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in *Drosophila* Imaginal Discs via *Broad-Complex*

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One of the most significant morphogenic events in the development of *Drosophila melanogaster* is the elongation of imaginal discs during puparium formation. We have shown that this macroscopic event is accompanied by the formation of Golgi stacks from small Golgi larval clusters of vesicles and tubules that are present prior to the onset of disc elongation. We have shown that the fly steroid hormone 20-hydroxyecdysone triggers both the elongation itself and the formation of Golgi stacks (V. Kondylis, S. E. Goulding, J. C. Dunne, and C. Rabouille, 2001, *Mol. Biol. Cell,* 12, 2308). Using mRNA *in situ* hybridisation, we show here that ecdysone triggers the upregulation of a subset of genes encoding Golgi-related proteins (such as *dnsf1*, *dsec23*, *dsed5*, and *drab1*) and downregulates the expression of others (such as *dergic53*, *dβ'COP*, and *drab6*). We show that the transcription factor Broad-complex, itself an "early" ecdysone target, mediates this regulation. And we show that the ecdysone-independent upregulation of *dnsf1* and *dsnap* prior to the ecdysone peak leads to a precocious formation of large Golgi stacks. The ecdysone-triggered biogenesis of Golgi stacks at the onset of imaginal disc elongation offers the exciting possibility of advancing our understanding of the relationship between gene expression and organelle biogenesis.

Key Words: **Golgi stacks; Golgi proteins; ecdysone; Broad-complex; gene expression; *in situ* hybridisation; FISH; immunofluorescence.**

INTRODUCTION

The Golgi apparatus has a striking morphology. It comprises a series of flattened membrane-bound compartments (cisternae) that are stacked to form the so-called Golgi stack. There are multiple stacks per cell. In mammalian systems, they are connected to each other to form a single copy organelle capping the nucleus. In *Drosophila melanogaster,* the stacks are dispersed throughout the cytoplasm (Kondylis *et al.,* 2001). On each side of the Golgi stack is a membrane network, the Cis Golgi Network (CGN) and the Trans Golgi Network (TGN).

Modulation of Golgi apparatus architecture has to date been shown to be mediated via posttranslational modifications and influenced by the translational status of secretory cargo passing through the organelle. Only recently have developmental cues and processes become a parameter in this modulation.

Protein phosphorylation plays a crucial role in the remodelling of the Golgi apparatus at the onset of mitosis. Several Golgi-associated proteins, such as mammalian GM130 (Golgi matrix 130) and GRASP55 (Golgi reassembly stacking protein 55), are substrates of the phosphorylation cascade catalysed by CDC2 (Lowe *et al.,* 1998) and MEK1 (Colanzi *et al.,* 2000; Jesch *et al.,* 2001), respectively. Phosphorylation of these proteins leads to the extensive fragmentation of the Golgi complex into small clusters of vesicles and tubules (Preisinger and Barr, 2001). Conversely, GM130 dephosphorylation by protein phosphatase 2A is essential in allowing organelle reassembly at telophase (Lowe *et al.,* 2000). Lipid modifications, such as the *N*-myristoylation of mammalian GRASP65, is thought to influence the stacking of Golgi cisternae (Barr *et al.,* 1997). Lipid kinases are also seemingly involved. TGN38 is a target for PIP3 kinase and modulate TGN dynamics (Ponnambalam *et al.,* 1999). Lastly, protein processing and degradation could be other factors as they control, for instance, the number of peroxisomes in yeast (Veenhuis *et al.,* 2000) and the biogenesis of trichocysts in Paramecia (Vayssie *et al.,* 2001).

A fine example of translationally influenced Golgi mor-

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phology is observed during larval development in *D. melanogaster*. The salivary gland glue synthesis that commences during mid-third instar larvae (TIL) is accompanied by a marked enlargement of the Golgi stacks (Thomopoulos *et al.*, 1992).

Salivary glue synthesis also provides an example of where development influences Golgi structure (Biyasheva *et al.*, 2001). Other examples are provided by mammalian hormones. For instance, prolactin is known to accelerate the production of breast milk, and the Golgi complex in the mammary gland cells is dramatically enlarged (Rambourg *et al.*, 1993). In both cases, however, the effect of the developmental cues on the enlargement of the Golgi complex is indirect and poorly understood.

Here, we provide evidence for a novel transcriptionally mediated mechanism directly modulating Golgi architecture in a developmentally controlled fashion. We have recently reported that a dramatic change in the morphology of the Golgi apparatus takes place in the cells of the *D. melanogaster* imaginal wing and leg discs at the onset of puparium formation (Kondylis *et al.*, 2001). This is the period during development when the imaginal discs begin their first phase of elongation in order to become the future adult appendages. During mid-TIL, the Golgi apparatus consists of small clusters of vesicles and tubules (called larval clusters), which become larger as the TIL progress towards puparium formation and are converted into typical Golgi stacks in white pupae.

Puparium formation is tightly controlled by the steroid hormone 20-hydroxyecdysone (ecdysone), which triggers all morphogenic events within the developing *Drosophila* larvae, including imaginal disc elongation (Fristrom and Fristrom, 1993). Many studies have focused on the prepupal peak of ecdysone at 6–8 h prior to puparium formation. This peak has been shown to activate the expression of the "early puff genes" via the heterodimeric receptor complex comprising the ecdysone receptor, EcR, and the gene product of *ultraspiracle* (Koelle *et al.*, 1991). At least three of these encode transcription factors, such as Broad complex (DiBello *et al.*, 1991; Andres *et al.*, 1993), E74 (Burtis *et al.*, 1990), and E75 (Segraves and Hogness, 1990). They self attenuate their own transcription and activate the transcription of more than 100 "late puff genes."

In light of the known activity of ecdysone as a gene expression regulator, we investigated and show that a subset of genes encoding Golgi-associated proteins are transcriptionally regulated by this hormone via the Broad-Complex transcription factor. This regulation leads to Golgi stack biogenesis during disc elongation.

MATERIALS AND METHODS

Fly Stocks

Overexpression of dNSF1 and dSNAP. The W1118 and OreR fly stocks were obtained from Andrew Jarman's lab (Edinburgh) and are both referred to as WT.

The HSN stock carries on the first chromosome a transgene comprising dNSF1 cDNA under the control of a heat shock promoter (gift from Leo Pallanck), whereby exposure at 33–37°C drives the expression of *dnsf1*.

UAS SNAP/UAS SNAP (III) is a transgenic fly stock carrying on the third chromosome a dSNAP cDNA transgene downstream of a UAS sequence (gift from Leo Pallanck). Homozygote females were crossed to hsGAL4/Bc, El, Gla to produce a genotype whereby we could drive dSNAP expression using the hsGAL4 driver. Black cell larvae (Bc) were used as control.

WT, HSN, hsGAL4/+; UAS SNAP/+ (hsGAL4 UAS SNAP) mid-TIL maintained at 22 ± 1°C were collected and heat shocked for 40 min at 37°C followed by 1 h at 33°C. The wing imaginal discs were then dissected and fixed as described below. As controls, wing imaginal discs from each fly stock above, maintained at 22°C, were dissected and processed in the same fashion.

The npr homozygote mutant clones. The *npr*/*npr* mosaic imaginal discs were generated as follows: Virgin female nrp l[3] w FRT18A/FM7; +/+; +/+ (Gift from Kevin Moses) were crossed to male yw/Y; +/+; hsflp, sb/TM6b,Tb. The F_1 virgin female nrp l[3] w FRT18A/+; +/+; hsflp, sb/+ were then crossed to male w[1118] P{w + piMyc} P{w + Ubi-GFP nls} P{ FRT18A}/Y for 24 h. After days 3, 5, and 6, a 20-min 37°C heat shock was given to the larvae to induce the homozygote clones. Late TIL female larvae were dissected and one in four had the desired genotype, nrp l[3] w FRT18A/W[1118] P{w + piMyc} P{w + Ubi-GFP nls} P{ FRT18A}; +/+; hsflp, sb/+.

Alkaline phosphatase RNA *in situ* hybridisation and FISH were performed by using mRNA probes for *dsec23*. The wing imaginal discs were mounted in glycerol in the case of alkaline phosphatase staining and in Vectashield in the case of FISH, and viewed under an Axioskop II epifluorescence microscope equipped with an Axiocam digital camera (Zeiss). Clones were detected by their lack of Ubi-GFP fluorescence (green channel). The *dsec23* expression was estimated within the clones by comparison with the surrounding tissue.

