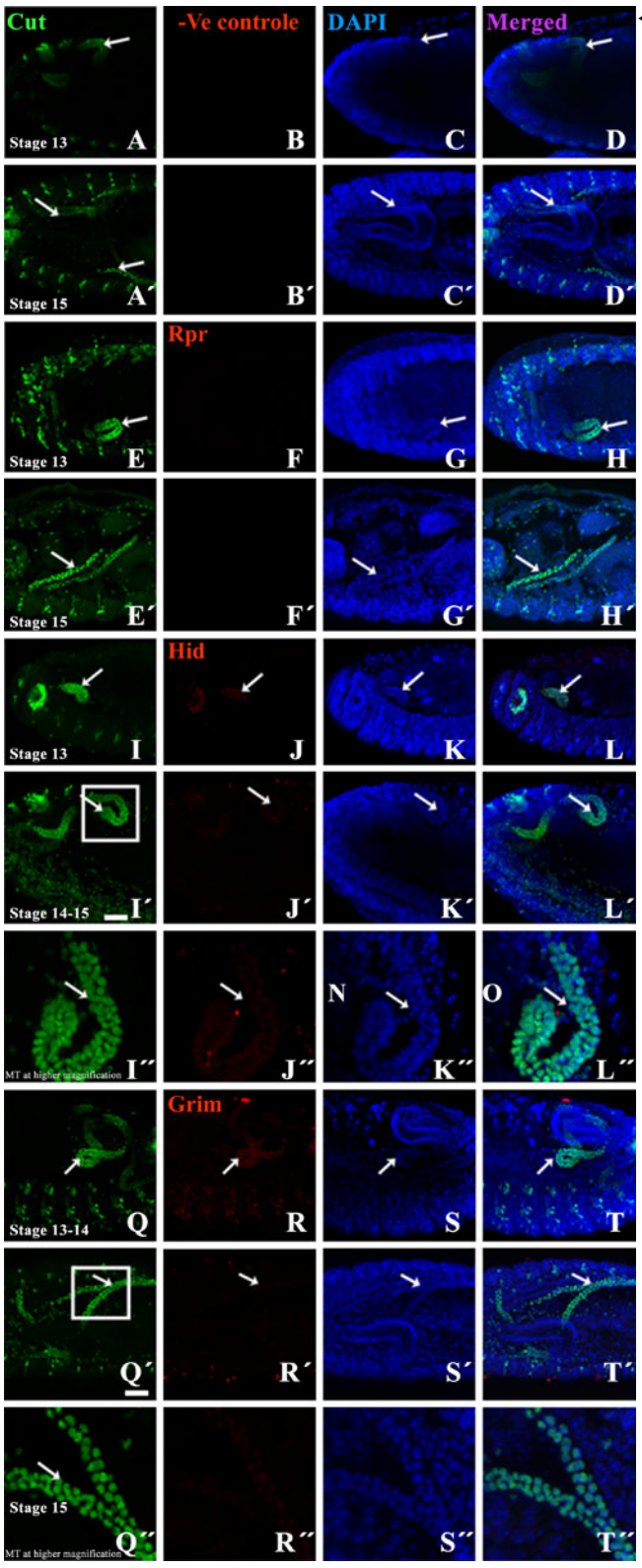

Figure 1. Expression of Rpr, Hid and Grim in MTs of wild-type embryos. The embryos are oriented with their posterior end on the left side. The MTs are identified by the expression of Cut protein (green) at stage 13 (**A**, **E**, **I**) stage 13–14 (**Q**), stage 15 (**A′**, **E′**, **Q′**, **Q″**) and stage 14–15 (**I′**, **I″**). Rpr expression is not observed in embryonic stage 13 (**F**) or 15 (**F′**), which is similar to the negative control (**B**, **B′**), while expression of Hid is observed (red) at stage 13 (**J**) and stage 14–15 (**J′**). The magnified view of the selected area of panel (**I′**) shows clear expression of Hid in MTs (**I″**). Grim (red) expression is observed at stage 13–14 (**Q**) and stage 15 (**Q′**) and in the magnified view (**Q″**). The signals of Hid and Grim are more than in the negative control (**B**, **B′**) The nuclei were counterstained with DAPI (blue) in all the embryos (**C**, **C′**, **G**, **G′**, **K**, **K′**, **K″**, **S**, **S′**, **S″**) and their merged images show the co-localization of Cut and the apoptotic proteins (**D**, **D′**, **H**, **H′**, **L**, **L′**, **L″**, **T**, **T′**, **T″**). All the images are captured by Zeiss Meta 510 confocal microscope (scale bar 10 μm). Scanning was done at the setting at which negative control does not shows any background.

overexpress *rpr*, *hid* and *grim* in MTs. Table 1 shows the different Gal4 driver lines with their domains of expression and the different UAS responder stocks used. We crossed Gal4 *c42* and Gal4 *c724* stocks (Rosay *et al.* 1997) with different UAS responder lines (table 1) and observed F1 progeny. Complete lethality was observed when each of the primary pro-apoptotic genes was expressed either in the PCs (*c42*) or in the SCs (*c724*) of MTs. These progeny died as early larvae shortly after hatching. The stage of lethality was estimated by examining the mouth-hook serrations of the dead larvae through which it was confirmed that larvae from different crosses (table 1) died at early 1st instar (figure 3A, B, C, A′, B′, C′). Next, we examined the length and diameter of MTs from these larvae. The mean lengths of the anterior and posterior tubules of the wild type were 712.9± 06.3 μm and 646.4±16.1 μm, respectively (table 2), whereas the length of the tubules expressing apoptotic proteins, Rpr, Hid and Grim either with *c42* or *c724* appeared to be reduced, varying from 459.8±09.3 μm to 598.1±12.3 μm of the anterior tubule and from 372.3± 12.0 μm to 472.8±17.6 μm of the posterior tubule. Statistical analysis showed that this reduction in length was significant when compared with the wild type ($p<0.05$). The diameter of the tubules of the main segment (table 3), which was 15.7±0.3 μm and 14.6±0.2 μm for the anterior and posterior tubules, respectively, in the wild type, also appeared to be reduced following overexpression. The diameter of the anterior tubule of larvae overexpressing *rpr*, *hid* and *grim* was in the range from 10.1±0.2 μm to 13.0±0.3 μm and that of the posterior tubule was from 9.0±0.2 μm to 11.4± 0.1 μm. Statistical analysis also confirmed that the MTs are significantly thinner when apoptotic proteins are expressed. In conclusion, overexpression of apoptotic genes *rpr, hid* and *grim* significantly reduces the length and diameter of larval MTs.

