The CrebA/Creb3-like transcription factors are major and direct regulators of secretory capacity

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Secretion occurs in all cells, with relatively low levels in most cells and extremely high levels in specialized secretory cells, such as those of the pancreas, salivary, and mammary glands. How secretory capacity is selectively up-regulated in specialized secretory cells is unknown. Here, we find that the CrebA/Creb3-like family of bZip transcription factors functions to up-regulate expression of both the general protein machinery required in all cells for secretion and of cell type–specific secreted proteins. *Drosophila* CrebA directly binds the enhancers of secretory pathway genes and is both necessary and sufficient to activate expression of every secretory pathway component gene examined thus far. Microarray profiling reveals that CrebA also up-regulates expression of genes encoding cell type–specific secreted components. Finally, we found that the human CrebA orthologues, Creb3L1 and Creb3L2, have the ability to up-regulate the secretory pathway in nonsecretory cell types.

Introduction

The human pancreas secretes liters of enzymes daily to aid in food digestion, and bovine mammary glands produce eight liters of milk each day, largely for human consumption. To do this, secretory organs must adapt to the increased need for protein secretion that occurs during development, differentiation, or changing physiological conditions. An important question is how changes in secretory capacity are coordinated to allow for efficient targeting, folding, modification, and delivery of secreted products. A few transcription factors have been discovered to up-regulate genes in the secretory pathway, including Xbp1, which is expressed and required in B cells as they differentiate into antibody secreting plasma cells (Shaffer et al., 2002), and which also regulates secretory function in a subset of specialized secretory organs (Shaffer et al., 2004; Lee et al., 2005). The bZip transcription factor ATF6 activates expression of chaperone proteins required for efficient protein folding (Adachi et al., 2008) as well as many of the lipid components of secretory organelles (Bommiasamy et al., 2009). Two other bZip transcription factors, Creb3L1/OASIS and Creb3L2/BBF2H7 (herein referred to as Creb3L1 and Creb3L2), are required for efficient bone deposition and cartilage matrix secretion, respectively (Murakami et al., 2009; Saito et al., 2009). A major question is whether these transcription factors function more broadly to up-regulate the entire secretory pathway in multiple specialized cell types or if their function is restricted to the up-regulation of only a subset of secretory genes in a few specialized cells.

The *Drosophila* salivary gland (SG) provides an excellent model for identifying and studying the factors required for secretory function. The SG is the largest secretory organ in *Drosophila*, and the processes of morphogenesis and differentiation have been well characterized (Kerman et al., 2006). The SG comprises two large secretory tubes, each containing ∼100 polarized epithelial cells that are specialized for the production and delivery of secreted proteins. Consistent with the high-level secretory activity of the SG, at least 34 secretory pathway component genes (SPCGs) are highly expressed in the secretory cells (Abrams and Andrew, 2005), and this expression requires at least two transcription factor genes, *fork head* (*fkh*) and *CrebA* (Andrew et al., 1997; Myat et al., 2000).

SG expression of *fkh* and *CrebA* is activated in the most posterior head segment (parasegment two) by the homeotic gene *Sex combs reduced* (*Scr*) and two more generally expressed homeotic cofactor genes *extradenticle* (*exd*) and *homothorax* (*hth*; Henderson and Andrew, 2000). Dpp signaling in dorsal cells blocks expression of *fkh* and *CrebA*, limiting their activation to only the ventral cells of parasegment two (Henderson et al., 1999).

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Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; *fkh*, *fork head*; GO; gene ontology; SG; salivary gland; SPCG, secretory pathway component gene; TEM, transmission electron microscopy.

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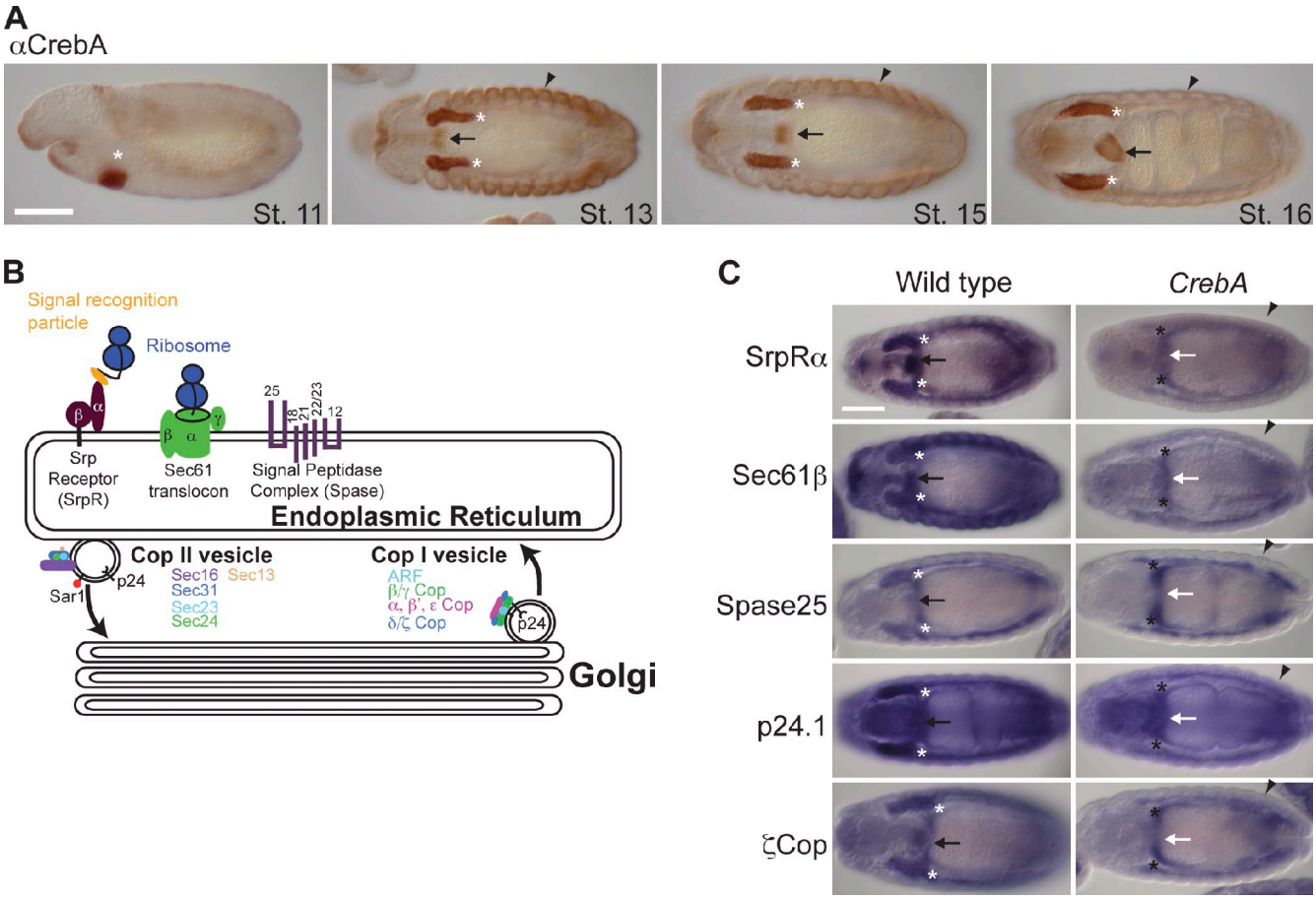


Figure 1. **CrebA is expressed in secretory tissues and regulates SPCG expression.** (A) Endogenous CrebA protein is detected in the SGs (asterisks), epidermis (arrowheads), and proventriculus (arrows) beginning at stage 11 (lateral view) and continuing through stage 16 of embryonic development (images of stage 13, 15, and 16 are ventral views). Elevated expression is also detected in the trachea, but is not visible in these images. (B) Cartoon representation of the protein complexes represented by the five enhancers characterized in this study. (C) In situ hybridizations show loss of SG (black asterisks), proventriculus (arrows), and epidermal expression (arrowheads) in the *CrebA* mutant. Bars, 125 µm.

Shortly after activation of *fkh* and *CrebA*, expression of *Scr*, *exd*, and *hth* disappears in the SG (Henderson and Andrew, 2000); continued expression of both *fkh* and *CrebA* is maintained by Fkh (Abrams and Andrew, 2005). Thus, we propose that Fkh plays a primarily indirect role in SPCG expression through its role in maintaining expression of *CrebA* (Abrams and Andrew, 2005). Consistent with this idea, the loss of *fkh* affects only late SPCG expression, whereas loss of *CrebA* affects both early and late SPCG expression. It is unknown, however, if CrebA directly regulates SPCG expression or if additional downstream factors are also involved.