Tissue Preparation

Mid- and late TIL were selected in a way such that the two experimental groups straddled the ~3-h prepupal ecdysone pulse. We used salivary gland morphology as a precise and convenient means of larval staging. Glue synthesis commences during third instar in response to the slow ecdysone titre increase and continues until the prepupal ecdysone pulse (Boyd and Ashburner, 1977; Biyasheva *et al.*, 2001). This is manifest as a gradual swelling of the salivary gland cells, until the surface of the gland becomes enlarged and bumpy. The cell borders become highly visible during this stage. This salivary gland morphology was taken as an indication of mid-TIL (see Results; Fig. 1A). The prepupal ecdysone pulse triggers the rapid expulsion of the glue product into the lumen of the gland, an event that is strictly under the control of ecdysone. This is manifest as a swelling of the entire gland and a concomitant stretching of the gland cells such that they become flattened and smooth. The cell borders become considerably less visible at this stage (see Results; Fig. 1B). White pupae displayed characteristic morphology with a soft white pupal case.

Each larvae or white pupae was transected posterior to the anterior/posterior midline and the anterior portion everted such that the imaginal structures were exposed and visible under a dissecting stereoscope. These larvae are what we term "semidissected." When used for *in situ* hybridisation, the carcasses were immediately fixed in 3.7% formaldehyde in PBT (DEPC treated

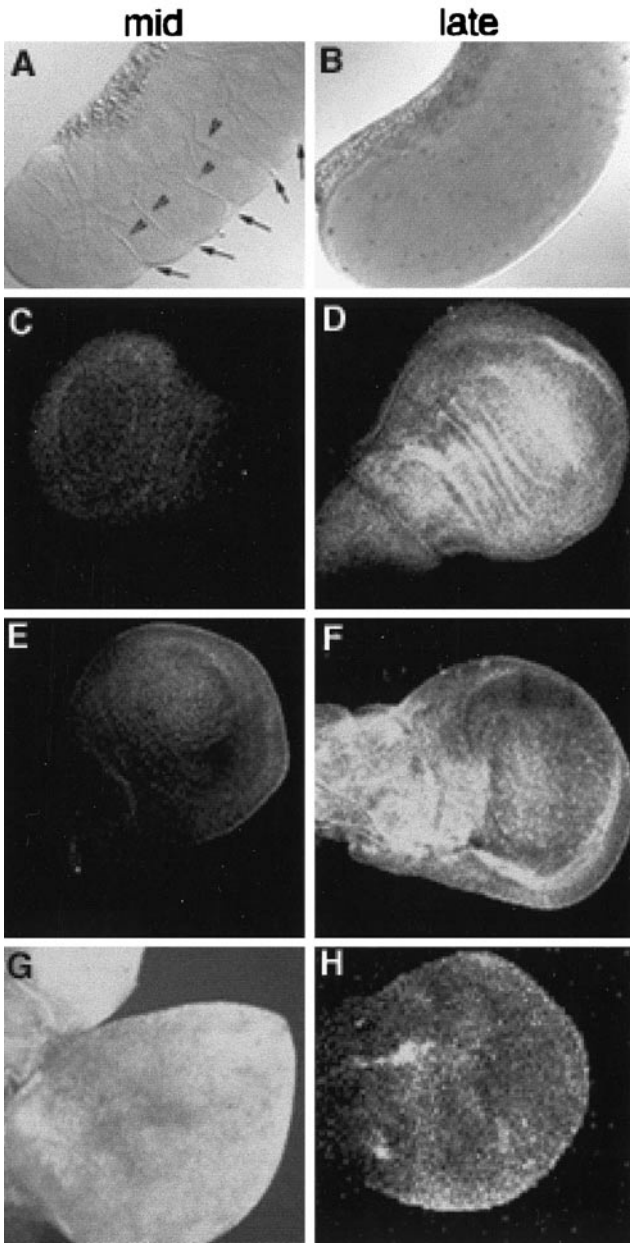

FIG. 1. Expression of Golgi proteins in mid- and late third instar larvae. WT mid- and late TIL were selected by using salivary gland morphology as a precise and convenient means of larval staging. During mid-third instar (A), the salivary gland cells are slightly swollen and as such the gland appears bumpy and the cell borders are clearly visible (arrows). The prepupal ecdysone pulse triggers the rapid expulsion of the glue product into the lumen of the gland. At late third instar, the entire gland becomes swollen and each cell becomes flattened and smooth (B). The cell borders become considerably less visible. WT mid (C, E, G)- and late TIL (D, F, H) were processed for indirect immunofluorescence by using a rabbit anti-dGMII antiserum (C, D), a rabbit anti-human Sec23 antibody (E, F), and a rabbit anti-rat β'COP (G, H) followed by an anti-rabbit IgG coupled to FITC. The wing imaginal discs were dissected, mounted in Vectashield, and observed under a Leica confocal microscope. A section projection encompassing the full disc thickness is displayed.

PBS supplemented by 0.1% Tween) for 1 h on a gently rotating wheel. This was followed by sequential dehydration to 100% EtOH. Tissue was then stored at −20°C until required for *in situ* hybridisation. *In situ* hybridisation for each mRNA probe tested consisted of a single group of tissue from each developmental time point (defined as an experimental group). In three cases, the everted larval carcasses from all three developmental time points were fixed and processed in the same tube so as to check avoidance of processing artifacts (see Results).

Indirect Immunofluorescence

WT mid- and late TIL were semidissected and fixed as described in Kondylis *et al.* (2001) and labelled with either an anti-Sec23 antibody that cross reacts with dSec23 (Kondylis *et al.*, 2001), an anti-dGMII antiserum (Rabouille *et al.*, 1999), or an anti-rat β'COP antibody (gift from Martin Lowe) followed by an anti-rabbit IgG antibody conjugated to FITC. The wing imaginal discs were dissected, mounted in Vectashield containing DAPI, and viewed under a confocal LEICA microscope. A series of optical sections (2-μm thickness) were obtained from mid- and late TIL imaginal discs at 40× by using identical laser settings. The appropriate laser intensity was chosen, using the late TIL so as to avoid optical saturation. A section projection was created, and the resulting image is presented.

In Situ Hybridisation

The templates used to synthesise the mRNA probes are described in Table 2. Each digoxigenin (Dig)-labelled mRNA antisense probe was synthesised with an *in vitro* transcription kit (Roche) using the 3′ digested template (see Table 2), the appropriate T7 or T3 promoter (see Table 2), and a nucleotide mixture including Dig-UTP. The *in situ* hybridisation protocol was as previously described (Goulding *et al.*, 2000). Anti-Dig-alkaline phosphatase Fab fragments (Roche) were used to visualise the Dig-labelled mRNA, and we used NBT (4-nitroblue tetrazolium chloride) and X-phosphate (5-bromo-4-chloro-3-indolyl-phosphate) as substrates. Anti-Dig-peroxidase Fab fragments were also used in the FISH experiments. In this case, we used the Cyanine 3-labelled Tyramide Signal Amplification System (NEN) to visualise the Dig-labelled mRNA. Following *in situ* hybridisation, the wing imaginal discs were dissected free from the carcasses and mounted in 80% glycerol in PBS or Vectashield (containing DAPI) and viewed under a Zeiss Axioskop II (as above) using DIC or fluorescence microscopy.

Disc Culture Assay

For this experiment, mid-TIL were required to be old enough to respond to exogenous ecdysone but not so old as to have been exposed to endogenous ecdysone. These larvae were again selected on the basis of their salivary gland morphology, which exhibited bumpy salivary glands with moderate cell size, slightly larger than the mid-TIL used for the *in vivo* experiments but not too large (for fear of selecting larvae that have been exposed to endogenous ecdysone) but have not yet extruded the glue into the canal there is approximately a 6-h gap between the two events.

They were semidissected and incubated at 22°C for 16 h in a 30-mm plastic culture dish containing 1.4 ml Schneider medium supplemented with 2 μM 20-hydroxyecdysone (Sigma; 5 mM stock in 10% ethanol). As a control, equivalent larvae were incubated in the same conditions in the absence of ecdysone. Upon conclusion of the incubation period, the carcasses were removed from the

culture medium and fixed immediately as above, sequentially dehydrated to 100% EtOH, and stored at −20°C.