To examine whether the ectopic expression of apoptotic proteins initiated apoptosis in MTs, we stained the MTs with Acridine Orange (AO). We did not observe any AO-positive cells, indicating that cell death does not occur in MTs (supplementary figure 1). Since the Gal4 drivers used

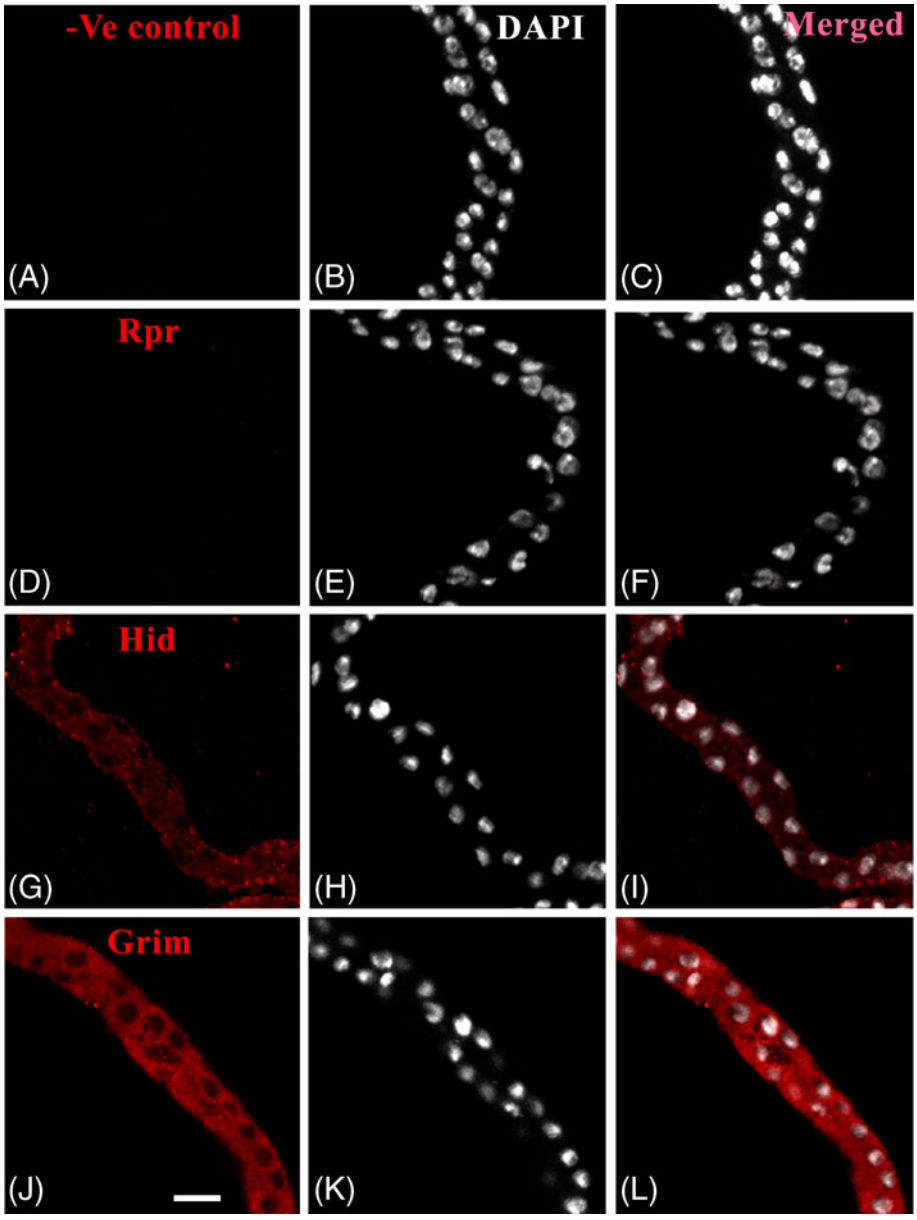

Figure 2. Expression of Rpr, Hid and Grim in the MT of 1st instar wild-type larvae. Immunostaining with antibodies (red) against Rpr (D) shows that it does not express in the MTs and is the same as the negative control (**A**). Hid (**G**) and Grim (**J**) expression is observed in the MTs. Nuclei (**B**, **E**, **H**, **K**) were counterstained with DAPI (pseudocolour white) and, in the merged images, show that Hid (**I**) and Grim (**L**) are cytoplasmic. All the images were captured by Zeiss LSM Meta 510 confocal microscope (scale bar 20 μm). Scanning was done at the setting at which negative control does not shows any background.

also express in the salivary gland, we did AO staining in the 1st instar larval salivary glands (SGs, the tissue destined for apoptosis) and observed that, unlike in the MTs, we observed AO staining in the nucleus, indicating the initiation of apoptosis (supplementary figure 2). To confirm apoptosis in SGs, we used a salivary-gland-specific driver, *Sgs 3*, to express the apoptotic genes. *Sgs3* expresses in the 3rd instar larvae, and hence we checked AO staining in the SGs of wandering 3rd instar larvae. We observed severe apoptosis in the SGs (supplementary figure 3B, C and D) when compared with the wild-type control (supplementary figure 3A). These data strongly suggest that developmental defects caused due to overexpression of Rpr, Hid and Grim in MTs is not because of cell death, but could be due to other unknown functions of these proteins.

Table 1. The different Gal4 lines, their domains of expression and UAS transgenes expressing apoptotic genes. The crosses conducted are indicated

Gal4 driver	Domain of expression	UAS Responder	Crosses
Gal4 *c42*	Principal cells, bar type cells of initial and transition segment, salivary gland, cuticle	*UAS-Rpr*	*c42 X UAS-Rpr*
		UAS-Hid^{14}/CyO	*c42 X UAS-Hid^{14}/CyO*
		UAS-Grim$^{wt9.8}$/CyO	*c42 X UAS-Grim$^{wt9.8}$/CyO*
Gal4 *c724*	Stellate cells, bar shaped stellate cells, salivary glands, visceral musculature cells		*c724 X UAS-Rpr*
			c724 X UAS-Hid^{14}/CyO
			c724 X UASGrim$^{wt9.8}$/CyO

