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Ecdysone signaling is required for proper organization and fluid secretion of stellate cells in the Malpighian tubules of *Drosophila melanogaster*

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ABSTRACT *Drosophila* development is a tightly regulated process involving metamorphosis of a relatively less mobile larva to a highly motile adult, triggered by secretion of 20-hydroxyecdysone. Under the influence of ecdysone, most of the larval tissues degenerate, while the imaginal cells differentiate and form adult specific structures. Although the larval Malpighian tubules do not seem to be affected by ecdysone during metamorphosis, we show that ecdysone signaling plays an important role in the early development and functioning of Malpighian tubules. Disruption of ecdysone receptor function, using targeted expression of dominant negative ecdysone receptor in stellate cells, results in disruption of organization of Malpighian tubules. The number of stellate cells is reduced in such Malpighian tubules. Further, they get clustered rather than distributed in their characteristic wild type pattern. We also demonstrate that expression of *Drosophila* integrin protein (DRIP), an aquaporin responsible for trans-cellular water transport, is also reduced in stellate cells when ecdysone signaling is disrupted. Our results show that of the three ecdysone receptor isoforms, only EcR-B2 rescues these phenotypes. A similar pattern of stellate cell clustering and reduced expression of DRIP is observed in *ecd*^{1}, a temperature sensitive mutant, under non-permissive conditions. These results suggest that ecdysone signaling is required for proper patterning and functioning of stellate cells and that EcR-B2 may be the primary isoform required for ecdysone signaling in stellate cells.

KEY WORDS: *Drosophila, ecdysone receptor, Malpighian tubule*

Introduction

The Malpighian tubules (MTs) in *Drosophila* are simple epithelial structures, which float in the hemocoel and which perform excretory and osmoregulatory functions analogous to vertebrate renal tubules. MTs comprise of two pairs of tubules which do not open to the outside, but are joined to alimentary canal at the junction of endodermal midgut and ectodermal hindgut. On the basis of the reporter gene expression in different enhancer trap lines, each pair of tubules has been divided into six segments, viz., the initial, transitional, main, lower tubule, upper ureter and lower ureter (Sozen *et al.*, 1997). MTs are made up of 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). Recent studies have shown that the PCs and SCs of MTs originate from different cell lineages, with PCs originating from ectodermal epithelial buds in the hindgut and the SCs from the caudal mesoderm cells which lie above the hindgut in the region from where MTs bud (Denholm *et al.*, 2003). The SCs undergo mesenchymal to epithelial transformation during intercalation

Abbreviations used in this paper: AF-1, activation function-1; AS, anterior spiracle; AP, anal plate; DAPI, 4, 6-diamidino-2 phenylindole dihydrochloride; DABCO, 1,4 diazobicyclo octane; DRIP, *Drosophila* integrin protein; EcR, ecdysone receptor; EcR-DN, dominant negative form of ecdysone receptor; Gal4, transcriptional activator protein in yeast; GFP, green fluorescent protein; MT, Malpighian tubules; PFA, paraformaldehyde; PBS, phosphate buffered saline; PCs, principal cells; RXR, retinoid X receptor; SCs, stellate cells; SG, salivary gland; USP, ultraspiracle; UAS, upstream activating sequence; VM, visceral masculature.

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and adopt epithelial characteristic once they are incorporated into the tubules and develop an apicobasal polarity. The primary function of MTs is secretion of isotonic fluid, which is accomplished by concerted action of physiologically distinct PCs and SCs. PCs are enriched with vacuolar-H^+-ATPase transporter which use Na^+/H^+ and K^+/H^+ exchanger to transport cations into the lumen (Davies *et al.*, 1996; Dow *et al.*, 1994, 1998; O'Donnell *et al.*, 1996; Sozen *et al.*, 1997) while SCs express channels which permit flow of Cl^- ions (O'Donnell *et al.*, 1998) and aquaporins which permit water flow into the lumen (Dow *et al.*, 1995; Kaufmann *et al.*, 2005). One of the *Drosophila* aquaporins, *Drosophila* Integral Protein (DRIP), a water specific aquaporin with very high transport rate is expressed in SCs of MTs (Kaufmann *et al.*, 2005). Expression of DRIP is significantly high during embryonic and adult stages in SCs, which suggests that water moves transcellularly through them (Kaufmann *et al.*, 2005).

MTs are unique in not undergoing extensive morphological changes during metamorphosis from larva to adult. *Drosophila* metamorphosis, which is coordinated by 20-hydroxyecdysone (referred to as ecdysone) hormone and is characterized by diverse events resulting in degeneration of most of the larval tissues (Jiang *et al.*, 1997) and proliferation and differentiation of imaginal cells into adult structures (Riddiford, 1993). Pulses of ecdysone also mark transition from one larval instar to the next.