Here, we show that CrebA is both necessary and sufficient for high level SPCG expression in the secretory tissues of the *Drosophila* embryo. We show that direct binding of CrebA to a consensus motif identified upstream of the 34 originally characterized SPCGs is required for elevated SPCG expression in the secretory tissues. Through microarray analysis, we find that over half of the 383 genes that require CrebA encode identifiable secretory pathway components. Surprisingly, CrebA targets include not only components of the general secretory machinery that function in all cells but also cell type–specific secreted cargo. Moreover, phenotypes associated with loss of *CrebA* are consistent with the role of this gene in secretion. Finally, we confirmed Creb3L1 and Creb3L2 as the closest mammalian orthologues to *Drosophila* CrebA and demonstrated that both human proteins have the same activities as their *Drosophila* counterpart.

Results

CrebA binds directly to SPCG enhancers in vitro and in vivo

Drosophila CrebA expression is elevated in many secretory organs in the embryo, with highest expression in the developing SG, proventriculus, late trachea, and epidermis (Fig. 1 A; Andrew et al., 1997). In these tissues, CrebA is required for the high level expression of 34 known SPCGs (Abrams and Andrew, 2005). A MEME analysis (http://meme.sdsc.edu/meme/) of the enhancer regions upstream of these genes revealed a conserved motif similar to the previously characterized CREB response element (Montminy and Bilezikjian, 1987) and unfolded protein response elements (Wang et al., 2000) that bind the mammalian CREB proteins (Fig. 2 A; Abrams and Andrew, 2005). To ask if the more distantly related CrebA protein binds these sites in vitro, we performed electrophoretic mobility shift assays (EMSAs) with ~30-nucleotide double-stranded oligomers corresponding

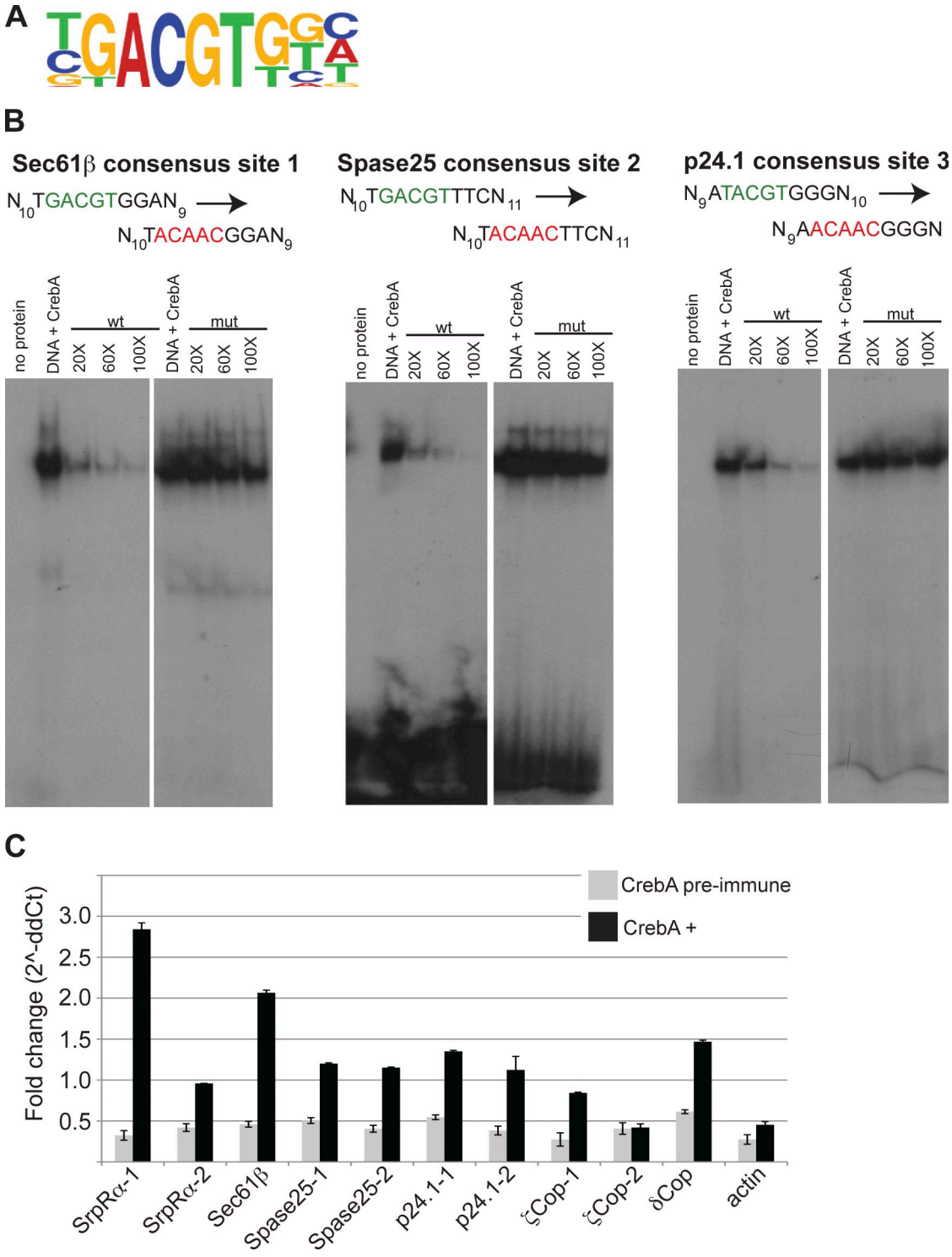
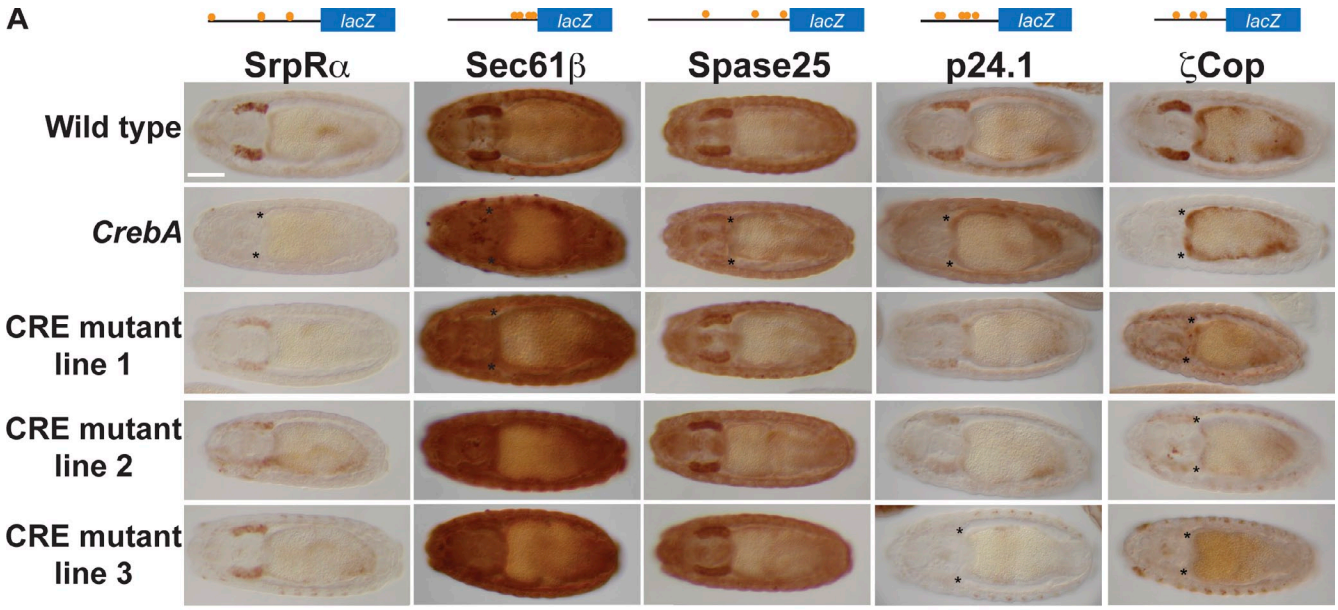


Figure 2. **CrebA directly activates SPCG expression.** (A) The CrebA consensus motif identified upstream of 34 SPCGs by MEME analysis (Abrams and Andrew, 2005). (B) EMSAs reveal that CrebA binds to radio-labeled double-stranded oligonucleotides containing the CrebA consensus motif (green sequence). Unlabeled wild type (wt) double-stranded oligonucleotides compete for CrebA binding, whereas unlabeled double-stranded oligonucleotides containing mutated consensus motifs (red sequence, mut) do not. (C) ChIP analysis using CrebA-specific antibodies, followed by quantitative PCR, revealed that SPCG enhancer regions exhibit increased CrebA occupancy when compared with control genes. This figure shows the mean from one representative trial (out of three). Of note, Spase25-2 showed lower occupancy in one trial, whereas ζCop-2 showed higher occupancy in the other two trials. ddCt indicates the relative abundance of a gene (or DNA fragment) normalized to a housekeeping gene (*actin5c*) and normalized to an IgG control. Error bars represent standard error of the mean.