Quantitation of Immunofluorescence and in Situ Hybridisation Labelling

An experimental group is defined as tissue of equal developmental age assayed for a given Golgi-related mRNA or protein. All the immunofluorescence and *in situ* hybridisation pictures used for quantitative purposes were captured at the same exposure settings within one experimental group. We used the 20× objective of the upright Zeiss Axioskop II equipped with epifluorescence and DIC and a Zeiss Axiocam digital camera. Images were transferred to Adobe Photoshop 5.5 and converted to black and white.

The labelling intensity was quantitated by using NIH Image Version 1.62 (publicly available at http://rsb.info.nih.gov/nih-image/). Approximately 20 boxes of various sizes were used to estimate the mean labelling intensity within at least 8 images per experimental group. In some cases, staining was more intense in specific areas of the wing imaginal disc, and as such an estimation of the labelling was performed for the specific region.

We also estimated the difference in labelling intensity by using FISH experiments. We achieved this by artificially matching labelling intensities through manipulation of camera exposure times. For each mRNA probe, we chose the sample that displayed the lower labelling intensity. We then set and recorded the exposure time required to obtain an image that is within the dynamic range of the camera. We then repeated the same procedure with the other samples such that the images displayed equivalent labelling intensities. The exposure time was also recorded. The ratio of the two exposure times provided an estimation of the ratio of labelling intensity. This technique gave very similar results to those obtained with the NIH Image software. When possible, the results from both techniques were combined and are expressed ± SD.

The quantitation of EM pictures was performed according to Kondylis *et al.* (2001).

RESULTS

Expression of Golgi Proteins in Mid- and Late Third Larval Instar

In the wing and leg imaginal discs of *D. melanogaster*, Golgi stack biogenesis takes place towards the onset of puparium formation and is dependent on the steroid hormone ecdysone (Kondylis *et al.*, 2001).

We reasoned that Golgi stack formation is not observed earlier (i.e., during mid-TIL) because the expression level of proteins necessary for building the Golgi apparatus is below a functional threshold. It is plausible to expect that the expression level of these proteins must reach this threshold before cisternae could be built and Golgi stacks assembled. Given the known activity of ecdysone as a gene expression regulator, we made the hypothesis that it triggers the expression of genes involved in this assembly.

We first set out to investigate whether ecdysone triggered the increased expression of Golgi-associated proteins. Mid- and late TIL and white pupae discs were labelled with specific antibodies to proteins of interest. Mid- and late TIL were selected in such a way that the two experimental groups straddled the ~3-h prepupal ecdysone pulse. We used salivary gland morphology as a precise and convenient means of larval staging (see Materials and Methods; Figs. 1A and 1B). White pupae displayed characteristic morphology with a soft white pupal case.

It was critically important to ensure that the above larval selection criterion was suitable for larval staging. Thus, we established the percentage homogeneity of each experimental group (Table 1). An experimental group was defined as tissue of equal developmental age assayed for a given Golgi-related protein or mRNA. Homogeneity was achieved when ≥75% of the discs displayed the same level of labelling intensity, which was the case for 44 of 52 experimental groups.

We first tested the protein expression of *Drosophila* Golgi mannosidase II (dGMII) and dSec23 (Table 2) in the imaginal discs of the different larval stages. There was a significant increase in labelling intensity between the mid- and late TIL wing imaginal discs (Figs. 1C–1F) that was estimated at 4.5 ± 1.0 fold in both cases. This result correlates nicely with that from our Western blots for dSec23 (Kondylis *et al.*, 2001), though changes in labelling intensity were not always uniform within individual wing imaginal discs.

The protein expression of dp115 and dGM130 (Kondylis *et al.*, 2001) was elevated 2.5- and 2.8-fold, respectively (not shown), between mid- and late TIL. This quantitation is somewhat tenuous in that only 65% homogeneity could be achieved for each experimental group using either anti-sera. The protein expression of dβ'COP (a COPI subunit; Table 2) was examined by using a rabbit polyclonal antibody raised against the rat protein (80% homology) and was found to be downregulated 4- to 5-fold from mid- to late TIL (Figs. 1G and 1H).

All proteins that display an increase in protein expression level between mid- and late TIL were observed to be correctly localised to the Golgi apparatus (not shown) as observed previously (Kondylis *et al.*, 2001).

Expression of mRNA Encoding Golgi-Associated Proteins in Mid- and Late Third Larval Instar

We investigated whether the expression dynamics of Golgi-related proteins were mimicked by changes at the transcriptional level. Using RNA *in situ* hybridisation coupled with alkaline phosphatase and/or FISH, we first assessed the expression dynamics of *dsec23* (see Figs. 4A and 4B) in WT mid- and late TIL and white pupae. Using the quantitative methods described in Materials and Methods and given the homogeneity of each group (Table 1), we found that *dsec23* expression increased 4.0 ± 0.6-fold between mid- and late TIL (see Fig. 3).

Encouraged by this result, we raised RNA probes to a host of other genes encoding Golgi proteins ("Golgi genes," a total of 15; Table 2). These are *Drosophila* homologues of mammalian genes whose products have been implicated in Golgi function and Golgi biogenesis (Table 2). Three expression patterns became apparent (see Fig. 3).

TABLE 1
Estimation of the Homogeneity of Labelling

	Immunofluorescence		Alkaline phosphatase *in situ* hybridisation		
Genes/gene products	Mid-TIL discs	Late TIL discs	Mid-TIL discs	Late TIL discs	White pupae discs
dGMII	78.1 ± 1.1	75.8 ± 0.8			
dnsf1			80.0 ± 0.0	85.0 ± 1.0	91.5 ± 8.5
dSec23/*dsec23*	90.0 ± 5.0	84.0 ± 4.5	100 ± 0.0	100 ± 0.0	93.7 ± 6.2
dsed5			97.2 ± 2.7	100 ± 0.0	96.6 ± 3.3
drab1			93.5 ± 6.5	76.0 ± 1	100 ± 0
dsnap			90.0 ± 1.0	84.0 ± 1	84.5 ± 1.5
dsar1			94.0 ± 2.0	85.0 ± 4.0	100 ± 0.0
dgos28			78.0 ± 5.0	83.0 ± 2.0	100 ± 0.0
dGM130/*dgm130*	65.2 ± 4.1	67.8 ± 3.0	92	80	66
dp115/*dp115*	66.0 ± 8.5	64.0 ± 7.9	71.2 ± 21.2	100 ± 0.0	95.0 ± 5.0
ter94			78.5 ± 11.5	86.65 ± 13.5	62.5 ± 12.5
dp47			87.5 ± 12.5	100 ± 0.0	66.0 ± 20.0
dergic53			75	81.25	100
drab6			80.0 ± 5.0	95.0 ± 5.0	74.5 ± 8.5
dβ′COP/*dβ′cop*	75.0 ± 6.0	60.0 ± 10.0	85	79	100

Note. Results are expressed as the percentage of imaginal discs that are of the same labelling intensity within one group (mid- or late TIL or white pupae) labelled for one mRNA probe. "100%" represents a result where all discs exhibit the same labelling intensity. No indication is made here as the to absolute level of staining intensity. "80%" represents a result where 80% of the discs are of the same labelling intensity and 20% are either above or below this intensity. 75% is deemed the lower limit for homogeneity of any one group.

The first is that exhibited by *dgos28* (Figs. 2A and 2B), *dsec23* (see Figs. 4A and 4B), *dnsf1* (see Figs. 6A and 6B), *dsnap* (see Figs. 6E and 6F), *dsed5*, *drab1*, and *dsar1*. These mRNA probes indicate a very low expression level within mid-TIL imaginal wing discs, and a 3.5- to 4-fold higher level within those of late TIL (Fig. 3). The results obtained for *dsec23* correlate nicely with those observed at the protein level by immunofluorescence (Figs. 1E and 1F) and Western blot (Kondylis *et al.*, 2001). The strong expression increase was most apparent within the leading edge, distal portion, and intermediate folds of the wing discs, which are the areas that mostly contribute to the first phase of disc elongation (Cohen, 1993). The mRNA expression within white pupal wing discs was found to be equal to (*dgos28*, *dsec23*, *dnsf1*, *dsnap*, *dsed5*, and *drab1*) or more intense than (*dsar1*) expression in late TIL discs. We also tested the expression of *dnsf2*, encoding the second *Drosophila* homologue of mammalian NSF (Pallanck *et al.*, 1995) in imaginal discs. We could not detect any labelling in this tissue, whereas muscles of late embryos were very positively labelled (not shown).