Ecdysone binds to heterodimeric complex made up of Ecdysone Receptor (EcR) and Ultraspiracle (USP) (Koelle *et al.*, 1992; Yao *et al.*, 1992; Thomas *et al.*, 1993) and up-regulates the primary response genes like *Broad-complex*, *E74*, *E75*, which code for transcriptional regulators for late response genes (Ashburner *et al.*, 1974; Burtis *et al.*, 1990; Thummel *et al.*, 1990). The *USP* gene produces a single protein which is the *Drosophila* homologue of vertebrate RXR protein (Henrich *et al.*, 1990; Oro *et al.*, 1990). In contrast the *EcR* gene encodes three functional isoforms, EcR-A, EcR-B1 and EcR-B2 (Koelle *et al.*, 1991; Talbot *et al.*, 1993). These isoforms differ in their N-terminal, A/B or activation function-1 (AF-1) regions, but share a common conserved carboxy-terminal domain harboring the C and D domains referred to as the DNA and ligand binding regions respectively (Talbot *et al.*, 1993). The unique AF-1 domains of EcR isoforms can activate genes in cell and target specific manner (Mouillet *et al.*, 2001; Hu *et al.*, 2003). Mutations that block all EcR isoforms are embryonic lethal (Bender *et al.*, 1997), but mutations in any one of the isoforms affect specific developmental processes. EcR-A is predominantly expressed in tissues that develop into adult structures (Davis *et al.*, 2005) while EcR-B1 predominantly expresses in larval structures which are destined to die (Bender *et al.*, 1997; Schubiger *et al.*, 1998). Due to the absence of EcR-B2 specific mutations, the functions of this isoform are less understood. Ectopic expression of wild type *EcR* can, to a certain extent, partially rescue *EcR* mutants, depending upon the EcR isoform involved. It is still not clear how the MTs remain more or less refractory to ecdysone and are carried over to the adults when all other larval tissues are degraded or remolded under the influence of ecdysone.

In the present study we have examined the possible role of ecdysone signaling in the development and function of MTs. Since SCs are incorporated at a later stage during MT development and are functionally different from PCs, we were interested in knowing the role of ecdysone in incorporation and function of SCs. We expressed a dominant negative mutant EcR (EcR-DN) (Cherbas *et al.*, 2003) using the UAS-GAL4 system (Brand and Perrimon., 1993) in SCs of Malpighian tubules to block ecdysone signaling. Blocking ecdysone signaling using two GAL4 lines, *c649* and *c724* results in developmental arrest at $1^{st}/2^{nd}$ instar transition and 1^{st} instar, respectively. We show that the number of SCs is reduced in these mutants. We also show that the intercalation of SCs into the MTs during elongation is disrupted as these larvae show clusters of SCs, unlike the regular arrangement in wild type. Expression of *Drosophila* aquaporin, DRIP, is also reduced in these SCs. These results were confirmed using the ecd^1 temperature sensitive hypomorph allele, (Garen *et al.*, 1977). In this case too, the SCs were irregularly distributed, with a reduced expression of DRIP. Co-expression of only EcR-B2 isoform with the EcR-DN in developing MTs, but not of EcR-B1 or EcR-A isoform, was able to rescue the lethal phenotype suggesting that EcR-B2 may be essential for SCs. A role of ecdysone in SC morphogenesis in MTs is further indicated by the fact that expression of EcR-DN with *c724* Gal4 driver which expresses in all the stellate cells results in a more drastic phenotype than with the *c649* Gal4 driver which expresses only in a subset of stellate cells.

Results

Disruption of ecdysone receptor results in morphological changes in Malpighian tubules

To examine the role of ecdysone in development of MTs, we used two dominant negative EcR proteins, EcR^{F645A} and EcR^{W650A}, carrying mutations in the ligand binding domain of EcR and thus affecting all isoforms of ecdysone receptors (Cherbas *et al.*, 2003). EcR^{F645A} binds to ecdysone, while EcR^{W650A} does not, although both form heterodimers with USP and bind to the ecdysone response elements. Since they are not able to activate the target gene expression normally, the ecdysone signaling is interrupted when any one of them is expressed (Cherbas *et al.*,

TABLE 1

EXPRESSION DOMAINS OF GAL4 DRIVERS USED IN THE PRESENT STUDY

GAL4 Driver	Domains of expression
c649	Bar- shaped stellate cell, salivary glands, proventriculus, mid gut, visceral musculature cells, anterior spiracles, anal plate
c724	Stellate cells, bar shaped stellate cells, salivary glands, visceral musculature cells

TABLE 2

MEAN DIAMETER OF MALPIGHIAN TUBULES IS AFFECTED BY EXPRESSION OF *ECR DN* TRANSGENES

Genotype	Mean (+ S.E.) diameter (in mm) of main segment (N=30 in each case)
Wild type	98.0 ± 2.5
c649 X EcR DN	80.0 ± 2.5*
c724 X EcR DN	50.1 ± 2.0*
c649 X EcR DN;EcR B2	95.2 ± 2.0
c724 X EcR DN;EcR B2	93.0 ± 2.8

* indicate significant differences in diameter of tubule (P<0.05).