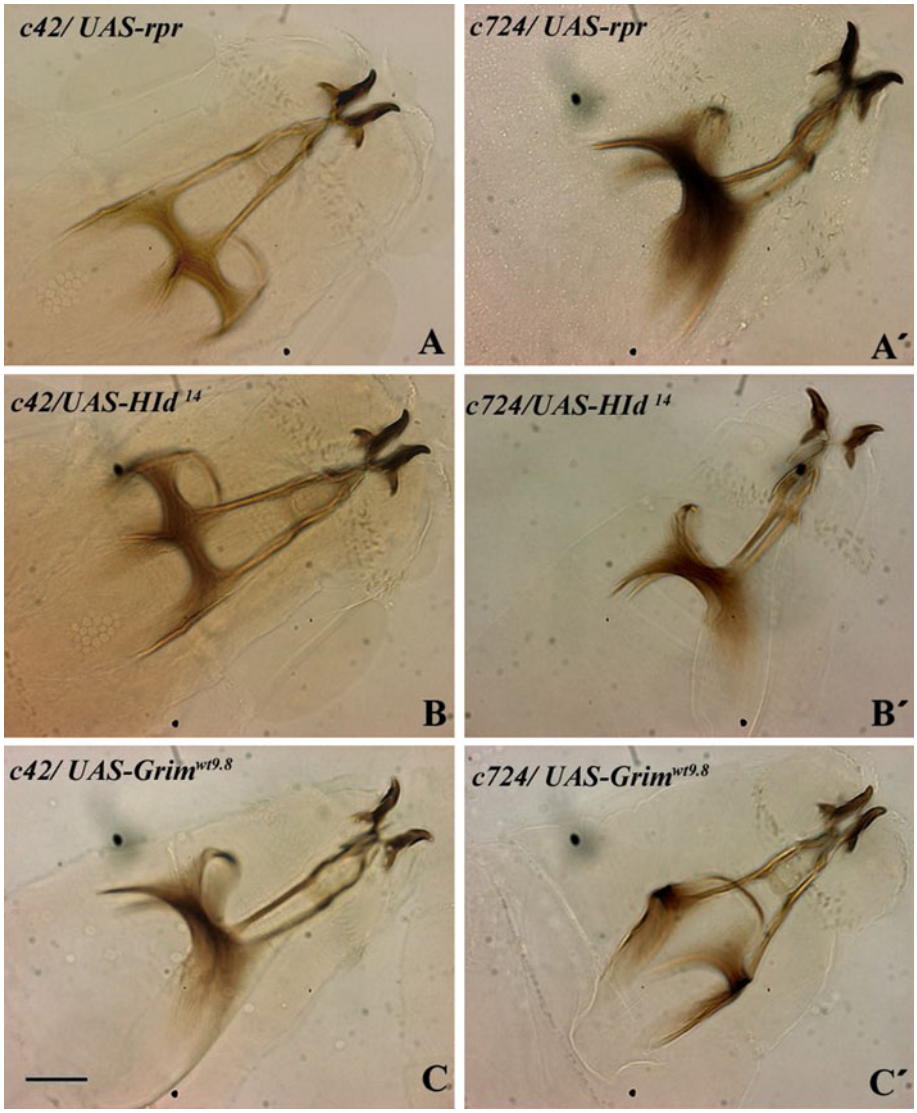

Figure 3. Larval cuticular preparations: mouth-hooks serrations of *c42/UAS-rpr* (**A**), *c42/UAS-hid* (**B**), *c42/UAS-grim* (**C**), *c724/UAS-rpr* (**A′**), *c724/UAS-hid* (**B′**) and *c724/UAS-grim* (**C′**) indicate that the larval death is occurring at 1st instar stage (scale bar 10 μm)

Table 2. Length (μm) of anterior and posterior Malpighian tubules of 1st instar larvae

Genotype (N=10)	Anterior tubules	Posterior tubules
Wild Type	712.9±06.3	646.4±16.1
c42/UAS-Rpr	598.1±12.3*	441.0±23.2*
c42/UAS-Hid^{14}	527.0±16.8*	467.4±15.9*
c42/UAS-Grim$^{wt9.8}$	520.9±11.5*	472.8±17.6*
c724/UAS-Rpr	524.4±05.5*	458.3±09.5*
c724/UAS-Hid^{14}	459.8±09.3*	409.6±10.1*
c724/UAS-Grim$^{wt9.8}$	529.2±03.0*	372.3±12.0*

* The mean difference is significant at $p<0.05$ level.

Dunnett *t*-tests treat one group as a control (WT), and compare all other group against it.

The regular arrangement of PCs and SCs in *Drosophila* MTs lends itself to identification of developmental defects affecting it. Having observed that the MTs are reduced in length and diameter, we examined the arrangement of PCs and SCs in the different overexpressing progeny. PCs and SCs were identified using the Cut and Teashirt antibody, respectively. We observed that in *c42/UAS-rpr* (figure 4E), *hid* (figure 4I) or *grim* (figure 4M), the regular arrangement of PCs are disrupted and they appear in clusters as compared with the regular arrangement in wild type (figure 4A). We observed that the arrangement of SC is also disrupted in *c42/UAS-rpr* (figure 4F), *hid* (figure 4J) or *grim* (figure 4N) and they too are present in clusters, unlike the wild-type arrangement of SCs (figure 4B). However, when the apoptotic proteins are expressed using SC-specific Gal4 as in *c724/rpr* (figure 4B′), *hid* (figure 4F′) and *grim* (figure 4J), only SCs' arrangement is affected and they get intercalated as clusters rather than the wild-type arrangement (figure 4B). We observed that the arrangement of PCs in these progeny (figure 4 A′, E′ and I′) are not disrupted. The nuclei were counterstained with DAPI, and the merged images distinctly show the disruption of nuclei arrangement in each case (figure 4H, L, P, D′, H′, L′). It is interesting to note that disruption of the PC arrangement also disrupted the SC arrangement; however, the reverse is not true. This is consistent with our previous observation that disruption of ecdysone signalling in PCs disrupts both PCs and SCs together, whereas disruption in SCs disrupts only the SCs and not the PCs (unpublished data). These observations confirm that the development of SCs in the MTs occur later than the development of PCs (Denholm *et al.* 2003; Jung *et al.* 2005). Another interesting phenomenon in MTs is that the number of PCs and SCs are determined during embryonic development and they remain nearly fixed throughout life (Wessing and Eichelberg 1978; Sozen *et al.* 1997). To find out if the loss of arrangement also affected the number of different cell types, we counted the number of PCs and SCs when pro-apoptotic genes were overexpressed. The mean number of PCs and SCs in anterior tubules of *c42/UAS-rpr*, *c42/UAS-hid* and *c42/UAS-grim* are 123.4±0.9 and 23.5±0.5, 113.2±1.4 and 13.1±0.5, and 112.0±1.3 and 14.4±0.7, respectively, while in posterior tubules, the numbers are 78.9±1.0 and 16.0±0.4, 74.4±1.0 and 13.9±0.4, and 81.1±0.9 and 14.0±0.6, respectively. Statistical analysis confirmed that the number of PCs and SCs are significantly reduced when compared with the wild type (table 4). Similarly, the number of PCs and SCs were counted when pro-apoptotic proteins were expressed in SCs. The number of PCs and SCs in anterior tubules of *c724/UAS-rpr*, *c724/UAS-hid*, *c724/UAS-grim* was 140.7±1.0 and 14.1±0.6, 139.9±0.6 and 12.3±0.3, and 139.8±1.1 and 15.1±0.6, respectively, while in posterior tubule the number of PCs and SCs was 108.3±0.8 and 12.1±0.5, 108.2±0.6 and 12.3±0.5, and 108.7±0.4 and 12.6±0.4, respectively. Statistical analysis suggests that there is significant reduction in the number of SCs in anterior and posterior tubules, while PCs remain nearly same as in the wild type (table 4). These results showed that disruption of PCs affect SCs as well; however, disruption of SCs do not affect PCs.