B

Enhancer	Number of lines	Reduced	Elevated	No Change
SrpRα	7	6	1	0
Sec61β	11	11	0	0
Spase25	10	2	0	8
p24.1	8	5	1	2
ζCop	12	10	0	2

Figure 3. **SPCG expression in vivo requires the CrebA consensus motif.** (A) The structures of the lacZ reporter gene constructs are represented above each gene name, with putative CrebA binding sites represented by orange circles. LacZ reporter lines show high-level SG expression in wild-type embryos (top row), which is significantly reduced in *CrebA* mutants (second row from the top). Mutation of the CrebA response element (CRE) results in significant reduction of β-gal expression for *SrpRα*, *Sec61β*, *p24.1*, and *ζCop* but not for *Spase25* (bottom) in otherwise wild-type embryos. Three individual transgenic lines are shown for each reporter. Asterisks denote SG location in the mutant lines. Bars, 125 µm. (B) Table showing the number of independent transgenic lines analyzed for each mutated enhancer and the relative level of lacZ expression compared with the wild-type reporter lines.

to 18 putative CrebA binding sites found within the CrebA-dependent enhancers of five representative SPCGs: *SrpRα*, *Sec61β*, *Spase25*, *p24.1*, and *ζCop* (Fig. 1 C). Each gene encodes a protein found in a distinct complex functioning at a different step in early secretion (Fig. 1 B). Using purified CrebA protein, we observed strong binding in all cases, as revealed by the decreased mobility of CrebA–DNA complexes relative to unbound DNA (Fig. 2 B and Fig. S1). The binding is specific: although unlabeled competitor oligomers corresponding to the same sequence as the labeled probe competed for CrebA binding in 17 of the 18 sites tested, unlabeled competitor oligos in which the five core nucleotides of the consensus motif were changed did not compete (Fig. 2 B and Fig. S1). We then performed chromatin immunoprecipitation (ChIP) followed by quantitative RT-PCR to determine if CrebA binds the enhancer regions upstream of these SPCGs in vivo. Chromatin was extracted from 0–24-h embryos and immunoprecipitated with either CrebA antiserum or CrebA preimmune serum as a negative control. All tested SPCG enhancers were preferentially pulled down with the CrebA antibody (Fig. 2 C). These findings indicate that CrebA binds to the conserved CrebA consensus site both in vitro and in vivo.

CrebA directly activates SPCG expression

To ask if the CrebA binding sites are required for CrebA-dependent expression of SPCGs in vivo, we generated multiple independent transgenic lines in which all of the CrebA consensus motifs within the SPCG enhancers were mutated. Transgenic lines carrying wild-type SPCG enhancers driving *lacZ* had robust β-gal expression in the SGs of wild-type embryos, which was significantly reduced in *CrebA* mutants (Abrams and Andrew, 2005). When stained in parallel with the wild-type constructs, most lines carrying mutated CrebA binding sites for *SrpRα*, *p24.1*, *Sec61β*, and *ζCop* had significantly reduced levels of SG β-gal expression when visualized in otherwise wild-type embryos (Fig. 3). Surprisingly, most of the mutant *Spase25* enhancer lines (8/10) had levels of β-gal expression similar to the wild-type *Spase25* enhancer. The residual SG β-gal expression observed with the mutated enhancers of *SrpRα*, *p24.1*, *Sec61β*, and *ζCop* and the strong β-gal

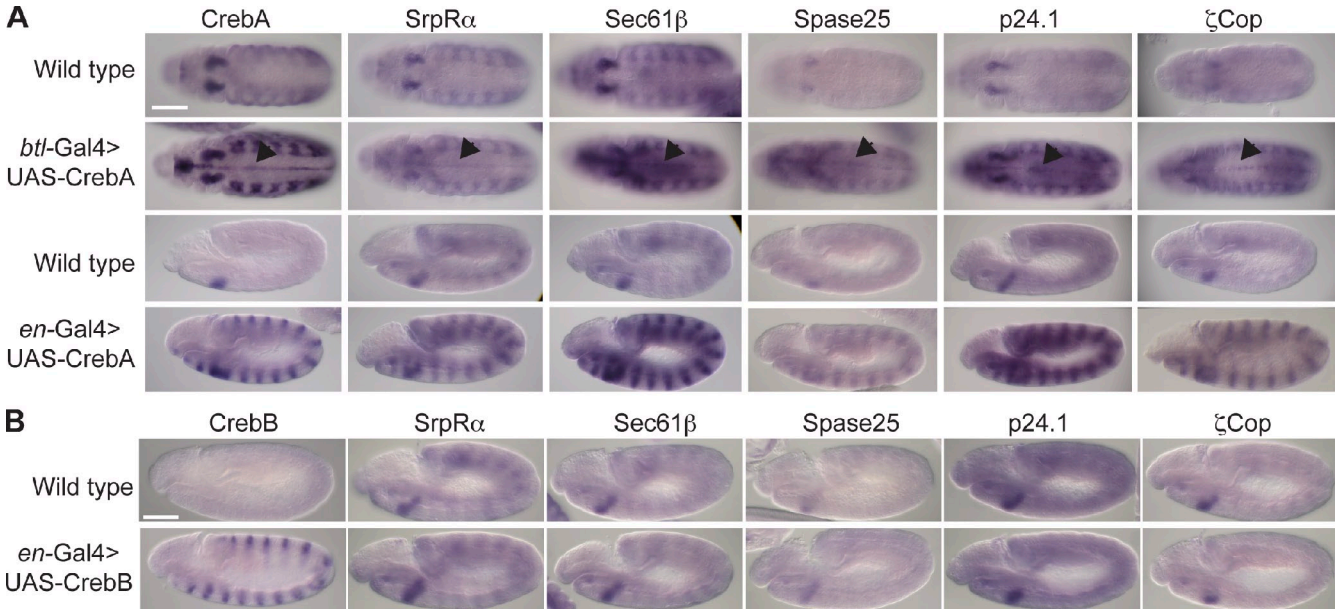


Figure 4. **CrebA is sufficient to induce SPCG expression.** (A) UAS-CrebA expression in ectopic domains using either the *btl*-Gal4 or the *en*-Gal4 driver results in activation of all tested SPCGs. *btl*-Gal4 drives UAS-*CrebA* expression and, consequently, SPCG expression in the trachea and central nervous system midline (arrowheads) of late stage 11 embryos (ventral views, first and second rows). *en*-Gal4 drives UAS-CrebA expression and, consequently, SPCG expression in epidermal stripes of early stage 11 embryos (lateral views, third and fourth rows). (B) Ectopic expression of UAS-CrebB by the *en*-Gal4 driver does not result in SPCG expression in the *engrailed* domain. Bars, 125 µm.

expression from the mutated enhancer of *Spase25* suggest that although CrebA directly activates expression of most SPCGs through the sites that bind CrebA in vitro, there may also be some activation by CrebA-dependent downstream transcription factors, or CrebA may also directly activate SPCG expression through divergent binding sites still contained within the enhancer regions of the mutated SPCGs. Taken together, these results indicate that CrebA is required for SPCG expression, and that full expression of most SPCGs is dependent on the CrebA consensus binding motif identified through the MEME analysis.

CrebA is sufficient to induce SPCG expression

We next asked if CrebA can induce high-level SPCG transcription in cells that normally only express low or undetectable levels of these genes. UAS-CrebA (Rose et al., 1997) was expressed using either the *breathless*-Gal4 (trachea, midline, and salivary duct) or *engrailed*-Gal-4 (epidermal stripes) drivers (Shiga et al., 1996; Weiss et al., 2001). In wild-type embryos, *CrebA* expression is not detected in the midline cells, the salivary duct, or in the engrailed domains, and only moderate levels are detected in the trachea (Fig. 4 A). Ectopic expression of CrebA resulted in elevated levels of expression of all five of the SPCGs in all locations (Fig. 4 A). Neither loss nor ectopic expression of *CrebB*, the most closely related *Drosophila* gene, had any effect on SPCG expression (Fig. 4 B). Thus, expression of CrebA alone is sufficient to up-regulate SPCG expression in multiple distinct embryonic cell types, which suggests a major role for this transcription factor in up-regulating secretory capacity.