2003). Since EcR^{F645A} and EcR^{W650A} exhibited identical results, we used EcR^{F645A} in the present study and refer to it as EcR-DN in the following text. The EcR-DN was expressed in SCs of MTs with *c649* and *c724* Gal4 drivers. To identify the different tissues in which these Gal4 drivers are expressed, we used UAS GFP as reporter and observed the expression pattern (see supplementary Fig. S1). It was seen that besides the SCs, these drivers also express in a few other tissues (see Table 1). As shown in Table 1, only the SCs, visceral musculature and salivary glands are common tissues in which both these Gal4 drivers express. Expressing EcR-DN with *c649* and *c724* Gal4 drivers resulted in slow development and larval lethality. The *c649/+*; *EcR-DN/+* (Fig. 1B) and *c724/EcR-DN* (Fig. 1C) larvae appeared smaller in size when compared to wild type larvae of similar age (Fig 1A). We looked at the MT as these drivers also express in SCs, we observed that the size of MTs from dying/dead larvae of *c649/+*; *EcR-DN/+* (Fig.1B; blue arrow and Fig. 1b; black arrows) or *c724/EcR-DN* (Fig.1C; blue arrow and Fig 1c; black arrows) was smaller when compared to wild type (Fig.1A; blue arrow and Fig 1a; black arrows). We measured the mean diameter of MTs (Table 2) from wild type across the main segment, which was 98.0±2.5µm, while that in the *c649/+*; *EcR-DN/+* and *c724/EcR-DN* larvae was 80.0±2.5µm and 50.1±2 µm, respectively. Larvae from *c724/EcR-DN* showed greater reduction in size than *c649/+*; *EcR-DN/+.* Larvae from these crosses also exhibited other gross abnormalities, like irregular tracheae (Fig. 1B and 1C; red arrow), low adipose tissue, and sluggish movements. Cuticular preparations of dying/dead larvae in these crosses showed defects in larval molting. The cuticular preparation of *c649/+*; *EcR-DN/+* larvae (Fig. 2B) revealed the presence of both 1st (Fig. 2B; black arrows) and 2nd (Fig. 2B; red arrow) instar larval mouth hooks, suggesting that these larvae retain the cuticle from previous instar in addition to their newly formed cuticle while the control wild type larvae had already reached 3rd instar stage as revealed by the mouth hooks (Fig 2A; black arrow). Cuticular preparations from *c724/EcR-DN* dying larvae (Fig. 2C; black arrows), on the other hand, showed mouth hooks characteristic of first instar stage suggesting their death during 1st instar itself.

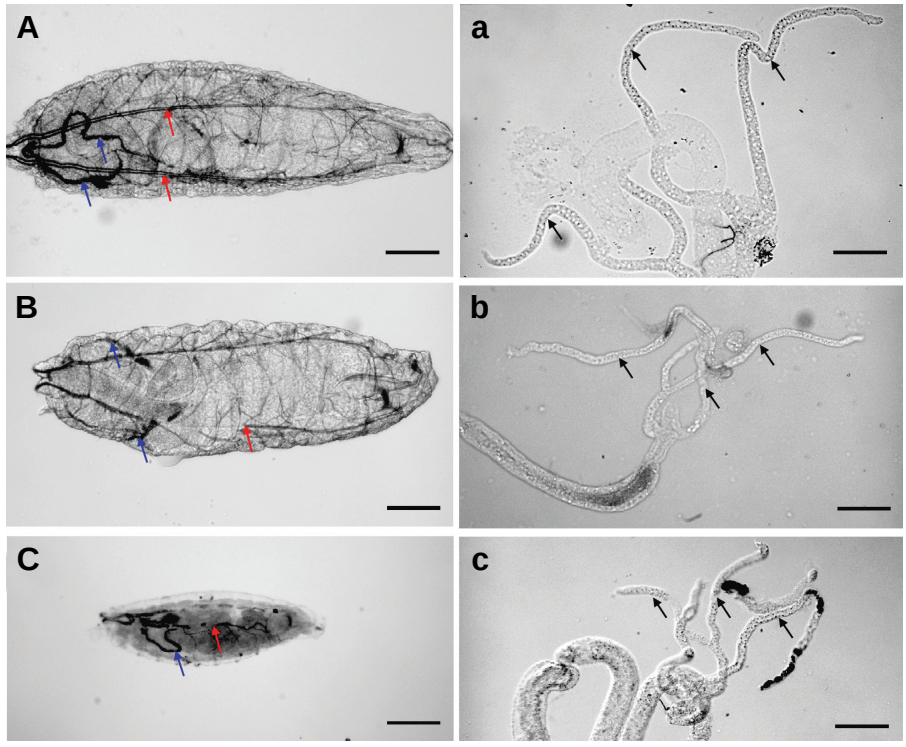

Fig. 1. Developmental defects of larvae and morphology of Malpighian tubules (MT). *Delayed development of larva from 649/+; EcR-DN/+* **(B)**, *c724/EcR-DN* **(C)** *when compared to wild type* **(A)**. *MTs and trachea are indicated by blue arrow and red arrows respectively. Malpighian tubules of wild type* **(a)** *are developed completely when compared to c649/+; EcR-DN/+* **(b)** *and c724/EcR-DN* **(c)**. *Bar represents 100 µm in each case.*

Disruption of ecdysone receptor leads to reduction in the number of stellate cells

A remarkable feature of MT is that the number of PCs and the SCs remain fairly constant throughout development (Wessing and Eichelberg, 1978; Sozen *et al.*, 1997). Since following expression of EcR-DN, the MTs were smaller in size, we counted the number of different cell types in MTs from wild type, *c649/+*; *EcR-DN/+* and *c724/EcR-DN* larvae. For an unambiguous identification of the SCs, the MTs were immuno-stained with teashirt antibody, which is a specific marker for SCs (Jung *et al.*, 2005; Singh *et al.*, 2007). Data in Table 3 show that the numbers of PCs in the anterior and posterior tubules of *c649/+*; *EcR-DN/+* and *c724/EcR-DN* genotypes were similar to that in the wild type. However, while the mean numbers of SCs in the wild type anterior and posterior tubules were 31.8±0.2 and 21.4±0.1, respectively, those in anterior and posterior tubules of *c649/+*; *EcR-DN/+* larvae were 24.9±0.4 and 17.5±0.2, respectively and in *c724/EcR-DN* MTs, these were 18.5±0.6 and 13.7±0.4, respec-