The second expression pattern is characterised by *dgm130* (Figs. 2C and 2D), *dp115*, *ter94*, and *dp47*. This group of genes exhibited an mRNA expression increase between 1.5- and 2-fold (Fig. 3). The mRNA expression in white pupae wing discs for 3 out of 4 groups tested was mixed. Some discs exhibited as strong a labelling intensity as in late TIL while others were clearly not, and as a consequence, homogeneity within white pupae wing discs for these probes was reduced (Table 1).

The third expression pattern is that exhibited by *dergic53* (Figs. 2E and 2F), *dβ′cop*, and *drab6*. These mRNA are downregulated by ~4-fold between mid- and late TIL (Fig. 3). This expression pattern is in stark contrast to that of the first group described above. Interestingly, mRNA expression levels increased between late TIL and white pupae for all three genes tested.

The expression dynamics of this last group of mRNA indicate that transcriptional upregulation between mid- and late TIL is not a general feature of Golgi genes in the imaginal wing discs of *D. melanogaster*.

The possibility that these observations are a result of discrepancies in the *in situ* hybridisation protocol was addressed by combining samples of all three developmental time points, processing as one, and restaging them using salivary glands morphology prior to visualisation. Three mRNA were tested in this manner (*drab1*, *drab6*, and *dp115*), and in all cases, the expression dynamics were identical to previous results.

The possibility that the results observed here are a result of ubiquitous and thus nonspecific mRNA expression dynamics is negated by the observation that in all but one instance (*dnsf1*) the larval brain does not mimic the expression dynamics seen in imaginal wing discs (data not shown). This is also the case in larval salivary glands for all but two of the mRNA tested (*dgm130* and *dsec23*).

The Role of Ecdysone

The temporal dynamics of Golgi gene expression suggested to us a possible involvement of the ecdysone in the transcriptional regulation of these genes. To address this

TABLE 2
Reagents Used for the *in Situ* Hybridisation Experiments

Genes	EST[a]/cDNA	Vector	Antisense Dig-labelled probe	Gene products	References
dβ′cop (CG6699/P35606)	LD 07733	pBS	*Kpn*I/T7	dβ′COP: Putative subunit of the COPI coat	Gad fly/Fly base
dergic53 (CG6822/*rhea*)	GH 16748	pOT2	*Xho*I/T7	dERGIC53: Putative mannose binding protein cycling between the ER and the cis Golgi cisternae	Gad fly/Fly base; Prout *et al.* (1997)
dgm130 (CG11061)	LD 07754	pBS	*Eco*RI/T7	dGM130: Putative tethering protein	Gad fly/Fly base; Kondylis *et al.* (2001)
dgos28 (CG7700)	RE 64493	PFlc1	*Xho*I/T3	dGos28: Putative Golgi v-SNARE	Gad fly/Fly base
dnsf1 (CG1618/comt)	clone dN20	pBS	*Xba*I/T7	dNSF1: Fusion ATPase	Ordway *et al.* (1994); Kondylis *et al.* (2001)
dnsf2 (CG9931)	clone dN2.14	pBS	*Xba*I/T3	dNSF2: Fusion ATPase	Pallanck *et al.* (1995)
dp115 (CG1422)	clone 1.P2	PBK-CMV	*Sal*I/T7	dp115: Putative tethering protein	Kondylis *et al.* (2001)
dp47 (CG11139)	GH 01724	pOT2	*Xho*I/T7	dp47: Putative cofactor for TER94	Gad fly/Fly base
drab1 (CG3320)	LD 14676	pBS	*Sma*I/T7	dRab1: Small GTPase Rab1 involved in ER to Golgi transport	Gad fly/Fly base; Satoh *et al.* (1997)
drab6 (CG6601)	GH 09086	pOT2	*Xho*I/T7	dRab6: Putative small GTPase Rab6 involved in intra-Golgi transport	Gad fly/Fly base
dsar1 (CG7073)	GH 06356	pOT2	*Xho*I/T7	dSar1: Putative GTPase involved in the formation of the COPII complex	Gad fly/Fly base
dsec23 (CG1250)	GH 23373	pOT2	*Xho*I/T7	dSec23: Putative Sar1-specific GTPase activating protein part of COPII machinery	Gad fly/Fly base; Kondylis *et al.* (2001)
dsed5 (CG4214)	clone SD1	subcloned in pBS	*Eco*RI/T7	dSed5: Golgi t-SNARE	Banfield *et al.* (1994)
dsnap (CG7809)	LP 04493	pOT2	*Xho*I/T7	dSNAP: Cofactor of dNSF1	Gad fly/Fly base; Ordway *et al.* (1994)
ter94 (CG2331)	clone Ab14	pBK CMV	*Bam*HI/T7	TER94: Homologue of p97, a fusion ATPase	Pinter *et al.* (1998); Leon and McKearin (1999)

Note. The references for the inferred function are in Klumperman (2000); Rabouille and Warren (1997); Pfeffer (1999); Roche and Monsigny (2001); Dunne and Rabouille (2001). See also Discussion.
[a] All the EST were from Research Genetics.

possibility, we turned to a disc culture assay (Kondylis *et al.*, 2001) in which semidissected mid-TIL are incubated *in vitro* in the presence or absence of exogenously applied ecdysone. An important aspect of this culture assay was the requirement of the TIL imaginal discs to be responsive, yet not endogenously exposed, to ecdysone. We again used salivary gland morphology to select suitable larvae and selected mid-TIL larvae that exhibited moderate but not extreme salivary gland cell swelling (see Materials and Methods). These larvae were semidissected and incubated in Schneider medium between 9 and 16 h in the presence or absence of 2 μM 20-hydroxyecdysone.

Using RNA *in situ* hybridisation, we assessed the mRNA expression of *dsec23* as a representative of the first expression group as described above. *In vitro* incubation up to 9 h elicited no significant increase in *dsec23* mRNA expres-

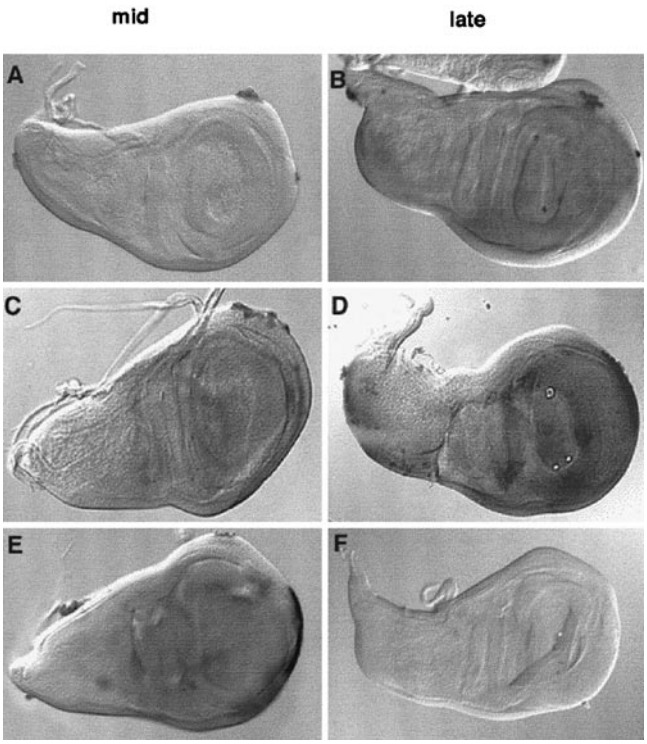

FIG. 2. mRNA expression in WT mid- and late third instar larvae. WT mid- and late TIL were selected according to salivary gland morphology, semidissected, and processed for RNA *in situ* hybridisation by using digoxigenin (DIG)-labelled mRNA probes for *dgos28* (A, B), *dgm130* (C, D), and *dergic53* (E, F). Wing imaginal discs were dissected, mounted, and viewed under a Zeiss Axioskop II microscope equipped with DIC using the 20× objective. Pictures were captured by using the Axiocam digital camera at equal settings for mid- and late TIL discs labelled for the same probe. Note the clear difference in staining intensity between mid- and late TIL for two of the three mRNA probes.

sion. A 12-h incubation led to a small but insignificant increase, and after 16 h, the increase in labeling intensity was equivalent to that observed *in vivo* (5.2-fold) (Figs. 4C and 4D). This mRNA expression increase was accompanied by a change in the disc morphology that was not observed *in vivo*. In view of the fact that the prepupal ecdysone pulse is ~2–3 h in duration, we mimicked this pulse by incubating the semidissected mid-TIL in the presence or absence of ecdysone for 3 h followed by 16 h of ecdysone-free incubation. This protocol elicited equivalent *dsec23* mRNA expression increase and wing disc morphological changes as continuous ecdysone exposure (data not shown).