CrebA regulates additional secretory pathway genes as well as secreted cargo

To ask if CrebA is a general regulator of SPCGs, we performed microarray analyses comparing RNA from wild-type and *CrebA* mutant embryos (Fig. 5 and Table S3). Not only did we observe a significant reduction in the expression levels of the majority of the SPCGs previously analyzed in *CrebA* mutant embryos (changes of 1.5-fold or greater) but we discovered that many additional genes encoding proteins known or suspected to function in secretion were similarly affected. Gene ontology (GO) clustering was performed using DAVID, a program that weighs the enrichment of a specific GO term in a given dataset relative to the frequency of that term in the entire genome (Dennis et al., 2003; Huang et al., 2009). This analysis revealed that in the absence of CrebA, the GO terms associated with secretory pathway function (i.e., cotranslational protein targeting to the membrane, secretory pathway, and protein transport) are the most highly enriched in the dataset, with enrichment of at least threefold compared with the entire *Drosophila* genome (Fig. 5 B and Table S1). Unexpectedly, many genes predicted to encode secreted cargo also showed significantly reduced expression in *CrebA* mutants, including larval cuticle proteins, mucins, and several secreted enzymes. Indeed, more than half of the ∼40 genes most affected by loss of *CrebA* encode known or predicted secreted proteins, including mucins and constituents of the larval cuticle. These data indicate that CrebA not only up-regulates genes encoding the general secretory machinery found in all cells but also activates genes encoding cell type–specific secreted proteins. DAVID analysis of the genes up-regulated in *CrebA* mutants did not reveal enrichment for any specific pathway or biological function, which suggests that CrebA functions primarily as a transcriptional activator of genes in the secretory pathway.

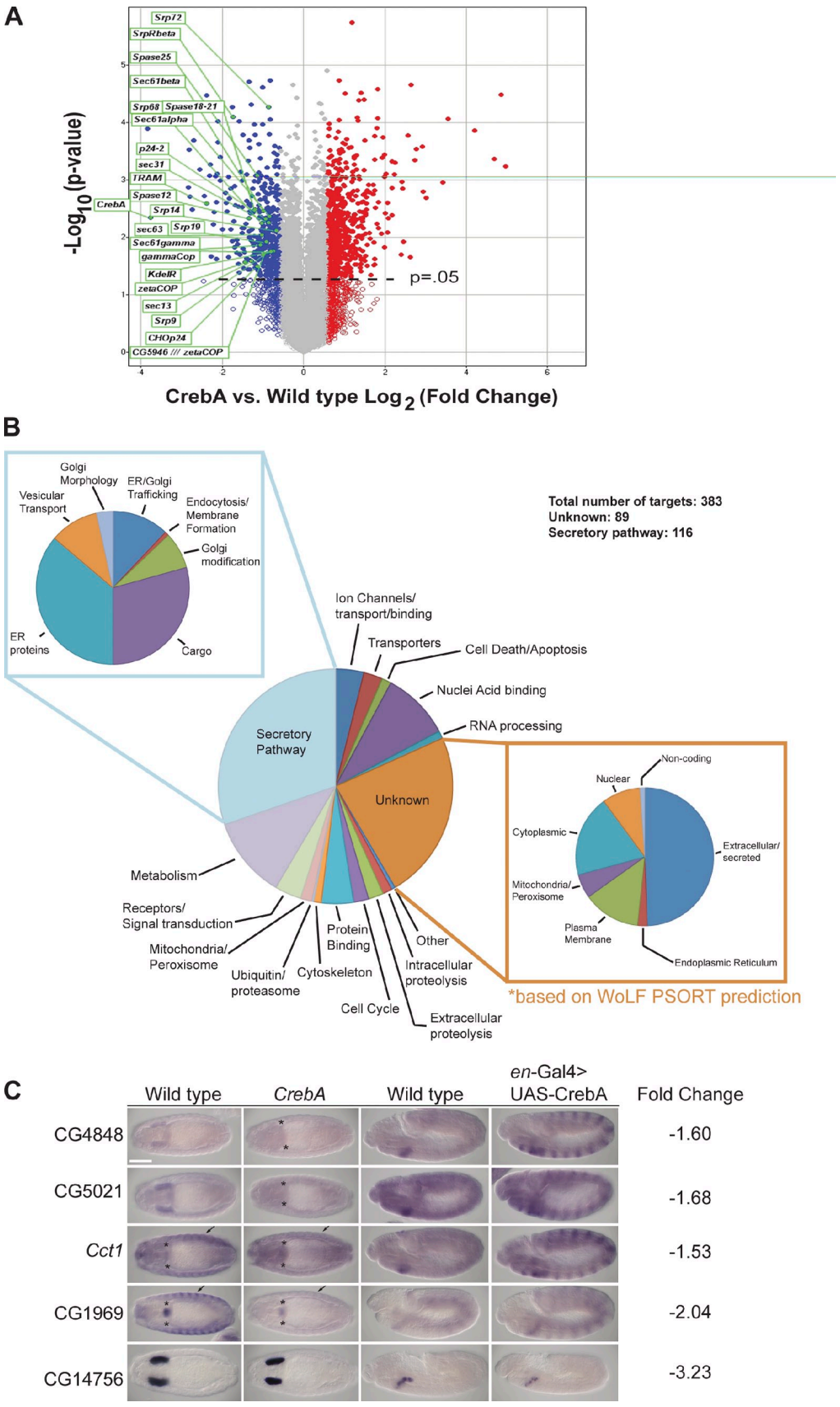


Figure 5. **CrebA activates additional secretory pathway genes as well as secreted cargo.** (A) Volcano plot showing changes in expression level and statistical significance of CrebA target genes. Transcripts elevated 1.5× or more in *CrebA* mutants are labeled red. Transcripts reduced 1.5× or more are labeled blue or green. Closed circles indicate significance with a P-value ≤ 0.05. Open circles indicate nonstatistically significant changes. (B) Pie charts showing

In addition to known secretory pathway genes, we identified 89 CrebA target genes encoding proteins of unknown function, i.e., with no associated GO terms. A WoLF PSORT analysis (http://wolfpsort.org/) of these proteins revealed that almost half (44) contain a signal sequence and are predicted to be secreted, with an additional 14 containing transmembrane domains and predicted to localize to the ER or plasma membrane (Fig. 5 B). Thus, it is likely that many of these uncharacterized genes encode either novel secretory pathway components or secreted gene products.

Using in situ hybridization, we confirmed that several genes identified as potential CrebA targets in the microarray analysis require CrebA for expression in the SG, proventriculus, and/or epidermis (Fig. 5 C). Interestingly, many of them have predicted roles in the secretory pathway. Although *CG4848* does not contain GO annotation, it encodes a Vps51/Vps67 domain protein closely related to mammalian Cog1, a member of the conserved oligomeric Golgi complex required for protein glycosylation. Cct1 encodes choline phosphate cytidyltransferase, an enzyme that functions in the lipid biogenesis pathway. *CG1969* encodes a glucosamine 6-phosphate *N*-acetyltransferase, a cytoplasmic enzyme involved in the metabolism of glutamate. *CG5021* and *CG14576* encode unknown proteins, but CG5021 has significant hydrophobic stretches that suggest multiple membrane spans, and CG14576 has an N-terminal signal sequence indicating that it likely travels through the secretory pathway and is secreted. Similar to the previously tested SPCGs, expression of CrebA using the *en*-Gal4 driver was sufficient to induce ectopic expression of a majority of these genes; the exception is *CG14576*, which we know requires additional cell type–specific transcription factors for its expression (see Discussion). Thus, the microarray screen has revealed many new CrebA target genes known or likely to encode components of the secretory pathway as well as the specific protein products that are processed and delivered via this pathway.