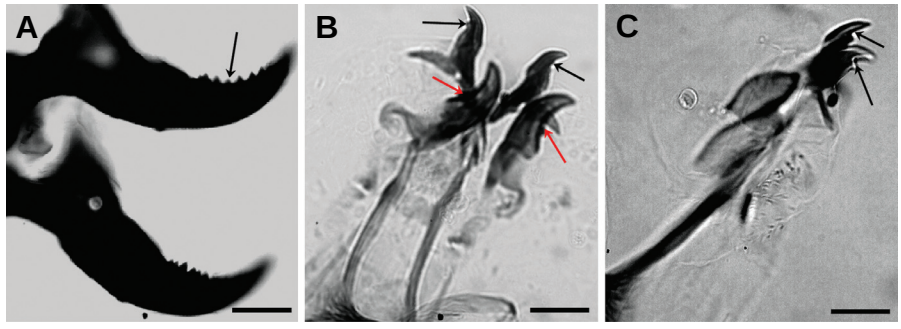

Fig. 2. Larval cuticular preparations. *Showing 3rd instar mouth-hooks of wild type* **(A)**, *1st and 2nd instar mouth hooks of c649/+; EcR-DN/+* **(B)** *and 1st instar mouth hooks of c724/EcR-DN* **(C)**. *Black arrows represent the 1st instar and red arrows show the 2nd instar larval mouth-hooks. Bar represents 20 µm.*

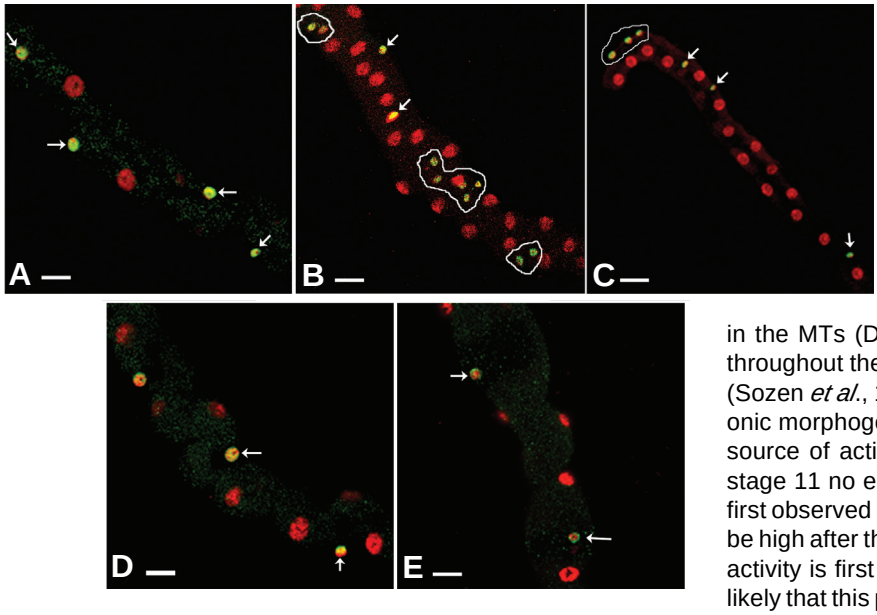

Fig. 3. Aberrant intercalation of stellate cells. *Expression of EcR-DN in SCs using c649* **(B)** *and c724* **(C)** *drivers leads to clustering of these cells when compared to regular arrangement of wild type* **(A)** *Co-expression of EcR-B2 rescues the clustering phenotypes induced by EcR-DN when driven by c649* **(D)** *and c724* **(E)** *SCs are identified by anti- teashirt antibody (green fluorescence) and chromatin is stained with DAPI (Pseudocolour red). Arrows indicate single stellate cells while encircled area shows clustering of stellate cells. Bar represents 20 μm.*

tively. We subjected this data to one way anova followed by post hoc test and found that the number of SCs in *c649/+*; *EcR-DN/+* and *c724/EcR-DN* was significantly reduced when compared to wild type ($p<0.05$). It was also seen that the *c724* Gal4 driver caused greater reduction in numbers of SCs than the *c649* Gal4 driver (Table 3).

Disruption of ecdysone signaling leads to aberrant pattern of integration of stellate cells