The disc culture assay did not completely mimic the *in vivo* situation and we therefore investigated the involvement of Juvenile hormone (JH), the other hormone present *in vivo* in TIL (Riddiford, 1996). Using the disc culture assay in the presence of exogenous JH for 16 h, we verified that JH played no role in the increased expression of *dsec23* (not shown).

Broad Complex

The response duration of the ecdysone induced *dsec23* mRNA expression increase *in vitro* was suggestive of an intermediate factor that mediates the ecdysone signal. Ecdysone is known to bind to its nuclear heterodimer receptor (Koelle *et al.*, 1991) and induce the transcriptional activation of a set of genes known as "early genes"(Ashburner, 1990; Thummel, 1990), most them being transcription modulators themselves. *Broad Complex* (*Br-C*) is among these early genes (Kiss *et al.*, 1988), has a number of known tissue-specific isoforms (containing one to four Zinc finger domains; DiBello *et al.*, 1991), and has been shown to mediate ecdysone-regulated gene expression (Karim *et al.*, 1993; Fletcher and Thummel, 1995).

We first used a hypomorphic viable allele of *Br-C* called *br-1*. We semidissected *br-1* homozygote mid- and late TIL and processed them for RNA *in situ* hybridisation by using a *dsec23* mRNA probe. *dsec23* mRNA expression was considerably reduced when compared with WT larvae of equivalent developmental status. More importantly, no increase in *dsec23* mRNA expression was observed between *br-1* mid- and late TIL (Figs. 4E and 4F). This result is in stark contrast to that obtained using WT larvae (Figs. 4A and 4B), where an ~4-fold increase was observed. Similar results were obtained with *drab1* mRNA expression (not shown).

We next utilised the *npr* (*non puparіating*) mutant, that lacks all the isoforms of Br-C (Brennan *et al.*, 2001). *npr* is homozygous lethal. Thus, we generated mosaic imaginal discs using the FRT/Flp technology (Dang and Perrimon, 1992; see Materials and Methods). The majority of the disc cells are heterozygous mutant for npr (*npr*/ubi-GFP) and are thus marked by the expression of ubi-GFP. Within these discs were clonal clusters of cells (marked by DAPI staining; Fig. 5E) that were homozygous mutant for *npr* where the ubi-GFP is absent (Figs. 5A and 5C). Alkaline phosphatase RNA *in situ* hybridisation and FISH were performed on these discs by using an mRNA probe for *dsec23* (Figs. 5B and 5D). The mRNA labelling of *dsec23* was significantly reduced in the *npr* homozygous clones by using both visualisation techniques, indicating that the increased expression of *dsec23* observed in late TIL is mediated by *Broad complex*.

dNSF1 and dSNAP

The ~4-fold mRNA expression increase for *dnsf1*, *dsnap*, and others of the "first expression pattern" (Fig. 3) is in good temporal correlation with the formation of Golgi stacks *in vivo* (Kondylis *et al.*, 2001). The larval clusters that are present in mid-TIL begin enlarging during late third instar; cisternae become visible and Golgi stacks are only observed in white pupae discs.

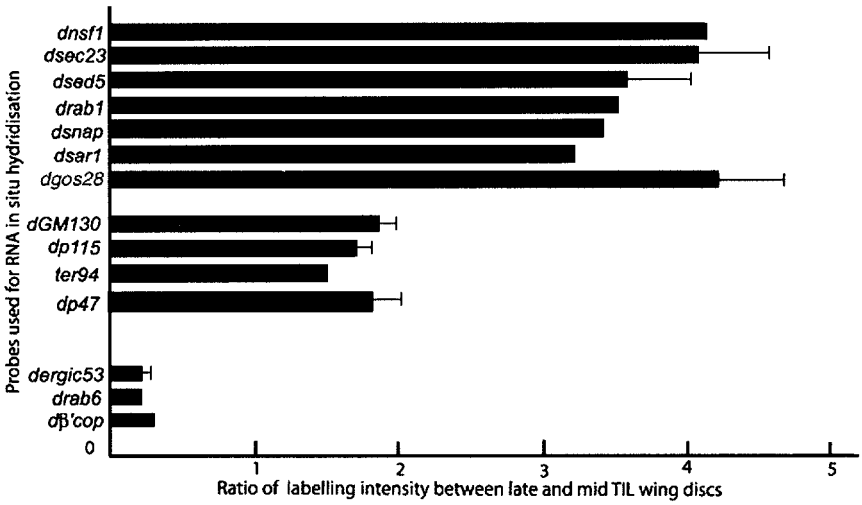

FIG. 3. mRNA expression dynamics in the wing imaginal discs of developing WT TIL. Wing imaginal disc labelling intensity was quantitated as described in Materials and Methods. Results are expressed ± SD. A ratio of 1 indicates no change in labelling intensity between mid- and late TIL. A ratio greater than 1 indicates an increased expression from mid- to late TIL. A ratio less than 1 indicates an expression decrease from mid- to late TIL.

Our mRNA expression dynamics observations support the possibility that the Golgi morphology observed at the onset of puparium formation is not observed earlier (i.e., during mid-TIL) because the expression level of proteins necessary for building the Golgi apparatus is below a functional threshold. It is plausible to expect that the expression level of these proteins must reach this threshold, via ecdysone-induced upregulation, before cisternae could be built and Golgi stacks assembled.

We tested this hypothesis by investigating whether the experimental upregulation of Golgi proteins could trigger Golgi stack biogenesis independently of ecdysone.

We first used a transgenic fly that carries a transgene comprising dNSF1 cDNA under the control of a heat shock promoter, HSN (gift from Leo Pallanck; Ordway *et al.*, 1994). We heat shocked WT and HSN mid-TIL and assessed the level of *dnsf1* mRNA expression using FISH. *dnsf1* expression was 7-fold increased in HSN over WT (compare Figs. 6C and 6D). *dnsf1* expression was 2-fold increased in heat shocked HSN vs HSN late TIL maintained at 21°C (compare Figs. 6B and 6D). The heat shock also led to a 1.3-fold increase in the *dnsf1* expression in WT mid-TIL (compare Figs. 6A and 6C).

As a control, we monitored the expression of *dsec23* mRNA. Heat shock treatment of WT and HSN mid-TIL elicited a 1.8-fold increase in *dsec23* expression compared with the same larvae maintained at 21°C (vs 1.3 for *dnsf1*), but there was no difference between WT and HSN. This contrasted markedly to the 7-fold increase observed for *dnsf1*.

A similar experiment was performed by using a transgenic fly stock carrying the cDNA of dSNAP under the control of UAS (gift from Leo Pallanck; Ordway *et al.*, 1994). This stock was crossed to hsGAL4 (see Materials and Methods). We heat shocked the WT and hsGAL4 UAS-SNAP mid-TIL and assessed the level of *dsnap* mRNA expression using FISH. *dsnap* expression was 5-fold increased in hsGAL4 UAS-SNAP vs WT (compare Figs. 6G and 6H). *dsnap* expression was 1.25-fold increased in heat shocked vs non heatshocked late TIL hsGAL4 UAS-SNAP (compare Figs. 6F and 6H). The heat shock also led to a 1.4-fold increase in the *dsnap* expression in WT mid-TIL (compare Figs. 6E and 6G).

We then observed the morphology of the Golgi area in the WT, HSN, UAS SNAP, and hsGAL4 UAS SNAP mid-TIL discs subjected to temperature treatment. Nontreated WT mid-TIL were used as control. The Golgi area in WT mid-TIL disc cells comprised clusters of vesicles and tubules with only about 10% of membrane in cisternae (Fig. 7A; Table 3), none of them stacked. After exposure to the temperature treatment, the percentage of Golgi membrane in cisternae increased to 30.3% (Fig. 7B; Table 3). A similar percentage was achieved in the noninduced UAS SNAP larvae (34.3%; Table 3). The percentage of stacked cisternae was 30 and 29%, respectively (Table 3).