Loss of CrebA leads to defects consistent with secretory dysfunction

Despite the critical role of CrebA in up-regulating secretory function, CrebA mutants do not display major morphological SG defects; the SGs form normally and are only mildly crooked at late stages (Andrew et al., 1997). Transmission EM (TEM) of *CrebA* mutant SGs revealed three overt changes: (1) the lumen size was significantly smaller than that of wild-type glands and was accompanied by reduced amounts of electron-dense luminal material (Fig. 6 A); (2) the mitochondria were concentrated to a region apical to the nucleus in contrast to wild-type SGs, where mitochondria were distributed throughout the cell (Fig. 6 B); and (3) the secretory vesicles were much fewer and smaller than those of wild-type SGs (Fig. 6, C–E). The TEM analysis also suggested reduced levels of ER, a change that could not be quantified from the TEMs. Although most of the SG defects revealed by TEM analysis are fully consistent with a role for CrebA as a general activator of secretory function, the change in mitochondrial localization was unexpected; whether or not this change is linked to CrebA secretory function remains to be determined.

To ask if CrebA is provided maternally and functions earlier in embryogenesis, we generated homozygous *CrebA* mutant germ-line clones using the FLP-DFS technique (Chou and Perrimon, 1996). Consistent with the absence of detectable germ-line expression (Smolik et al., 1992; Rose et al., 1997), maternal loss of *CrebA* did not exacerbate the *CrebA* zygotic loss-of-function phenotypes. Indeed, cuticle preparations of $CrebA^{mat\text{-}zyg\text{-}}$ animals revealed the same phenotypes as observed with zygotic mutants (Fig. 7, A–D). Also, staining with the apical marker Crumbs revealed that, as observed with only zygotic loss of *CrebA*, maternal-zygotic loss of *CrebA* did not result in overt defects in early embryos (Fig. 7, E–H). Thus, CrebA is not required for the basal levels of secretion that occur in most cell types and instead functions to selectively up-regulate secretory capacity in specialized secretory cells, both through its effects on genes encoding the general machinery and on genes encoding secreted cargo.

CrebA is related to the mammalian proteins Creb3L1 and Creb3L2

BLAST analysis of the *Drosophila* CrebA protein against the human genome revealed its two closest orthologues to be Creb3L1 (Honma et al., 1999; Nikaido et al., 2001) and Creb3L2 (Fig. 8 A; Kondo et al., 2007; Saito et al., 2009), which is consistent with earlier findings that Creb3L2, originally named BBF2H7, is highly related to *Drosophila* CrebA, originally named BBF-2 (Abel et al., 1992; Smolik et al., 1992; Storlazzi et al., 2003; Kondo et al., 2007). Creb3L1 and Creb3L2 share ∼25% overall similarity with CrebA, with 97% similarity (84% identity) and 79% similarity (71% identity) within the DNA binding domains of CrebA with Creb3L1 and Creb3L2, respectively (Fig. 8 B). Both human proteins also contain C-terminal transmembrane domains and are bound to the ER membrane (Omori et al., 2002; Kondo et al., 2007). During ER stress, Creb3L1 and Creb3L2 undergo regulated intramembrane proteolysis (RIP), wherein their transcription factor domains are released into the cytoplasm and subsequently translocated to the nucleus to activate transcription (Kondo et al., 2005, 2007; Saito et al., 2007). The roles of both Creb3L1 and Creb3L2 as sensors and mediators in the unfolded protein response have been described, and both are expressed in many secretory organs (Omori et al., 2002; Kondo et al., 2005, 2007; Saito et al., 2007, 2009; Murakami et al., 2009).

Creb3L1 and Creb3L2 activate SPCG expression in *Drosophila* and human cells

To determine if human Creb3L1 and Creb3L2 have the same activity as CrebA, we generated UAS lines that allow for Gal4 driven expression of both the full-length (FL) and truncated (T) forms

predicted functions of all 383 genes down-regulated in *CrebA* mutants in the microarray experiments. The 116 secretory pathway genes are further categorized according to known function and/or localization (blue box). Unknown genes (89) were further subdivided according to predicted localization as ascertained by WoLF PSORT analysis. (C) In situ hybridizations validate CrebA regulation of new target genes identified by microarray analysis. Overexpression of CrebA using the *en*-Gal4 driver is sufficient to induce ectopic expression of four of the five newly identified target genes. Asterisks represent SG position, and arrows point to epidermis expression. Bars, 125 µm.

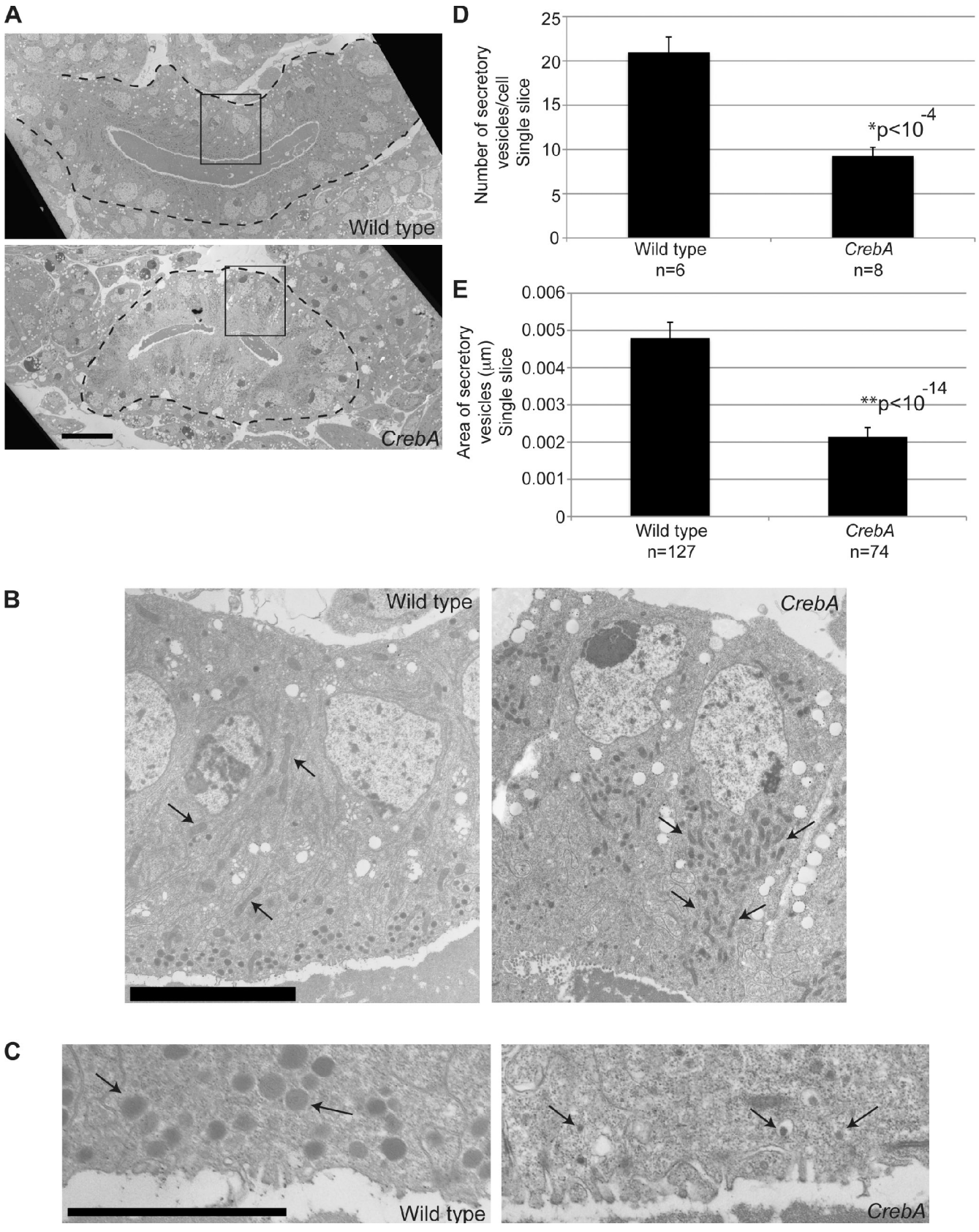


Figure 6. **Characterization of CrebA mutant SGs revealed decreases in secretion and changes in organelle positioning.** (A) Low-magnification (980×) TEM images of stage 15 SGs (top, wild-type; bottom, *CrebA* mutant) show a reduction in lumen size and content in the *CrebA* mutant (bottom). Boxes indicate region magnified in B, and the dotted line outlines the SG. Bar, 10 µm. (B) Higher-magnification images (2,850×) of individual cells, with arrows indicating the position of the mitochondria. Note the clustering of mitochondria in the right panel (*CrebA* mutant). (C) High-magnification (15,000×) images of the apical regions of a single SG cell with arrows pointing to secretory vesicles (left, wild-type; right, *CrebA* mutant). Bar, 1 µm. (D) Quantification of secretory