Drosophila MTs are derived from the ectodermal epithelial buds and the mesenchymal mesodermal tissues (Denholm *et al.*, 2003). During embryogenesis, the principal cells are formed by stage 13 (Skaer, 1989). The SCs are found near the vicinity of MTs at stage 11/12 and at stage 13 the SCs have just started intercalation, but only by stage 15 the SCs are fully incorporated in the MTs (Denholm *et al.*, 2003). SCs are distributed evenly throughout the anterior and posterior tubules at regular intervals (Sozen *et al.*, 1997). Ecdysone plays an important role in embryonic morphogenesis. Maternally deposited ecdysteroids are the source of active ecdysone (Hoffman *et al.*, 1985), however till stage 11 no ecdysone activity is observed. Ecdysone activity is first observed at stage 12 and peaks at stage 13 and continues to be high after that (Kozlova and Thummel, 2003). Since ecdysone activity is first observed just prior to SCs intercalation, it is very likely that this process is governed by ecdysone. We investigated the role of ecdysone in arrangement of the SCs. In wild type larvae (Fig. 3A), the SCs were distributed in the characteristic pattern as reported earlier (Sozen *et al.*, 1997). However, in larvae expressing EcR-DN under the *c649* (Fig. 3B) and the *c724* (Fig. 3C) Gal4 driver, the regular arrangement of SCs in MTs was lost, instead they were found to be present in clusters suggesting that the intercalation of SCs into the MTs during embryogenesis was disrupted. We counted the number of SC clusters in wild type and compared them with EcR-DN mutant expressing MTs (Table 4). It was found that in 25 wild type MTs examined, there were only five instances when SCs were present in clusters, whereas in similar numbers of *c649/+*; *EcR-DN/+* and *c724/EcR-DN* MTs, majority of the tubules showed clusters of SC (Table 4). Further, the number of SCs in each cluster in wild type was never more than two, but the clusters in EcR-DN expressing MTs showed

TABLE 3

THE NUMBER OF STELLATE CELLS IS REDUCED IN ECR-DN EXPRESSING MALPIGHIAN TUBULES

	Principle cells		Stellate cells	
Genotype (N=30 in each case)	**Anterior tubule**	**Posterior tubule**	**Anterior tubule**	**Posterior tubule**
Wild type	142.9 ± 0.3	110.0 ± 0.3	31.8 ± 0.2	21.4 ± 0.1
c649X EcR DN	140.0 ± 0.6	104.1 ± 0.8	24.9 ± 0.4*	17.5 ± 0.2*
c649X EcR DN;EcR B2	142.4 ± 0.4	106.3 ± 0.5	28.5 ± 0.4	19.8 ± 0.3
c724X EcR DN	141.9 ± 0.4	109.3 ± 0.5	18.5 ± 0.6*	13.7 ± 0.4*
c724X EcR DN; EcR B2	141.1 ± 0.5	102.4 ± 0.9	27.5 ± 0.6	20.2 ± 0.2

* shows significantly reduced stellate cells number ($P<0.05$).

TABLE 4

THE NUMBER OF STELLATE CELL CLUSTERS AND THEIR RANGE IN ANTERIOR AND POSTERIOR TUBULES

	Anterior tubules (N=25)		Posterior tubule (N=25)	
Genotype	**Total No. of Clusters**	**Range of cells in a cluster**	**Total No. of Clusters**	**Range of cells in a cluster**
WT	6	2	4	2
c649 X EcR DN	128*	2-6*	25*	2-3*
c724 X EcR DN	95*	2-5*	52*	2-4*
c649 X EcR DN; EcR B2	7	2	4	2
c724 X EcR DN; EcR B2	10	2	8	2

N- total number of MTs counted. * shows the significant increase in cluster number and Size ($P<0.05$).

between two to seven SCs per cluster.

The B2 ecdysone receptor isoform is required for maintaining normal stellate cell number and integration

The three EcR isoforms are known to be involved in distinct functions depending upon their spatial and temporal expression (Kim *et al*., 1999; Snug and Robinow., 2000; Truman *et al*., 1994). It is known that expression of wild type EcR can to a certain extent, rescue phenotypes resulting from EcR-DN expression (Schubiger, 2003; Cherbas *et al*., 2003). Therefore, we individually co-expressed each of the wild type EcR isoforms (EcR-B1, EcR-B2 or EcR-A) along with EcR-DN to examine possible rescue. To find out if over-expression of EcR-B1, EcR-B2 or EcR-A with any of the two *Gal4* drivers by itself had any detectable phenotype, we expressed each of these isoforms in the wild type background. We did not observe any effect on the viability and phenotype following over-expression of any of these EcR isoforms in the target tissues (data not shown). Then we co-expressed each of these isoforms individually with EcR-DN using the two Gal4 drivers as above. It was found that expression of EcR-B2 isoform only was capable of rescuing the lethality caused by EcR-DN. The morphology and pattern of intercalation of SCs in MTs co-expressing EcR-DN and EcR-B2 under the *c649* (Fig. 3D) or the *c724* (Fig. 3E) drivers was similar to that in wild type. The numbers of SCs in EcR-B2 co-expressing in MTs were restored to almost to the level in wild type (Table 3). Likewise the mean diameter of these MTs was also found to be not significantly different from that in wild type (Table 2). Co-expression of EcR-B1 and EcR-A along with EcR-DN with any of the two Gal4 drivers, however, was not able to suppress the altered MT phenotype (data not shown).

Expression of dominant negative form of the ecdysone receptor reduces DRIP expression in stellate cells

Expression of DRIP, which facilitates water transport (Dow *et al*., 1995, Kaufmann *et al*., 2005), has been reported only in the SCs during embryogenesis and in adults (Kaufmann *et al*., 2005). To investigate if DRIP is expressed in larvae and if ecdysone signaling is required for DRIP expression, we immunostained wild type larval MTs using anti-DRIP antibody. It was seen that as in embryos and adults (Kaufmann *et al*., 2005), DRIP is also expressed in the larval MTs and the expression is limited to only SCs (Fig. 4A). Expression of DRIP was greatly reduced in SCs following expression of EcR-DN under the *c649* (Fig. 4B) or *c724* Gal4 driver (Fig. 4C). For exact comparison we measured DRIP intensity using Profile Display Function of Zeiss LSM Meta 510 software which displays the intensity of flurochrome. The fluorescence intensity of DRIP and DAPI is depicted on the Y axis and the marked line in Fig. 4 (A,B,C,D) starting from tail to arrow head and is plotted on the X axis. We found that the intensity of DRIP (Green colour graph) in *c649/+*; *EcR-DN/+* (Fig. 4b) and *c724/EcR-DN* (Fig. 4c) was much less than wild type (Fig. 4a). Co-expression of EcR-B2 in MTs expressing EcR-DN under *c649* (Fig. 4E,e) and *c724* (Fig. 4F,f) Gal4 driver restored the DRIP expression to near wild type levels. As noted with MT morphology, co-expression of *EcR-B1* or *EcR-A* did not elevate the expression of DRIP in SCs (data not shown).