Table 3. Diameter (μm) of anterior and posterior Malpighian tubules of 1st instar larvae

Genotype (N=10)	Anterior tubule	Posterior tubule
WT	15.7±0.3	14.6±0.2
c42/UAS-Rpr	13.0±0.3*	10.3±0.3*
c42/UAS-Hid^{14}	10.7±0.3*	10.5±0.2*
c42/UAS-Grim$^{wt9.8}$	10.1±0.2*	11.4±0.1*
c724/UAS-Rpr	11.2±0.3*	09.9±0.3*
c724/UAS-Hid^{14}	11.2±0.2*	09.0±0.2*
c724/UAS-Grim$^{wt9.8}$	10.9±0.2*	09.0±0.2*

* The mean difference is significant at $p<0.05$ level.

Dunnett *t*-tests treat one group as a control (WT), and compare all other group against it.

2.3 *Alteration in the localization of β-tubulin, F-actin and Dlg on overexpression of apoptotic proteins*

The integrity of epithelial structures greatly depends on the proper assembly of polarity complexes like apico-basal, basolateral and the adherens junction protein complexes (Lee *et al.* 2006; Lynch and Hardin 2009). During MT development, at stage 13 the cylindrical buds of MTs undergo convergent extension to reduce the tubular circumference from four cells to just two cells (Liu 1998). The caudal mesodermal cells undergo mesenchymal to epithelial transition giving rise to the SCs, which get intercalated in between the PCs. The polarity complexes play an important

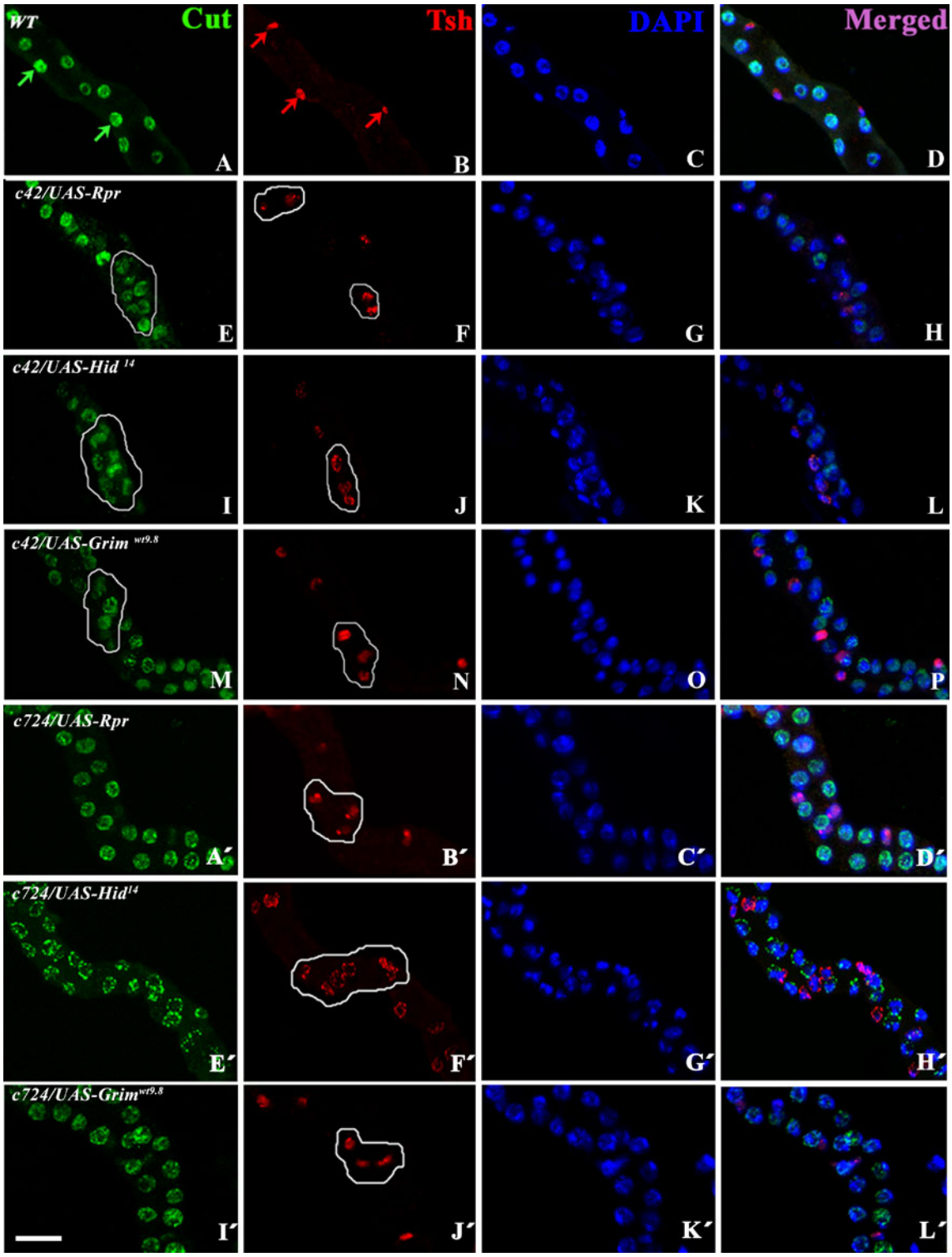

Figure 4. Random organization of PCs and SCs after overexpression of apoptotic genes: The arrangement of PCs identified by the Cut antibody in the wild type (**A**) and SCs identified by the Teashirt antibody (**B**) shows regular organization of the two cell types. However, this organization is lost when expression of *rpr, hid* and *grim* is driven by *c42*. Clusters of PCs and SCs were observed in the progeny of *c42/UAS-rpr* (**E**, **F**), *c42/UAS-hid* (**I**, **J**) and *c42/UAS-grim* (**M**, **N**). When these genes are driven by *c724*, only the arrangement of SCs is altered and they appear in clusters as observed in *c724/UAS-rpr* (**B′**), *c724/UAS-hid* (**F′**) and *c724/UAS-grim* (**J′**). However, the organization of PCs is not disrupted in *c724/UAS-rpr* (**A′**), *c724/UAS-hid* (**E′**) and *c724/UAS-grim* (**I′**). Nuclei were stained by DAPI (**C**, **G**, **K**, **O**, **C′**, **G′**, **K′**) and the merged images are shown (**D**, **H**, **L**, **P**, **D′**, **H′**, **L′**) (scale bar 20 μm).