When HSN mid-TIL were subjected to the temperature treatment, the Golgi area comprised 56.3% of membrane in cisternae, 70% of them stacked (Fig. 7C; Table 3). Similar results was obtained with the hsGAL4-induced UAS SNAP larvae (Fig. 7D). The percentage of membrane in cisternae reached 55.5%, 68% of them in stacks (Table 3). These latter figures are very similar to those obtained in the white pupae *in vivo* (Kondylis *et al.*, 2001). Furthermore, the volume density of Golgi membrane in the overexpressing discs after temperature treatment was increased by 2.7 ± 0.4 times when compared with the WT under the same conditions. This was not

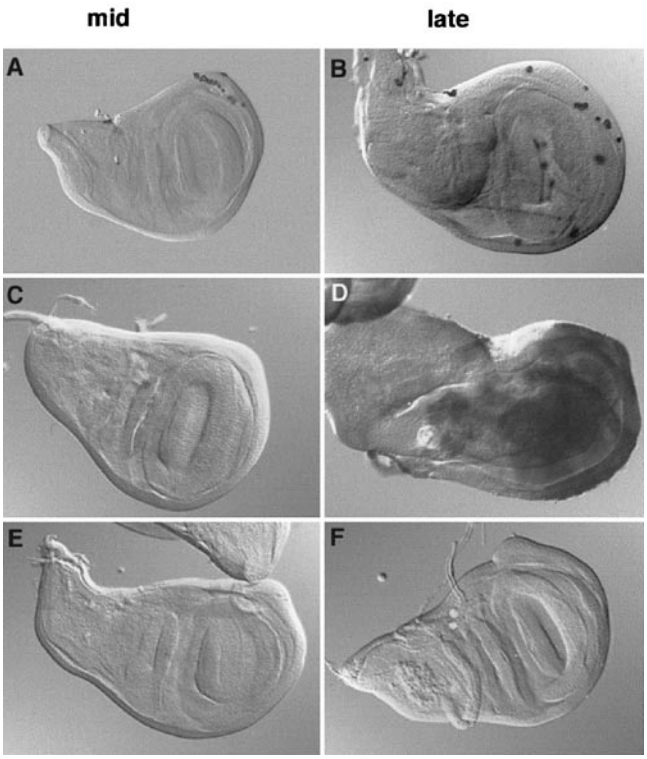

FIG. 4. The role of ecdysone and *Broad-Complex* in *dsec23* mRNA expression regulation. *In vivo dsec23* mRNA expression in WT mid (A)- and late TIL (B) was assayed as described in the legend for Fig. 2. WT mid-TIL were semidissected and incubated at 22°C for 16 h in a 30-mm culture dish containing 1.4 ml of Schneider medium supplemented with (C), or free of (D), 2 μM ecdysone. *In situ* hybridisation was performed on the incubated larvae by using a *dsec23* mRNA probe. Broad-Complex homozygous mutant *br1* mid (E)- and late TIL (F) were selected and processed for *in situ* hybridisation by using a *dsec23* mRNA probe. Note the significant *dsec23* mRNA expression increase in the presence vs absence of ecdysone. Also note the abolishment of *dsec23* mRNA increase between mid- and late TIL in the *br-1* homozygous mutant.

due to a change in the density of the Golgi membrane, but to an increase in the total membrane present.

This 2.7-fold increase, together with the doubling in the percentage of membrane in total and stacked cisternae, shows that Golgi biogenesis can be driven by increasing the expression of dNSF1 and dSNAP. This may also be the case for other Golgi genes of the "first expression pattern." Since the expression level of these is under the control of ecdysone, this suggests that ecdysone provides a developmental timing mechanism so that Golgi stack biogenesis takes place during, and not prior to, puparium formation.

DISCUSSION

Here, we show that transcriptional regulation of a number of *Drosophila* Golgi genes directly controls Golgi apparatus architecture. We believe this provides evidence of a novel mechanism of developmentally controlled organelle biogenesis, mediated by ecdysone and the transcription factor Broad-Complex.

Developmental Implications

We show that Golgi genes are subjected to developmental control by ecdysone, adding to the long list of known ecdysone targets (White *et al.*, 1999). Ecdysone activates the transcription of early puffs genes (see Introduction; and Riddiford, 1993), such as Broad complex (DiBello *et al.*, 1991; Andres *et al.*, 1993), E74 (Burtis *et al.*, 1990), and E75 (Segraves and Hogness, 1990), which in turn activate the transcription of more than 100 "late puff genes."

A recent microarray analysis (White *et al.*, 1999) examined the expression pattern of 4500 *Drosophila* genes in the third larval instar and pupae. The expression of approximately 12% of these was modulated by ecdysone and approximately 6% were upregulated (170 out of 4500). Ecdysone-responsive genes were grouped into clusters according to their pattern of regulation. Among these are genes that we also have identified, such as *dsec23* (cluster 28) and *ter94* (cluster 29). We found *dpdi* to have a constant expression within the time frame of our experiment, and that was confirmed by the microarray analysis (cluster 26). *dβ'cop* was found in cluster 27 of genes whose mRNA expression is reduced at the onset of pupation, supporting our results. Interestingly, their study also revealed that *clathrin* (cluster 0) is strongly upregulated, suggesting an increased endosomal activity. This raises the possibility that ecdysone also controls the biogenesis of the endocytic pathway in addition to Golgi stack biogenesis.

We have shown that ecdysone triggers its transcriptional activity through *Broad-complex*, itself a well-known ecdysone target (Karim *et al.*, 1993; Fletcher and Thummel, 1995). *Br-C* encodes a family of transcription factors differing in their zinc finger motif (Z1 to Z4) (DiBello *et al.*, 1991). We have utilised a BR-C null mutant *npr* lacking all four Br-C isoforms, to show that the *dsec23* mRNA expression increase observed between WT mid- and late TIL is dependent on the activity of at least one of the four Br-C isoforms. A switch from the Z1 to Z2 isoform has been implicated in the progression of the morphogenetic furrow in *Drosophila* eye imaginal discs (Brennan *et al.*, 2001). Further experiments will be needed to clarify the activity of one or more isoforms in the increased expression of Golgi genes.

We cannot exclude the possibility that Br-C does not directly regulate the expression of Golgi genes but rather induces the synthesis of another transcription factor(s), such as the *crooked leg* gene product (D'avino and Thummel, 1998). This issue will need to be investigated further.

There is one other example of developmentally associated Golgi remodelling, though the regulation of this event is not known. The expression of alpha-mannosidase II and TGN 38 (two Golgi resident proteins) has been shown to be

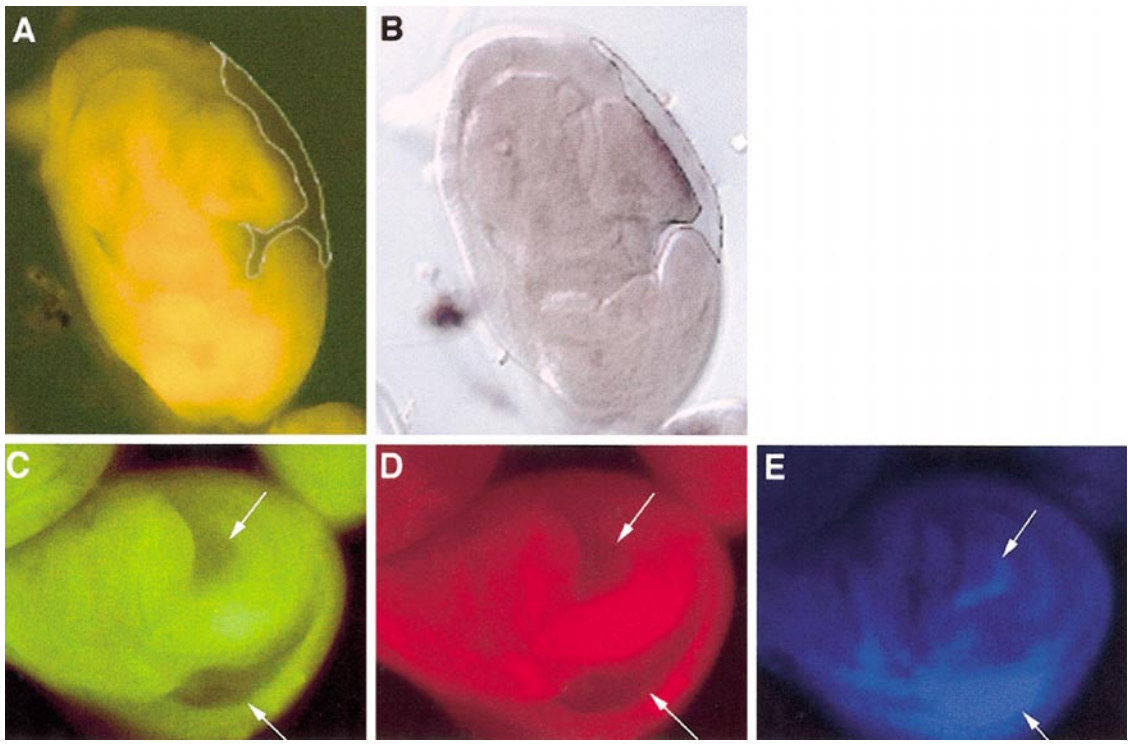

FIG. 5. Broad-Complex is the transcription factor mediating ecdysone-induced mRNA expression. Broad Complex (*npr*) homozygote mutant clones were induced as described in Materials and Methods. The *npr*/*npr* clones were marked by the absence of ubi-GFP marker (A, C) (white arrows, and doted line in A). *dsec23* mRNA expression was assessed by using alkaline phosphatase (B) and FISH (D). DAPI staining (E) was used to assess the viability of the homozygous mutant clonal tissue. Note that the clone borders are slightly different. This is due to differences in the visualisation techniques (epifluorescence picture, A) encompassing the full thickness of the disc and the clone, and DIC (B) creating an optical section. Note the absence of *dsec23* mRNA expression within the homozygote mutant clones.