Malpighian tubules of larvae homozygous for temperature-sensitive ecd^{1} mutant allele exhibit reduced stellate cell numbers and defects in stellate cell intercalation at non-permissive temperature

ecd^{1} (Garen *et al*., 1977) is a temperature sensitive allele for

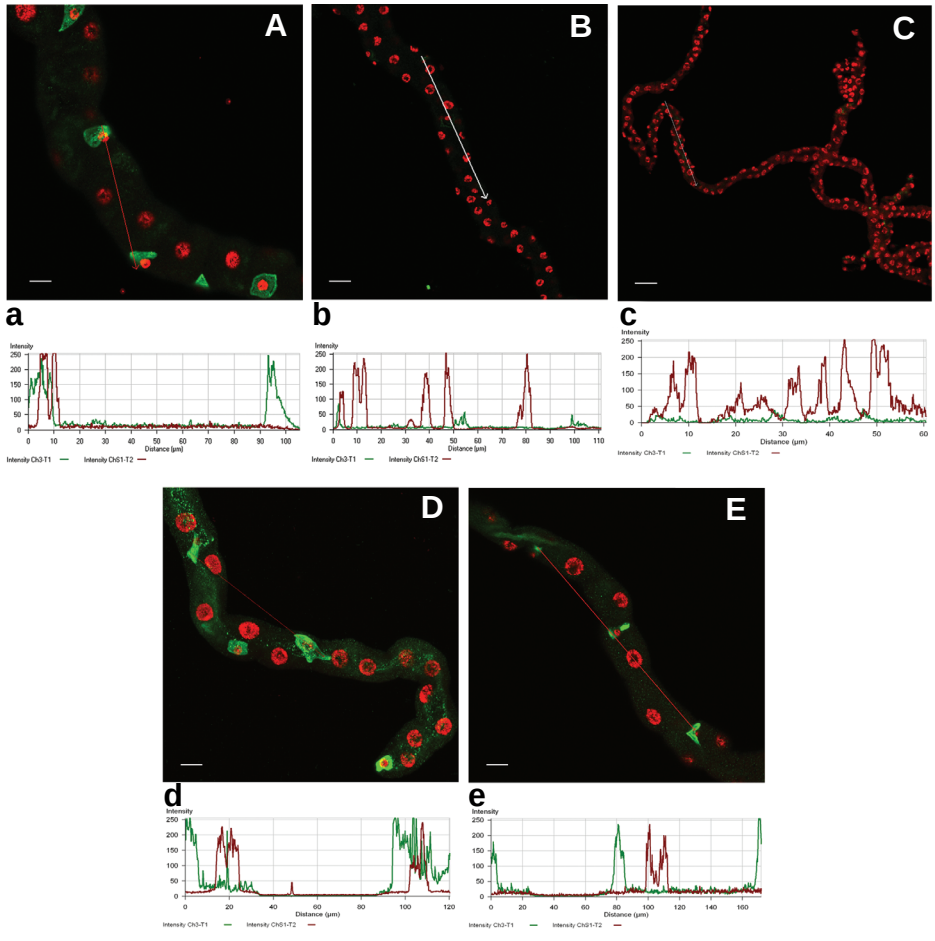

Fig. 4. *Drosophila* integrin protein (DRIP) levels are reduced in stellate cells when EcR-DN is expressed. *Compared to wild type* **(A)**, *DRIP expression is less in c649/+; EcR-DN/+* **(B)** *and c724/EcR-DN* **(C)**. *Coexpression of EcR-B2 restores DRIP expression to nearly wild type levels when driven by c649* **(D)** *and c724* **(E)**. *DRIP is labeled with FITC (green fluorescence) and chromatin with DAPI (pseudocolour red). The graph below each slide* **(a,b,c,d,e)** *represents the Profile display function of LSM 510 Meta which measures the intensity of fluorescence along the line drawn. In Graph green colour shows intensity of DRIP while red shows the DAPI. Bar represents 20 μm in each case. Arrows passing through different cells show DRIP expression only in stellate cells and intensity in these cells is measured by the Profile display function.*

ecdysone production and is unable to synthesize ecdyosne when shifted to 29ºC. It's a hypomorph allele showing lethality at non-permissive conditions. In order to confirm if the observed phenotypes following disruption of EcR is because of ecdysone signaling, we grew *ecd*^{1} homozygous embryos at non-permissive temperature, and examined the arrangement of SCs in MTs from 1st instar larvae. The SCs in these MTs were indeed clustered (Fig. 5A) similar to that seen when EcR-DN is expressed in MTs (Fig. 3). The expression of DRIP in these SCs was also found to be reduced (Fig. 5D) than in wild type (Fig. 4A). The fluorescence intensity profile of DRIP also showed reduced intensity levels (Fig. 5d,f).