Table 4. Number of PCs and SCs in anterior and posterior tubule

Genotype (N=25)	Principal cells		Stellate cells	
	Anterior tubule	Posterior tubule	Anterior tubule	Posterior tubule
WT	142.8±0.2	110.4±0.2	31.9±0.2	21.5±0.1
c42/UAS-Rpr	123.4±0.9*	78.9±1.0*	23.5±0.5*	16.0±0.4*
c42/UAS-Hid^{14}	113.2±1.4*	74.4±1.0*	13.1±0.5*	13.9±0.4*
c42/UAS-Grim$^{wt9.8}$	112.0±1.3*	81.1±0.9*	14.4±0.7*	14.0±0.6*
c724/UAS-Rpr	140.7±1. 0	108.3±0.8	14.1±0.6*	12.1±0.5*
c724/UAS-Hid^{14}	139.9±0.6	108.2±0.6	12.3±0.3*	12.3±0.5*
c724/UAS-Grim$^{wt9.8}$	139.8±1.1	108.7±0.4	15.1±0.6*	12.6±0.4*

* The mean difference is significant at the $p<0.05$ level.

Dunnett *t*-test treats one group as a control, and compare all other groups against it.

N denotes the number of tubules counted.

role in these morphogenetic changes and formation of the tubules. In the present study, we examined the expression and localization of cytoskeletal elements, actin and β-tubulin and Disclarge (Dlg), an adherns junction protein, in the progeny overexpressing apoptotic genes. Apparently, overexpression of *rpr, hid* and *grim* with *c42* results in abnormal distribution of β-tubulin (figure 5D, G, J), F-actin (figure 5E, H, K) and Dlg (figure 5F, I, L) when compared with the wild type (figure 5A, B, C). Similarly, when apoptotic genes were overexpressed under *c724* Gal4, we observed the disruption of β-tubulin (figure 5D′, G′, J′), F-actin (figure 5E′, H′, K′) and Dlg (figure 5F′, I′, L′) when compared with the wild type (figure 5A, B, C). These results suggests that the apoptotic proteins act as a scaffold in the assembly of the cytoskeletal elements, and thus imbalance in their levels could be responsible for the destabilization and loss of integrity of MTs, resulting in lethality.

2.4 *Overexpression of apoptotic proteins affects the physiological functioning of MTs*

MTs function in generating primary urine and selectively reabsorbing essential ions (Gäde 2004). Ion transport is primarily carried on by vacuolar-type H^+-ATPase, which is responsible for driving the net movement of Na^+ and K^+ between cells of MTs and lumen (Maddrell and O'Donnell 1992). The balance between the cellular and luminal levels of Na^+ and K^+ is driven by a Na^+/K^+-ATPase-type pump, which is present in the basolateral membrane of the PCs (Ianowski and O'Donnell 2004). Water transport is accomplished by *Drosophila* aquaporin, DRIP, which is specifically expressed in the SCs (Kaufmann *et al.* 2005; Spring *et al*. 2007). Since we observed that overexpression of pro-apoptotic genes in the MTs resulted in improper development of MTs, we wanted to examine whether the functioning is also disrupted as a result, and so, we examined the expression of Na^+/K^+-ATPase and DRIP in PCs and SCs. The expression of Na^+/K^+-ATPase, when compared with that in the wild type (figure 6A), is slightly enhanced in *c42/UAS-hid* (figure 6G) and *UAS-grim* (figure 6J), while in *UAS-rpr* (figure 6D) there is no change. The nuclei were counterstained with DAPI (figure 6B, E, H, K) and the merged images are shown in figure 6C, F, I, L. We also observed a lowered expression of DRIP in larvae having more than normal levels of Rpr (figure 7D), Hid (figure 7G) and Grim (figure 7J) as compared with the wild type (figure 7A). The nuclei were counterstained with DAPI (figure 7B, E, H, K), and the merged images are shown in figure 7C, F, I, L. Fluid secretion can be directly correlated with the proper organization and expression of transport proteins of MTs, and this can be easily gauged by the deposition of uric acid in the MTs (unpublished data). We analysed the functioning of MTs in the light of disruption of Na^+/K^+-ATPase and DRIP by observing the deposition of uric acid in the tubules. The uric acid deposition was observed under birefringence light and the results clearly showed that the levels of uric acid were very much reduced in *c42/UAS-rpr* (figure 8B′), *c42/UAS-hid* (figure 8C′), *c42/UAS-grim* (figure 8D′), *c724/UAS-rpr* (figure 8B1′), *c724/UAS-hid* (figure 8C1′) and *c724/UAS-grim* (figure 8D1′), in comparison with the wild type (figure 8A′). The MTs were identified by observing larvae under the bright field light in wild type (figure 8A) and in *c42/UAS-rpr* (figure 8B), *c42/UAS-hid* (figure 8C), *c42/UAS-grim* (figure 8D), *c724/UAS-rpr* (figure 8B1), *c724/UAS-hid* (figure 8C1) and *c724/UAS-grim* (figure 8D1).

3. Discussion

The critical function of apoptotic proteins in programmed cell death has been unequivocally proved; however, several studies have recently indicated that they may have non-apoptotic functions as well and may be involved in different