regulated during vertebrate myogenesis. Both proteins were present in the embryonic myotubes but absent in the innervated muscle fibre (Antony *et al.*, 1995), during which time the Golgi apparatus is remodelled and relocated under the neuromuscular junction (Jasmin *et al.*, 1989). This and our results suggest that Golgi proteins could have a role in development.

There is mounting evidence that this is the case (reviewed in Dunne and Rabouille, 2001). In addition, genes encoding members of the p24 family (resident of the ER-Golgi intermediate compartment) have recently been shown to be genetic interactors of Dpp (Decapentaplegic), encoding a secreted protein that is crucial for proper *Drosophila* embryonic development (Bartoszewki *et al.*, 2001). The formation of the embryonic muscles and myoepidermal junction in *Drosophila* embryos has been shown to be significantly impaired in *rhea* mutants due to the strong genetic interactions between dERGIC53 (a mannose-binding protein resident of the *ER* to *G*olgi *I*ntermediate *C*ompartment) and PS integrins (Prout *et al.*, 1997). Further, the knockout of the mouse alpha-mannosidase II (a Golgi resident enzyme) results in dyserythropoietic anaemia (Chui *et al.*, 1997).

Taken together, our results strengthen the argument that Golgi gene expression is developmentally controlled and that Golgi stack formation at puparium formation could be implicated in development.

How Does Golgi Gene Expression Drive Golgi Stack Biogenesis?

At the onset of disc elongation, small larval Golgi clusters comprising vesicles and tubules grow in size to finally be converted to Golgi stacks in white pupae.

During this period, *dsar1*, *dsec23*, *drab1*, *dnsf1*, *dsnap*, *dsed5*, and *dgos28* genes and dSec23 protein were shown to be upregulated by ecdysone. To us, these results suggest a possible model involving the activation of three pathways leading to the formation of large Golgi stacks from small larval clusters: a COPII vesicle budding pathway (dSec23/dSar1) leading to increased ER-derived membrane (Barlowe *et al.*, 1994); a docking mechanism involving dRab1 (Allan *et al.*, 2000; Moyer *et al.*, 2001) leading to increased vesicle tethering prior to fusion; and a fusion pathway (dNSF1 and dSNAP, dSed5 and dGos28) mediating fusion of vesicles and tubules to form Golgi cisternae.

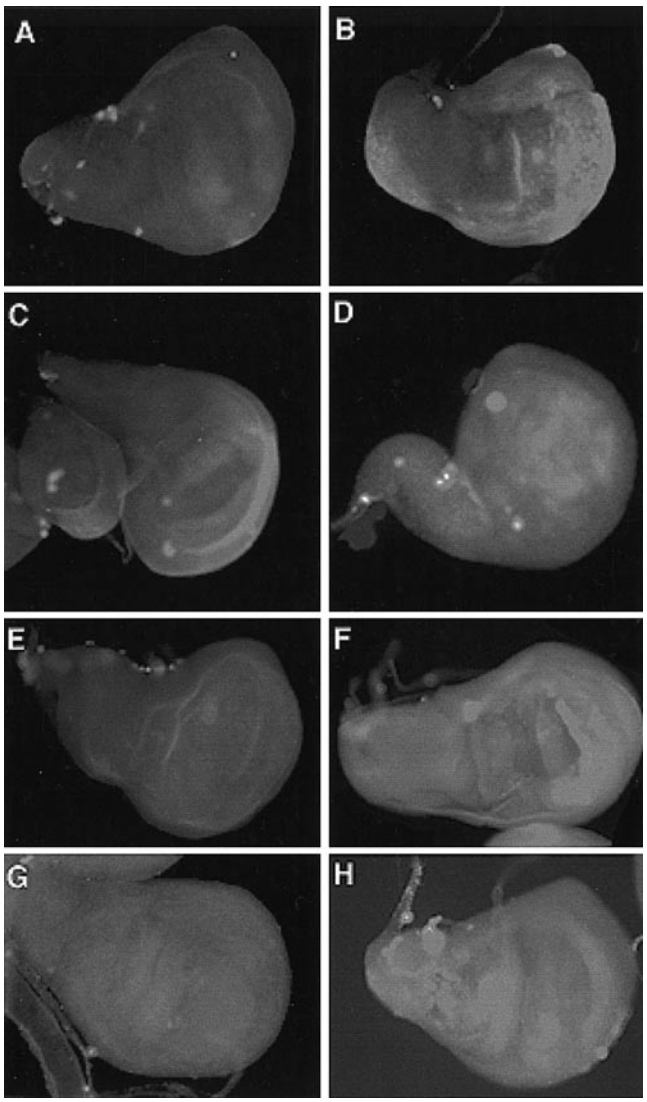

FIG. 6. Expression of *dnsf1* and *dsnap* in TIL and in transgenic flies. WT mid (A, E)- and late (B, F) TIL were semidissected and processed for FISH by using an mRNA probe for *dnsf1* (A, B) and *dsnap* (E, F). WT (C, G), HSN (D), and hsGAL4/+; UAS SNAP/+ (H) mid-TIL were heat shocked at 37°C for 40 min followed by 1 h at 33°C and processed for FISH by using a mRNA probe for *dnsf1* (C, D) and *dsnap* (G, H). Imaginal wing discs were viewed under an upright epifluorescence microscope AxioskopII by using the 20× objective. Note that the images in (B), (D), (F), and (H) were taken at 250-ms exposure time, whereas the photographs in (A), (C), (E), and (G) were taken at 800 ms.

We have shown previously that larval clusters grow in size between mid- and late TIL (their surface density increases ~3-fold; Kondylis *et al.*, 2001), and we have shown that ~60% of the larval clusters in the TIL are populated with COPII-derived vesicles. These findings could be explained by our present results. *dsec23* and *dsar1* are transcriptionally activated between mid- and late TIL (and dSec23 protein is being synthesised). This activation could be expected to activate the COPII budding pathway leading to more vesicles generated at ER exit sites. Interestingly, concomitant to the COPII budding mechanism being stimulated, observations of mRNA and protein expression dynamics of dβ'COP suggest that the COPI budding mechanism is downregulated from mid- to late TIL. Together, these results suggest that, during late TIL, the COPII budding mechanism dominates over the COPI. Con-