Discussion

In *Drosophila*, developmental events like molting at different larval instars and metamorphosis during pupal period is triggered by the ecdysteroids. Malpighian tubules is an interesting tissue to study developmental biology, because firstly it does not undergo ecdysone induced degeneration (Jiang *et al.*, 1997) and secondly it is made of cells from two different lineages (Denholm *et al.*, 2003) which have physiologically distinct functions (Davies *et al.*, 1996; Dow *et al.*, 1994, 1998; O'Donnell *et al.*, 1996; Sozen *et al.*, 1997; O'Donnell *et al.*, 1998). Though ecdysone signaling in larval MTs does not lead to cell death, we find that disruption of ecdysone signaling using *EcR-DN* in SCs during early development results in improper morphogenesis of MTs which affects their functioning as reflected in the reduced DRIP expression. These results clearly suggest that ecdysone has an important role in development and physiology of MTs. Earlier studies on the expression of *EcR-DN* with *Eip*, *GMR*, *Ser* or *dpp* Gal4 drivers revealed local as well as a global effects resulting in complete blockage of development (Cherbas *et al.*, 2003) suggesting that there is a molting checkpoint. It is likely that localized malfunctioning of EcR in important tissues is sensed by the surveillance system and in cases of irreparable defects, blocks development at the next ecdysone dependent event. Our data suggest that EcR dependent functioning of SCs in MTs could also be one of the critical functions whose malfunctioning can ultimately lead to larval death.

Our present studies show a new role of ecdysone in proper intercalation of SCs. The normal incorporation of SCs depends on the Hibris protein, which is an ortholog of mammalian protein NEPHRIN. Hibris helps to stabilize the intercalation of SCs in MTs (Denholm *et al.*, 2003, Artero *et al.*, 2006). Hibris acts in cell-cell recognition and attraction during embryogenesis (reviewed by Chen and Olson, 2004). We show that ecdysone signaling affects SC arrangement, leading to the possibility that ecdysone may be regulating *hibris* expression either directly or indirectly. Since disruption of ecdyosne signaling in SCs does not affect PC integration our present results further suggest that the arrangement of SCs and PCs are independent of each other. The arrangement of PCs is largely controlled by the product of *rols*, since in MTs of *rols* mutants while the PCs were in clusters; the SCs were normally integrated between the PCs (Putz *et al.*, 2005). Our results show that the intercalation requires ecdysone at some stage and that the EcR-B2 isoform may be the major isoform through which ecdysone signaling is transduced. It is notable in this context that migration of border cells in egg chambers requires C/EPB transcription factor encoded by *slow border cells* (*slbo)* locus and in *slbo* mutants, border cells migrate either very slowly or not at all (Rorth *et al.*, 1998). Expression of EcR-DN driven by *slbo-GAL4* in ovaries, results in failure of border cells migration which is subsequently rescued by the expression of EcR-B2 isoform (Cherbas *et al.*, 2003). In view of our present results, it is tempting to speculate that the developmental migration of different cells is triggered by ecdysone and EcR-B2 may be the key isoform that tranduces the signal.

SCs play an important role in fluid secretion and inhibition of chloride channels affects functioning of SCs and ultimately affects fluid secretion (Sozen *et al.*, 1997). In *hibris* mutants, where the number of SCs is reduced, the fluid transport is also affected, resulting in lethality (Denholm *et al.*, 2003). In agreement with some early reports that ecdysone plays a role in fluid secretion by altering membrane permeability (Gee *et al.*, 1977; Ryerse, 1978), we provide direct evidence that ecdysone, directly or indirectly controls the fluid secretion of SCs via its influence on the expression of