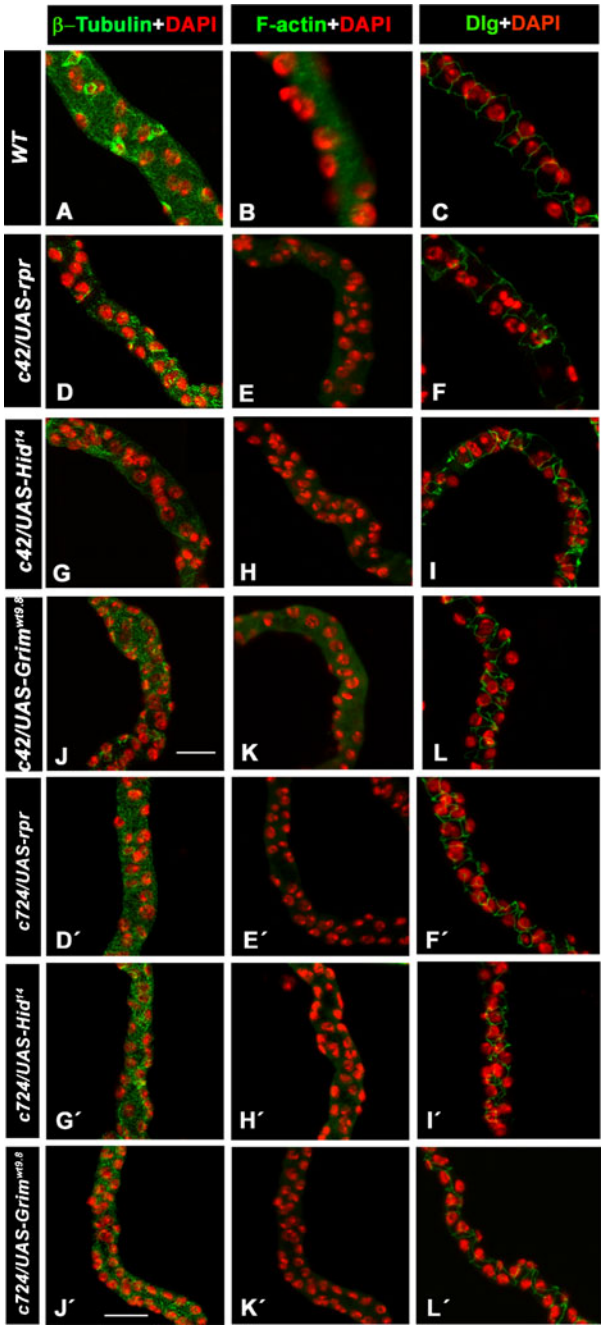

Figure 5. Altered expression pattern of β-tubulin, F-actin and Dlg in the MTs: Normal expression pattern of β-tubulin (**A**), F-actin (**B**) and Dlg (**C**) was observed in the wild type and compared with the disrupted expression pattern of β-tubulin (**D**, **G**, **J**), F-actin (**E**, **H**, **K**) and Dlg (**F**, **I**, **L**) in *c42/UAS-rpr*, *c42/UAS-hid* and *c42/UAS-grim*, respectively. Similarly, in the progeny of *c724/UAS-rpr* (**D′**, **E′**, **F′**), *c724/UAS-hid* (**G′**, **H′**, **I′**) and *c724/UAS-grim* (**J′**, **K′**, **L′**), the organization pattern of β-tubulin, F-actin and Dlg, respectively, is disrupted. The images in merged panels showing antibodies (green) and nuclei counterstained with DAPI (pseudo-colour red). All the images were captured by Zeiss LSM Meta 510 confocal microscope (scale bar 20 μm).

cellular processes. Caspase-1 is responsible for both the inflammatory and innate immune responses (Nadiri *et al.* 2006), Caspase-7 knockdown specifically inhibits cell cycle progression during apoptosis (Hashimoto *et al.* 2008). Caspase-8 can regulate lymphocyte proliferation (Salmena *et al.* 2003). Caspase-9 has been involved in non-apoptotic function in muscle differentiation (Murray *et al.* 2008) where reduction of caspase-9 prevents activation of caspase-3, thus preventing myoblast fusion. The results of the present study also indicate that expression of *hid* and *grim* in the MTs is functionally important for the spatially precise arrangement of the PCs and SCs; although *rpr* expression is not observed in embryonic and 1st instar larval MTs, its overexpression in PCs and SCs shows phenotypes similar to *hid* and *grim*. How apoptotic proteins regulate development of MTs is not known, but we assume that the balance of pro-apoptotic proteins is very important for the proper development and function of MTs. Although proper epithelial morphogenesis in development is essential, very few molecules that modulate the cytoskeleton and control cell shape changes and movement are known. Members of Rho GTPase family have been shown to modulate cytoskeleton elements (Reviewed by Hall 1998). There are reports of loss of directional movement of neutrophils upon disruption of polarized organization of actin and microtubule cytoskeleton (Xu *et al.* 2003). Several other components essential for epithelial morphogenesis have been identified (reviewed by Leptin 1995; Noselli 1998). However, there are other molecules that play a role in epithelia morphogenesis but are still unidentified. In the present results we show that disturbing the levels of apoptotic proteins affects the distribution pattern of cytoskeletal proteins and Dlg. In our previous report we have shown that the apoptotic proteins are also present on the basal membrane of MTs apart from the nucleus (Shukla and Tapadia 2011), and it could be possible that these apoptotic proteins could play a scaffolding role in maintaining the integrity of epithelial structures. Whether apoptotic proteins have a direct function in regulating the cytoskeletal elements and other membrane protein complexes and, in turn, controlling the organization is still to be understood.

MTs, by virtue of their development from cell proliferation to tubule morphogenesis, provide a useful system to identify molecules that modulate these processes. Having observed that the apoptotic proteins are expressed in the MTs despite these tissues escaping destruction (present results, Shukla and Tapadia 2011), and their imbalance causing MTs deformities, we suggest that the apoptotic proteins are as yet unidentified molecules in tubule morphogenesis. We believe that during early development these apoptotic proteins are involved in organizing the MTs, whereas later, when they show nuclear localization at pupal stages (Shukla and Tapadia 2011). They may serve two functions: first, helping MTs evade apoptosis