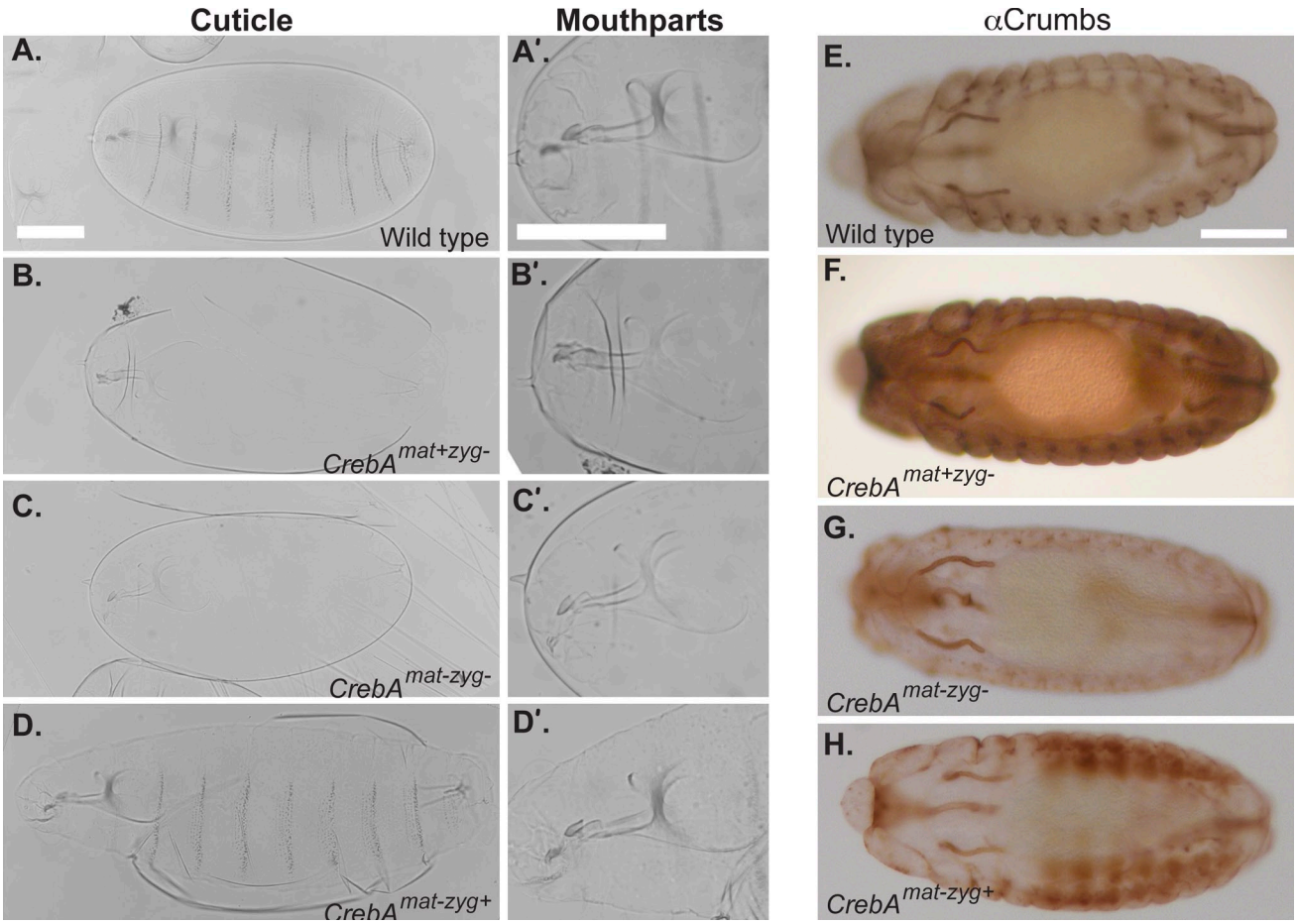


Figure 7. **CrebA is not supplied maternally.** (A–D) Cuticle preparations of larvae just before hatching from the vitelline membrane reveal that animals missing both the maternal (mat-) and zygotic (zyg-) contributions of *CrebA* are similar to animals missing only zygotic function (note the near complete absence of ventral denticles in B and C, and the loss of mouthpart pigmentation in the enlarged views in A′–D′). Paternally-supplied zygotic *CrebA*$^{+}$ is sufficient for maternal *CrebA* mutant larvae to finish embryonic development and hatch. (E–H) Staining with the Crumbs antibody reveals no overt morphological changes associated with either the maternal, zygotic, or maternal and zygotic loss of CrebA. All embryos are early stage 15. Bars, 125 µm.

of Creb3L1 and Creb3L2 in *Drosophila* embryos. The truncated forms correspond to the fully processed proteins and should be functionally analogous to *Drosophila* CrebA (Kondo et al., 2005, 2007). With the *en*-Gal4 driver, we detected high-level expression of both forms of Creb3L1 and Creb3L2 in the epidermal stripes corresponding to *engrailed* expression (Fig. 8 C). Importantly, we also detected robust expression of all five of the SPCGs we tested in the *engrailed* domain with expression of the truncated forms of both Creb3L1 and Creb3L2, but not with full-length Creb3L1 and Creb3L2; these findings are fully consistent with the robust localization of the truncated proteins to nuclei and of the full-length proteins to the ER (Kondo et al., 2005, 2007). We conclude that human Creb3L1 and Creb3L2 can activate SPCG transcription in a heterologous system, which suggests a general and direct role for this family of bZip transcription factors in mediating high-level secretory capacity.

To ask if Creb3L1 can also up-regulate SPCG expression in human cells, we performed a microarray analysis of HeLa cells expressing the truncated form of Creb3L1 and control nonexpressing cells. It should be noted that HeLa cells are not specialized for secretion; therefore, this experiment would determine if the overexpression of Creb3L1 alone can up-regulate the secretory pathway in cells that are not dedicated to high-level protein secretion. Thus, it was exciting to discover that the most highly up-regulated genes in Creb3L1 T expressing HeLa cells had GO terms including Golgi vesicle transport, secretory pathway, and secretion (Tables S2 and S4). The up-regulated set of genes exhibited at least threefold enrichment in the prevalence of these terms as compared with the human genome (Table S2). Thus, like its *Drosophila* orthologue, Creb3L1 can activate expression of multiple components of the secretory pathway.

vesicle number per cell in an individual slice for wild-type and *CrebA* mutant SGs. (E) Mean area of the secretory vesicles, determined using ImageJ software, for an individual slice for wild-type and *CrebA* mutant SG cells. P-values in D and E were determined using a two-tailed Student's *t* test. Error bars represent standard deviation.