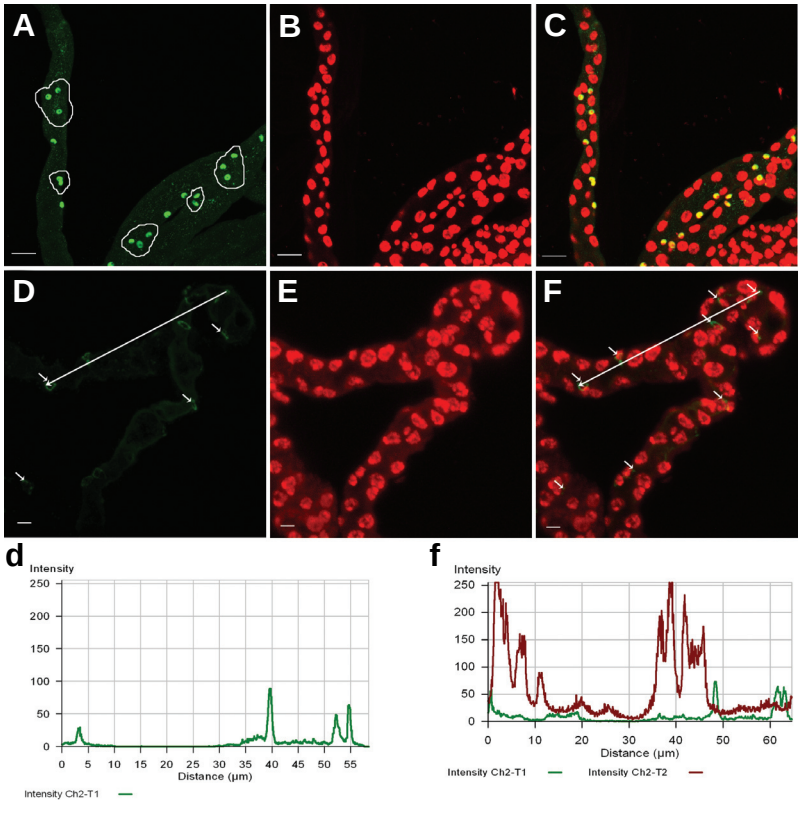

Fig. 5. Ecdysone levels affect stellate cell organization and *Drosophila* integrin protein (DRIP) levels. *Immunostaining with anti-teashirt antibody in ecd*^{1} *mutant shows clusters of SCs* **(A)**, *DAPI* **(B)** *and merged* **(C)**. *ecd*^{1} *mutants also show reduced expression of DRIP* **(D)**, *DAPI* **(E)** *and merged* **(F)**. *The intensity of DRIP* **(d,f)** *is measured by Profile display function of LSM Meta 510 software. DAPI is pseudocolour red. Bar represents 50 µm (A,B,C) and 5 µm in (D,E,F).*

DRIP.

Stellate cells have an important role and the appropriate number of SC is essential for proper development. This is evidenced by the fact that the phenotypes derived from *c724* are more drastic than similar phenotypes observed by *c649*. This could be because *c724* affects the entire stellate cells, where as the *c649* affects only a small proportion of bar shaped stellate cells.

Our present results thus show that proper ecdysone signaling via the EcR-B2 in MTs during embryonic development is required for correct integration of SCs in developing MTs and for their physiological functions involving DRIP expression.

Materials and Methods

Drosophila stocks

Following fly stocks used in the present study were obtained from the Bloomington stock centre unless mentioned otherwise. Wild type Oregon R^+, stellate cells specific GAL4 drivers *c724* and *c649* (Kind gift by Dr. J. A. T. Dow, Institute for Biomedical Sciences, University of Glasgow, UK), UAS responders, *P{UAS-EcR.B1-DeltaC655.F645A} (EcR^{F645A}) P{UAS-EcR.B1-DeltaC655.W650A} (EcR^{W650A}) P{UAS-EcR.B1} (EcR-B1), P{UAS-EcR.B2} (EcR-B2), P{UAS-EcR.A (EcR-A).* Temperature sensitive hypomorph allele for ecdysone (*ecd^1 st^1 red^1 e^4 ca^1*) (*ecd^1*) (Garen *et al*., 1977) was obtained from Dr. C. Thummel (Department of Human Genetics, University of Utah School of Medicine, UT). Appropriate crosses were set up to generate *P{UAS-EcR.B1-DeltaC655.F645A}; P{UAS-EcR.B1}, P{UAS-EcR.B1-DeltaC655.F645A};P{UAS-EcR.B2}, P{UAS-EcR.B1-DeltaC655.F645A; P{UAS-EcR.A}.* The references to these stocks in the paper are shown in bracket and bold. All flies and larvae were reared at 25±1^0 C on standard food containing maize powder, agar, dried yeast and sugar at 12 hours dark: 12 hours light cycle. For checking lethality, egg laying was set on agar plates and the number of eggs laid was counted. After hatching they were counted and checked for the stage of lethality. In the rescue experiments, similar procedure was done and finally the number of adults emerged was used to calculate the percentage of rescue. The *ecd^1* flies were allowed to lay eggs at 25±1^0 C and then shifted at 29^{0}C for further development.

Morphological analysis of larvae and Malpighian tubules

Larvae of different genotypes were collected from food plates and washed with distilled water. They were anaesthetized with ether and mounted on bridged slides in 50% glycerol and observed under bright field. The MTs were dissected in 1XPBS (18.6mM NaH_2PO_4, 84.1mM Na_2HPO_4, 17.5mM NaCl) from larvae of desired genotype and mounted in 50% glycerol and observed under DIC optics. Images were collected using Nikon Digital camera DXM 1200 fitted on the Nikon E800 microscope. For measuring the diameter Malpighian tubules, they were dissected and spread on a slide. The diameter at the main segment was measured with an ocular micrometer under 10X magnification in units of 16.7 µm. For examining the cuticle, mouth-hooks and spiracles, larvae of appropriate genotypes were washed in 1X PBS, fixed in 1:4 glycerols: acetic acid and incubated over-night at 60^{0}C. They were mounted in Hoyer's mounting medium covered with coverslips under a 20gm weight and incubated again at 60^0 C for 2 days. Images were captured as above.

Immunocytochemistry

Malpighian tubules from larvae of appropriate genotypes were dissected in 1X PBS, fixed in 4% formaldehyde for 20 min at RT, rinsed in PBST (PBS, 0.1% Triton X-100) and processed for immunostaining essentially as described earlier (Patel NH,1994). The MTs were mounted in antifadant, DABCO (Sigma). The primary antibodies used were anti-teashirt used at 1:3000 and anti-DRIP used at 1:1000. Goat-anti rabbit AF488 (Molecular probes, USA) was used as secondary antibody. Chromatin was stained with DAPI (1 µg/ml in 1XPBS). All preparations were analyzed on a Ziess LSM 510 Meta Confocal microscope and images were processed with Adobe Photoshop.

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