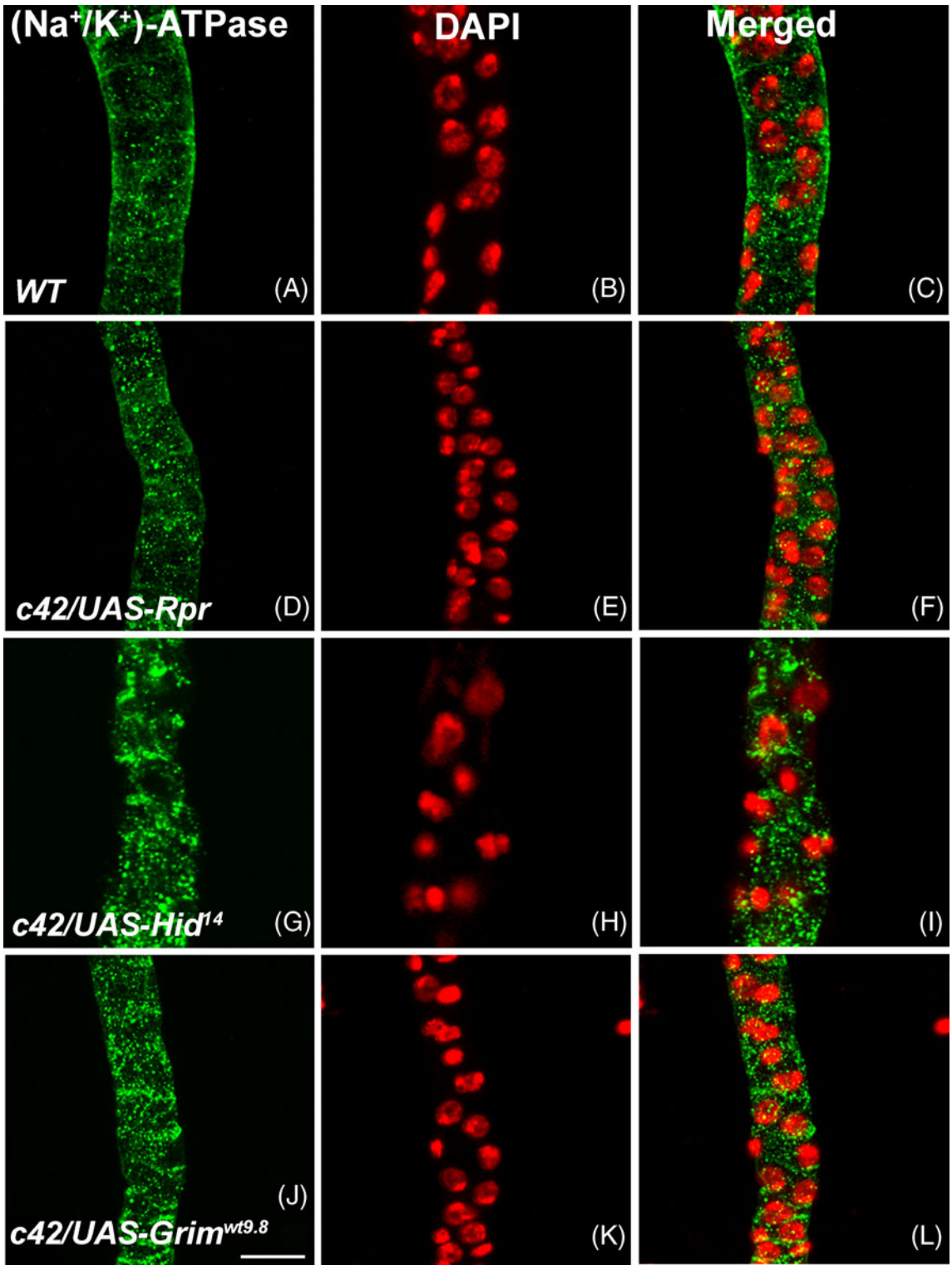

Figure 6. Expression of Na^+/K^+–ATPase in MTs: In wild-type MTs (**A**), the expression of Na^+/K^+-ATPase is similar to that of *c42/UAS-rpr* (**D**); however, in *c42/UAS-hid* (**G**) and *c42/UAS-grim* (**J**), the distribution pattern is altered and appears to be enhanced. The nuclei were counterstained with DAPI (pseudocolour red) (**B**, **E**, **H**, **K**), and the merged images are (**C**, **F**, **I** and **L**). All the images were captured by Zeiss LSM Meta 510 confocal microscope (scale bar 20 μm).

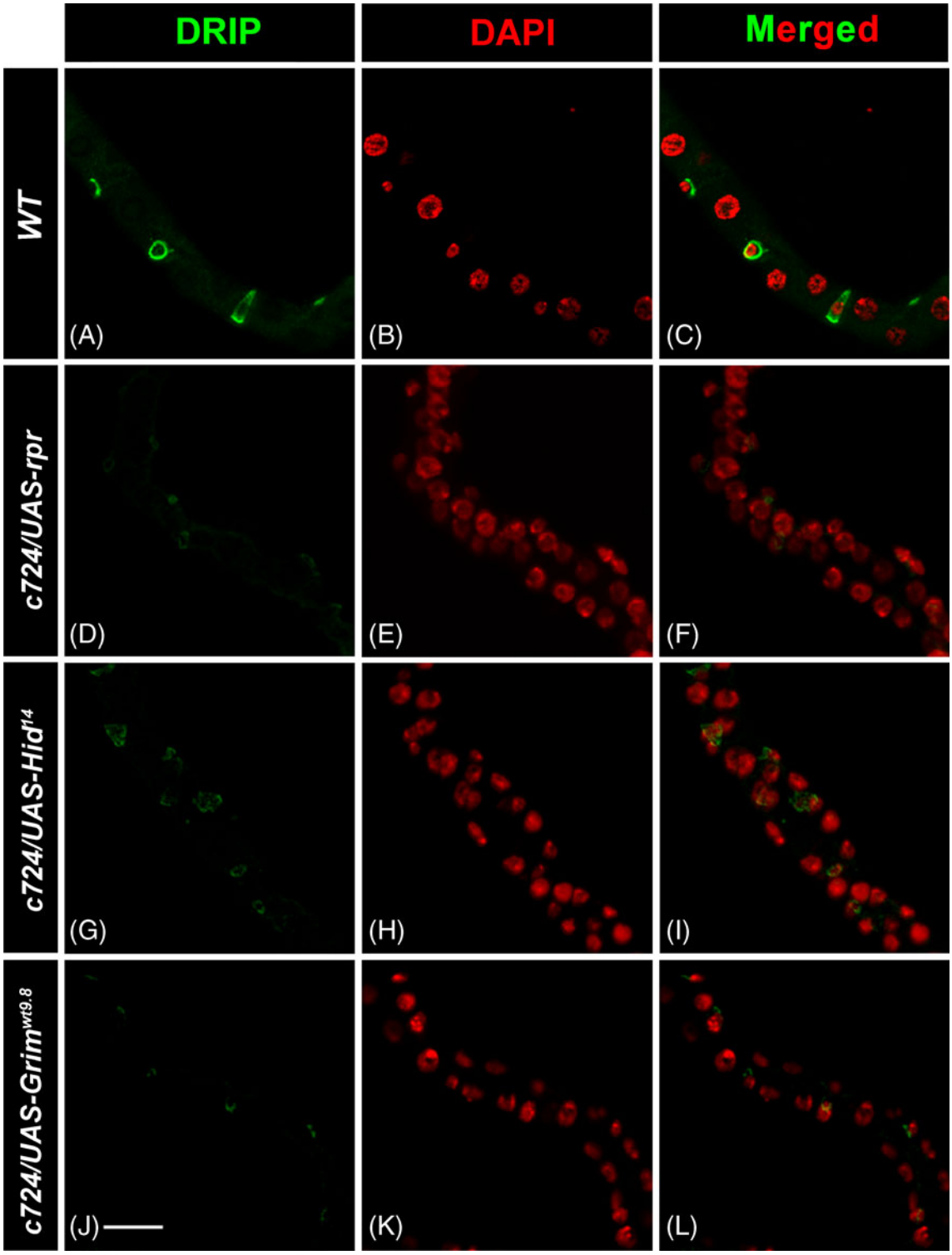

Figure 7. Expression of DRIP in the MTs: In wild-type MTs (**A**), the expression of DRIP is observed only in the stellate cells and is much higher than in MTs expressing apoptotic genes *c42/UAS-rpr* (**D**), *c42/UAS-hid* (**G**) and *c42/UAS-grim* (**J**). The nuclei were counterstained with DAPI (pseudocolour red) (**B**, **E**, **H**, **K**), and the merged images are (**C**, **F**, **I** and **L**). All the images were captured by Zeiss LSM Meta 510 confocal microscope (scale bar 20 μm).