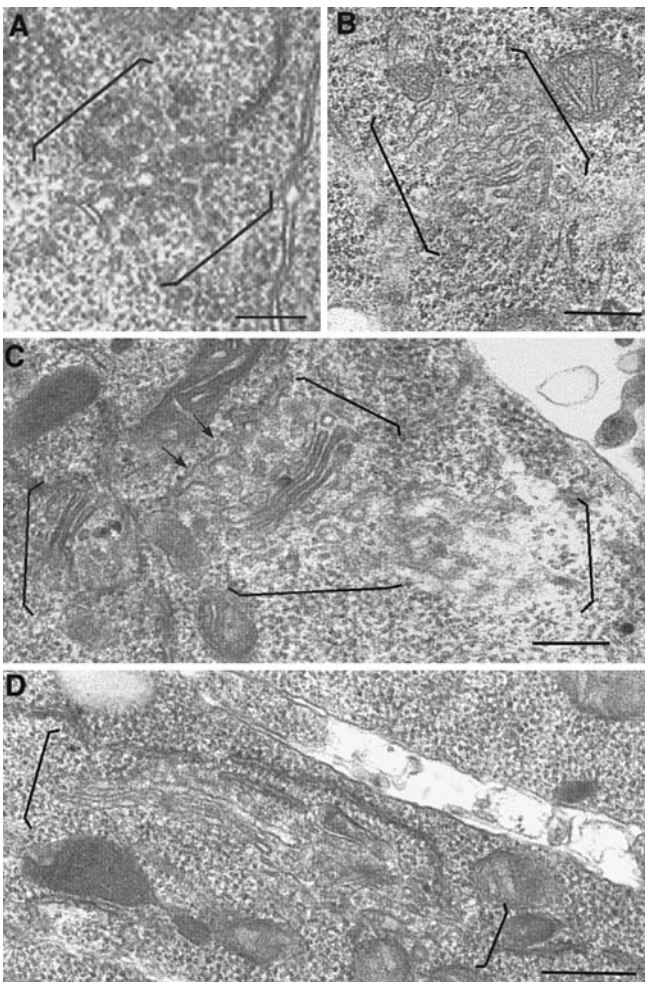

FIG. 7. Effect of dNSF1 and dSNAP overexpression on Golgi stack morphology. WT (A) mid-TIL were maintained at 21°C. WT (B), HSN (C), and hsGAL4/+; UAS-SNAP/+ (D) mid-TIL were heat shocked as described in the legend for Fig. 6. Wing and leg imaginal discs were immediately dissected, fixed, and processed for conventional electron microscopy as described in Kondylis *et al.* (2001). The Golgi area is marked between brackets. The arrow in (C) points to budding profiles from the surrounding ER cisternae. Note the considerable difference in the Golgi stack formation between the WT, HSN, and hsGAL4/+; UAS SNAP/+ cells after temperature treatment. Bars, 200 nm.

TABLE 3
Effect of dNSF1 and dSNAP Overexpression on the Formation of Golgi Stacks

	Percentage of total Golgi membranes in:			
	Total cisternae (%)	Tubules (%)	Vesicles (%)	Stacked/nonstacked
WT mid-TIL at 21°C for 1 h 40 min	10.0 ± 6.0	48.0 ± 17.5	38.0 ± 16.4	nd
WT mid-TIL after temperature treatment[a]	30.3 ± 8.2	42.1 ± 5.7	35.5 ± 4.9	0.30 ± 0.05
HSN mid-TIL after temperature treatment[a]	56.3 ± 10.1	23.4 ± 8.7	23.6 ± 7.7	0.70 ± 0.15
Noninduced UAS SNAP mid-TIL after temperature treatment[a]	34.3 ± 3.3	45.8 ± 5.6	19.8 ± 5.0	0.29 ± 0.06
hsGAL-induced UAS SNAP mid-TIL after temperature treatment[a]	55.5 ± 9.2	30.1 ± 5.3	14.4 ± 3.5	0.68 ± 0.13

Note. The percentage of membrane in each category has been estimated as described in Materials and Methods and Kondylis *et al.* (2001).
[a] The temperature treatment consists of exposing the larvae to 37°C for 40 min followed by 1 h at 33°C.

versely, the dβ'COP downregulation suggests that the COPI budding mechanism dominates during mid-third instar. This model requires that the Golgi larval clusters present in the mid-TIL would comprise predominantly COPI-derived vesicles and the enlargement of larval clusters observed in late TIL imaginal discs could be accounted for by COPII vesicles. Our immunofluorescence studies provide circumstantial evidence that this is the case, but further work needs to be undertaken to clarify the process.

Rab1 is part of the Ras superfamily of small GTPases and has been shown to have a role in vesicular transport between the ER and the cis face of the Golgi stack. Between mid- and late TIL, we found that *drab1* expression increases about 3.7-fold. This result represents the activation of a possible docking mechanism of the newly budded COPII vesicles. Recently, mammalian Rab1 has been shown to recruit p115 (Allan *et al.*, 2000) and GM130 (Moyer *et al.*, 2001), two proteins known to be involved in vesicle tethering and docking (Nakamura *et al.*, 1997; Sönnichsen *et al.*, 1998).

Although in *Drosophila* a similar interaction has yet to be characterised, the role for dRab1 could be as follows. The expression of *dp115* and *dGM130* was found to be elevated throughout the third larval instar. During mid-TIL, their gene products could serve a tethering mechanism (as it does in mammalian systems; Sönnichsen *et al.*, 1998; Pfeffer, 1999) for the COPI vesicles present in the larval clusters. During late third instar, dRab1 would switch dp115 and dGM130 function from tethering the COPI vesicles to docking and priming the newly formed COPII vesicles prior to their fusion to form Golgi cisternae.

The upregulation of *drab1* was specific. Indeed, *drab6* (involved in retrograde transport in mammalian systems; Martinez *et al.*, 1994) was downregulated ~4-fold.

The fusion machinery including the SNAREs dSed5 and dGos28, and the fusion ATPase dNSF1 and cofactor dSNAP is also upregulated ~4-fold at the mRNA level. This upregulation would provide the machinery necessary for COPII vesicle fusion and cisternae formation. This correlates well with our earlier results showing that dNSF1 is required for the conversion in Golgi morphology (Kondylis *et al.*, 2001). A similar activity has also been shown *in vitro* by using a Golgi stack reassembly assay from mitotic Golgi fragments (Rabouille *et al.*, 1995).

A second homologue of mammalian NSF was identified in *Drosophila* (dNSF2; Pallanck *et al.*, 1995) that is expressed from the end of embryogenesis to adulthood. Throughout our study, *dnsf2* was not detected in TIL imaginal discs, contrary to previous reports (Boulianne and Trimble, 1995).

We are therefore left with the possible role of TER94 and cofactor dp47 that we found are not substantially regulated but present at all stages of disc elongation. This second fusion machinery has also been shown *in vitro* to be involved in Golgi stack reassembly (Rabouille *et al.*, 1995; Acharya *et al.*, 1995). Nevertheless, because of the constant level of expression, we tend to favour the hypothesis according to which this complex is not involved in Golgi stack biogenesis at puparium formation. Our previous work indicates that Golgi clusters are not converted to Golgi stacks in the absence of dNSF1, using the *comt 17* mutant maintained at restrictive temperature (Kondylis *et al.*, 2001). Taken together, these results suggest to us that the dNSF1 fusion pathway is the major and possible sole contributor to the formation of Golgi stacks in disc cells. Only a hypomorphic ter94 mutant will solve the puzzle, and we are currently engaged in these experiments. p97, the mammalian homologue of TER94 has been shown to bind at least two other cofactors, ufd1 (required for ubiquitine degradation) and nlp4 (implicated in nuclear transport) (Meyer *et al.*, 2000). Its presence in imaginal disc cells might reflect the activity of these two other pathways.

Finally, we have shown that the overexpression of either dNSF1 or dSNAP leads to the precocious formation of Golgi

stacks from larval clusters that were comparable to pupal profiles (Kondylis *et al.*, 2001). That the overexpression of either proteins was sufficient to stimulate Golgi stack biogenesis encouraged us to investigate whether *dsec23* expression was influenced by the overexpression of dNSF1 or dSNAP. *dsec23* expression was 1.8-fold increased, but resulted from the heat shock and not from the overexpression of either protein. This result argues against a direct cross talk between dNSF1 and dSNAP (protein or mRNA), and *dsec23*. Other genes will be investigated.

The direct involvement of dNSF1 and dSNAP in the formation of active COP II vesicles could account for this biogenesis, especially the 2.7-fold increase in volume density. This has been shown before in mammalian systems for the COPI vesicles (Wattenberg *et al.*, 1992) and for clathrin-coated vesicles in the endocytic pathway (Steel *et al.*, 1996). The data presented here support the hypothesis that transcriptional regulation of a variety of Golgi genes is a significant factor contributing to Golgi stack biogenesis. The direct transcriptional activation of the budding, docking, and fusion pathways are likely to lead to the construction of Golgi stacks.

Until present, experimental evidence has indicated that modulation of organelle architecture is mostly influenced and/or regulated by modifications at the translational and posttranslational level (see Introduction). Here, we show for the first time that changes in the expression of genes known to participate in the building of the Golgi apparatus exert a direct effect on Golgi biogenesis. Furthermore, for the first time, we demonstrate that this gene expression regulation is developmentally controlled by the steroid hormone ecdysone. This novel mechanism is likely to be common to other organisms.

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