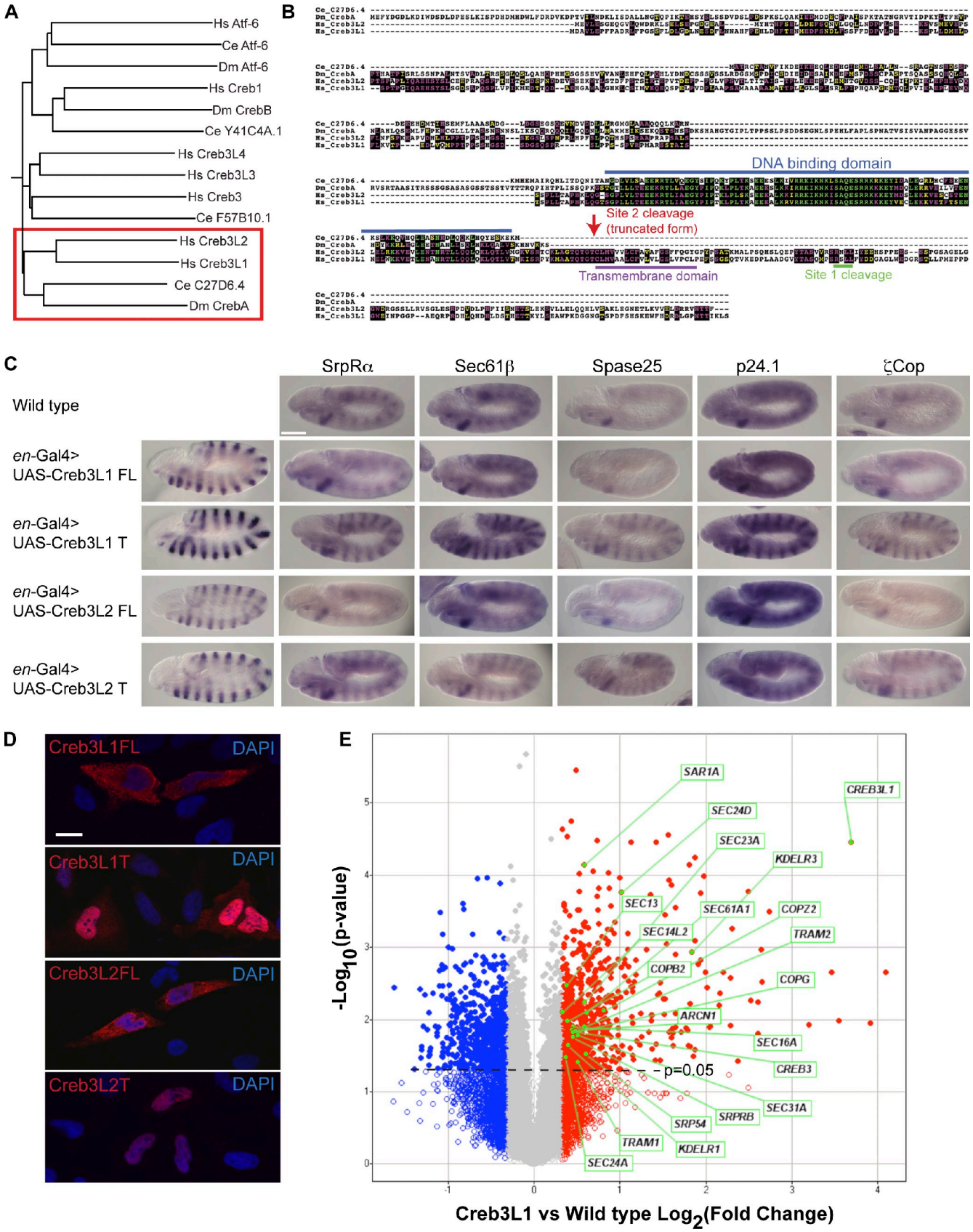


Figure 8. **The CrebA human orthologues Creb3L1 and Creb3L2 are sufficient to up-regulate the secretory pathway genes.** (A) A rooted phylogenetic tree reveals the relationships among members of the CREB/ATF family of transcription factors from *Drosophila melanogaster* (Dm), *Caenorhabditis elegans* (Ce), and *Homo sapiens* (Hs). The red box highlights *Drosophila* CrebA and its closest family members. (B) Sequence alignment of the CrebA family. The truncated forms of Creb3L1 and Creb3L2 contain all N-terminal residues up to the site 2 protease cleavage point. (C) Human Creb3L1 and Creb3L2

Discussion

Here, we provide evidence that the CrebA/Creb3-like bZIP transcription factors are direct and major regulators of secretory capacity. *Drosophila* CrebA directly activates high-level expression of SPCGs through a site we found to be conserved among the enhancers of 34 CrebA-dependent SPCGs. Moreover, ectopic expression of CrebA in multiple tissues is sufficient to activate high-level expression of every SPCG tested. Microarray analysis indicates that CrebA is required for full expression of ~400 genes, including almost 200 implicated in secretion. The secretory target genes include general machinery required for secretion in all cells as well as cell type–specific secreted cargo, such as the cuticle proteins and mucins. Phenotypic characterization of *CrebA* mutant SGs revealed a range of expected secretory defects, including reduced luminal secretory content and a decrease in the size and frequency of apical secretory vesicles, as well as unexpected changes in organelle distribution. We demonstrated that active forms of the closest vertebrate orthologues Creb3L1 and Creb3L2 activate the *Drosophila* SPCGs when expressed in embryos. Active Creb3L1 can also induce expression of multiple components of the secretory pathway when expressed in HeLa cells, a nonsecretory cell type.

CrebA is the single *Drosophila* member of the Creb3-like family of transcription factors that includes five different proteins in mammals (Creb3/Luman, Creb3L1/Oasis, Creb3L2/BBF2H7, Creb3L3/CrebH, and Creb3L4/Creb4) and two in worms (C27D6.4 and F57B10.1; Fig. 8 A). This singularity means that the fly protein is likely to play a more pivotal role in the regulation of secretion because there is no possibility of compensation for its activity by other family members. Each member of the Creb3-like family has a unique expression pattern, with some overlap among family members. *Creb3/Luman* is most highly expressed in the brain, with expression detected in the liver, intestine, colon, and skeletal muscles (Audas et al., 2008). *Creb3L1* is expressed in osteoblasts, prostate, pancreas, ovary, testis, the gut, lungs, kidney, and SGs (Nikaido et al., 2001; Omori et al., 2002). *Creb3L2* is expressed in chondrocytes, heart, lung, liver, kidney, adrenal gland, bladder, submandibular gland, brain, ovary, pancreas, spleen, testis, and prostate (Kondo et al., 2007). *Creb3L3/CrebH* is almost exclusively detected in the liver (Chin et al., 2005), whereas *Creb3L4/Creb4* expression is elevated in the prostate, thymus, brain, pancreas, skeletal muscle, and peripheral leukocytes (Cao et al., 2002). Unlike the *Drosophila* and worm orthologues, all five members of the Creb3-like family are ER-bound transcription factors previously implicated as sensors in the unfolded protein response (UPR; Kondo et al., 2005, 2007; Liang et al., 2006; Stirling and O'hare, 2006; Zhang et al., 2006; Saito et al., 2007). Recently published phenotypes of the knockout mutations in each of the two genes most closely related to *CrebA*, *Creb3L1*, and *Creb3L2* suggest a more physiological role for these genes during normal development, with a major defect being failure to secrete the extracellular matrix in the cell types expressing the highest levels of each gene (Murakami et al., 2009; Saito et al., 2009). These data support a model wherein one or more of the remaining members of the family may largely compensate for the loss of secretory capacity associated with the loss of any one family member. Indeed, our findings that the expression of only a single Creb3-like family member in HeLa cells, a nonsecretory cell type, is sufficient to activate expression of multiple components of the secretory machinery further supports this hypothesis. Among the many secretory genes induced in HeLa cells by Creb3L1 are genes encoding multiple components of CopII vesicles: *Sec16A*, *Sec23A*, *Sec24A*, *Sec24D*, *Sec31A*, and *Sar1A*. The reduced expression of one or more of these genes could explain the ER trapping of ECM proteins observed with the loss of either *Creb3L1* or *Creb3L2*.

Our microarray analysis of *CrebA* mutants revealed that CrebA up-regulates transcription of secretory cargo, specifically expression of multiple components of the insect cuticle, several mucin-like proteins (secreted highly-glycosylated proteins rich in serine and threonine), and multiple secreted proteins of unknown function. Although unexpected, this parallels the finding that mouse Creb3L1 directly up-regulates the type I collagen gene *col1a1*, a major secreted component of bone ECM (Murakami et al., 2009). Our data also suggests that CrebA may function in parallel with tissue-specific regulators to control high-level expression of organ-specific cargo. An example is *CG14756*, which encodes an SG-specific secreted protein of unknown function. Loss of *CrebA* results in a 3.2-fold decrease in the expression of this gene based on the microarray analysis, but unlike the CrebA targets that show more general expression in all secretory tissues, expression of *CG14756* could not be induced by CrebA in other cell types, which suggests the additional requirement for tissue-specific transcription factors for its activation (Fig. 5). Indeed, expression of *CG14756* is absolutely dependent on Fkh (Maruyama, R., personal communication, and unpublished data), and the region immediately upstream of *CG14756* contains a good consensus Fkh binding site ~150 bp upstream of three clustered CrebA consensus binding sites. Thus, we propose that the CrebA/Creb3-like family enhances secretory capacity by coordinately up-regulating expression of the general secretory machinery and of tissue-specific secreted cargo, with the expression of cargo genes likely mediated through cooperation with tissue-specific factors.