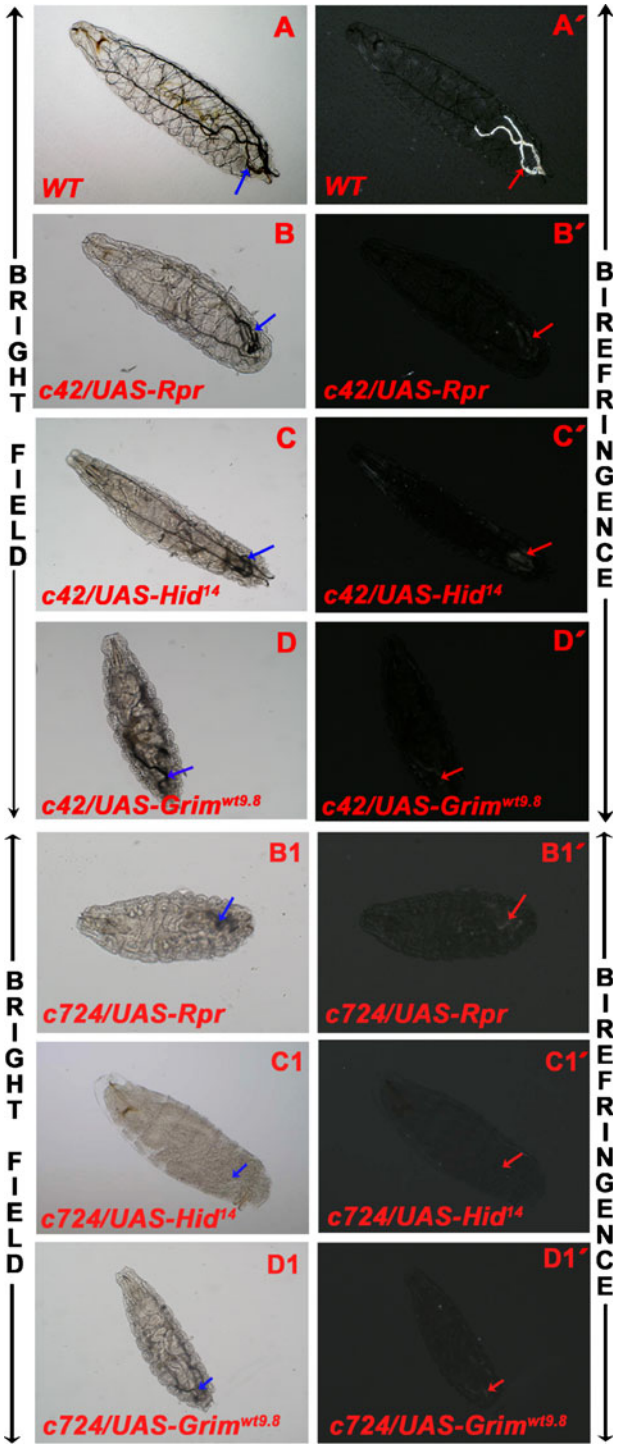

Figure 8. Uric acid deposition in MTs : MTs are observed under bright field in wild type (**A**), *c42/UAS-rpr* (**B**), *c42/UAS-hid* (**C**), *c42/UAS-grim* (**D**), *c724/UAS-rpr* (**B1**), *c724/UAS-hid* (**C1**) and *c724/UAS-grim* (**D1**) and are indicated by blue arrows. Uric acid deposition is observed under birefringence light in wild type (**A′**), which is much higher than in *c42/UAS-rpr* (**B′**), *c42/UAS-hid* (**C′**), *c42/UAS-grim* (**D′**), *c724/UAS-rpr* (**B1′**) *c724/UAS-hid* (**C1′**) and *c724/UAS-grim* (**D1′**) and indicated by red arrows in each case.

and, later, act as transcriptional regulators for genes specifically involved in metamorphosis.

The physiological properties of MTs are also important for proper fluid secretion and changes in membrane transporter affect uric acid deposition (unpublished data). The present results suggest that during normal development, changes in the apoptotic protein levels may correlate with the function of the tubules. We show that levels of DRIP and Na^+/K^+ ATPase are altered, leading to altered uric acid deposition in the tubules.

At present we do not have an answer to the question of whether the apoptotic proteins are the causal factors or indirectly affect these processes. However, we can say that they have a definite non-apoptotic role in the development of MTs. The mechanism by which apoptotic proteins control the development and function of MTs is unclear at present. The studies on non-apoptotic function of apoptotic proteins suggest that during evolution these proteins must have acquired multiple functions to control various aspects during development.

4. Materials and methods

4.1 *Fly stocks*

The fly stocks used for the study obtained from Bloomington Stock Center except where mentioned. Oregon R^+, wild-type stocks, were used as a control. GAL4 drivers *c724* and *c42* were used, which express in SCs and PCs, respectively (gifted by JAT Dow, Institute for Biomedical Sciences, University of Glasgow, UK). UAS responders used were: *UAS-reaper/UAS-reaper*, *UAS-hid^{14}/CyO* and *UAS-Grim$^{wt9.8}$/CyO* (Bloomington Stock Center). Appropriate crosses were set up to generate *c42/UAS-reaper*, *c42/UAS-hid^{14}*, *c42/UAS-Grim$^{wt9.8}$* and *c724/UAS-reaper*, *c724/UAS-hid^{14}* and *c724/UAS-Grim$^{wt9.8}$* individuals. All flies were reared at 23±1°C, unless otherwise mentioned, on standard food containing maize powder, agar, dried yeast and sugar in 12 h dark:12 h light cycle.

4.2 *Cuticle preparation*

To observe the stages of dead larvae the mouth-hook cuticle preparation were performed. Larvae of appropriate crosses were washed in 1× PBS (18.6 mM NaH_2PO_4, 84.1 mM Na_2HPO_4, 17.5 mM NaCl) and fixed in 1:4:: glycerol:acetic acid and incubated at 60°C overnight. Fixed larvae were mounted in Hoyer's mounting medium (gum arabic, chloral hydrate, glycerol), covered with a coverslip under a 20 gm weight and incubated again at 60° C for 2 days. Observations were carried out under the Nikon-800 microscope.

4.3 *Antibodies and immunocytochemistry*

Larvae from appropriate crosses were taken and MTs were dissected in 1× PBS, fixed in 4% formaldehyde for 20 min at RT, rinsed in PBST (PBS, 0.1% Triton X-100), blocked in blocking solution (0.1% Triton X-100, 0.1% BSA, 10% FCS, 0.1% deoxycholate, 0.02% thiomersol) for 2 h at RT. Tissues were incubated in primary antibody at 4°C overnight. After PBST (3×20 min each) washing, tissues were blocked for 2 h and incubated in the secondary antibody. Tissues were rinsed in PBST and counterstained with DAPI (1 μg/ml, Molecular Probe) for 15 min at RT. They were again washed in PBS and mounted in antifadant, DABCO (Sigma). Primary antibodies used were Teashirt (a kind gift from S Cohen, EMBL Heidelberg, Germany) and Cut (Developmental Studies Hybridoma Bank) at a dilution of 1:3000 and 1:50, respectively. For the study of the cytoskeleton of MTs, monoclonal anti-β-tubulin (dilution 1:50, DSHB), phalloidin (1 μg/ml) from Sigma were used to visualize F-actin organization. Organization of the septate junction protein in MTs were observed by using antibody anti-Dlg (dilution 1:50, DSHB). Secondary antibodies used were goat-anti-rabbit AF488, goat-anti-mouse AF488, goat-anti-rabbit Cy3 and goat-anti-mouse Cy3 (Molecular Probes, USA). All preparations were analysed on a Ziess LSM 510 Meta Confocal microscope and images were processed with Adobe Photoshop.

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