More than 30% (116 of 383) of genes identified in the CrebA microarray experiments had GO terms associated with

up-regulate SPCGs. The top row shows wild-type expression of each SPCG tested. Overexpression of UAS-Creb3L1 (full-length [FL] or truncated [T]) and UAS-Creb3L2 (FL or T) using the *en*-GAL4 driver resulted in robust mRNA expression in epidermal stripes (first column). Overexpression of the full-length forms did not affect SPCG expression, whereas expression of the active truncated forms induced high-level expression of every SPCG tested. Bar, 125 µm. (D) HeLa cells transfected with either the FL or T forms of the Creb3L1 or Creb3L2 cDNAs. For both, the full-length protein is detected in the ER, whereas the active truncated form is predominantly detected in nuclei. Bar, 20 µm. (E) Microarray analysis of HeLa cells expressing the active truncated form of Creb3L1 compared with control HeLa cells reveals a significant increase in the expression of many SPCGs. Genes represented in red (increased) or blue (decreased) show a fold change of at least 1.25. Open circles indicate nonsignificant changes.

roles in the secretory pathway (Fig. 5), and WoLF PSORT predictions suggested that more than half of the unknown targets are likely to have roles in secretion. Indeed, genes not implicated in the secretory pathway may, nonetheless, participate in secretion. Several of the ion channel/transporter genes have human orthologues known to function in secretory pathway organelles; for example, *CG10449* (*Drosophila catsup*, human SLC39A7) encodes a Golgi-localized zinc transporter (Huang et al., 2005). Also, 26 of the target genes that did not have GO annotations have highly conserved human orthologues, several of which are involved in secretion. For example, *CG4293* and *CG7011* encode proteins similar to ERGIC2 and ERGIC3, respectively, which are proteins localized to the ER–Golgi intermediate compartment that function in protein folding and trafficking (Nishikawa et al., 2007). Thus, it is likely that many of the newly identified CrebA target genes encode proteins that function in secretory organelles, highlighting the potential of the microarray studies to reveal new genes with key roles in the efficient production and delivery of products through the secretory pathway.

Altogether, our studies reveal that CrebA and its human orthologues Creb3L1 and Creb3L2 activate transcription of components that function at all steps in secretion. Coordinate up-regulation of secretory components by one (or a very few) transcription factors allows for easily adjustable levels of secretory capacity in a variety of cell types, as nicely exemplified in the *Drosophila* embryo, where levels of CrebA and corresponding SPCG expression correlate with the levels of secretory activity in the different tissues. Furthermore, our microarray analysis combined with the recent studies of Creb3L1 and Creb3L2 in specialized cell types (osteoblasts and chondrocytes; Murakami et al., 2009; Saito et al., 2009) suggest that CrebA family proteins also up-regulate expression of tissue-specific secreted content, highlighting the significance of this protein family in secretory cell specialization and function.

Materials and methods

Fly strains

The *CrebA*^{wR23} protein null allele was used for all loss-of-function analysis (Andrew et al., 1997). UAS-CrebB and *CrebB*^{S162} were obtained from the Bloomington Stock Center: HS-FLP and P{ovo^{D}} FRT2A (79D)/TM3 were obtained from E. Chen (Johns Hopkins University School of Medicine, Baltimore, MD). Recessive lethal lines were balanced over *lacZ* or GFP balancers to allow identification of homozygous mutants. UAS-Creb3L1-FL/T and UAS-Creb3L2-FL/T were generated by Gateway (Invitrogen)-mediated cloning and recombination into the pTW untagged UAS vector (Drosophila Gateway Collection, Carnegie Institute). *engrailed (en)*-Gal4 and *breathless (btl)*-Gal4 were used to express UAS-CrebA (Rose et al., 1997), UAS-Creb3L1 FL, UAS-Creb3L1 T, UAS-Creb3L2 FL, or UAS-Creb3L2 T constructs in epidermal stripes (*en*-Gal4; Weiss et al., 2001), or the trachea, salivary duct, and midline (*btl*-Gal4; Shiga et al., 1996).

EMSAs

Plus and minus strand oligonucleotides of ∼30 bases were designed and synthesized (IDT DNA) for each binding site, and included the nine-nucleotide consensus motif flanked by ∼10 nucleotides of genomic sequence at both the 5′ and 3′ ends. The plus strand of each oligo was labeled with γ-^{32}P using the manufacturer's protocol (Invitrogen) and was annealed to the minus strand by heating the DNA to 95°C for 5 min and then cooling to RT. Unlabeled double-stranded oligos were prepared similarly for competition experiments. DNA binding reactions were performed as described previously (Smolik et al., 1992), with the exception that all competitor oligonucleotides were added at concentrations of 20×, 60×, and 100×. Binding reactions were run on a 4% polyacrylamide gel at 30 mAmps for ∼2 h and prepared for autoradiography using standard methods.

Site-directed mutagenesis of SPCG enhancer-lacZ reporters

Primers for putative CrebA binding sites (Abrams and Andrew, 2005) were designed to mutate the five core nucleotides of the CrebA-binding motif G/TACGT to ACAAC using the Stratagene Primer Design program (Agilent Technologies). Sites were subsequently mutated using the QuikChange Multi Site-Directed Mutagenesis kit (Agilent Technologies), and the mutated constructs were injected into *w*^{1118} flies (Rainbow Transgenic Flies Inc.).

ChIP and quantitative PCR

The ChIP followed the protocol of Birch-Machin et al. (2005). Quantitative real-time PCR was performed using 1 µl of eluted sample DNA, 1.5 µM forward and reverse primers, and iQ SYBR Green Supermix (Bio-Rad Laboratories). Experiments were performed in triplicate, including those from an input control. Samples were normalized to *actin5c*, and fold change over the no-primary antibody control was calculated using the ddCt method (Livak and Schmittgen, 2001). Error bars represent the standard deviation of ddCt.

TEM

CrebA mutant embryos were identified by the absence of GFP staining from the TM3 balancer chromosomes. Stage 16 wild-type and *CrebA* mutant embryos were processed for TEM using standard protocols and examined on a microscope (EM120; Philips). To determine secretory vesicle size, area measurements were collected using ImageJ software (National Institutes of Health), and statistical significance was determined using a two-tailed Student's *t* test.

Immunohistochemistry and in situ hybridizations

In situ hybridization and immunohistochemistry were performed as previously described (Reuter et al., 1990; Lehmann and Tautz, 1994). Antibody concentrations used in this study were as follows: α-CrebA (1:1,000; Andrew et al., 1997), α-Crumbs (1:100; Developmental Studies Hybridoma Bank), and α-β-galactosidase (1:10,000; Promega). All secondary antibodies (Vector Laboratories and Invitrogen) were used at a 1:500 dilution. The fluorochrome Alexa Fluor 568 was used for HeLa cell experiments, and nuclei were labeled with DAPI. Confocal images were obtained using a confocal microscope (LSM 510 Meta; Carl Zeiss, Inc.), using a Plan-Neofluor 40×, 1.3 NA oil objective and the LSM software (Carl Zeiss, Inc.). All other images were obtained using an microscope (Axiophot; Carl Zeiss, Inc.) configured with a digital camera (Coolpix 4500; Nikon). Images were taken using a Plan-Neofluor 20×, 0.50 NA objective. Images were rotated and cropped using Photoshop (Adobe). All images were obtained at room temperature.

Generation of CrebA maternal-zygotic mutants

CrebA^{23w-} was recombined onto a third chromosome containing FRT2A (79D) and then crossed to flies carrying hsFLP; P{ovo^{D}} FRT2A (79D). After 72 h, larvae were heat-shocked for 1 h at 37°C on consecutive days until pupae formation. Female flies carrying both *CrebA*$^{23w-}$ FRT2A(79D) and P{ovo^{D}} FRT2A (79D) were crossed to *CrebA*$^{23w-}$*/TM6B*, *Ubx-lacZ* males, and progeny were collected and stained using the appropriate markers. *CrebA*$^{mat\text{-}zyg\text{-}}$ embryos were identified based on the absence of staining for β-gal from the lacZ-containing balancer.

Microarray experiments to identify CrebA target genes in *Drosophila*

Three samples of sorted stage 11–16 *CrebA* homozygous (GFP balancer negative) mutant embryos and wild-type OR embryos were isolated using a COPAS Select embryo sorter (Union Biometrica). Total RNA was isolated using TriZOL chloroform extraction and precipitated with isopropanol. The RNeasy kit (QIAGEN) was used for RNA clean up. Total RNA (100 ng) was labeled according to standard Affymetrix protocols and hybridized to the *Drosophila* genome 2.0 chip. After scanning, intensity values were normalized by RMA (Partek Inc.; Irizarry et al., 2003a,b), and statistical analysis was performed using Spotfire software (TIBCO). CrebA target genes were identified based on a 1.5-fold change in gene expression, with a p-value ≤ 0.05. This fold change was selected because many known, confirmed, CrebA target genes fall into the range of 1.5–2.0×.

HeLa cell culture, transfection, and immunofluorescence

cDNAs corresponding to the full-length Creb3L1 and Creb3L2 were obtained from Thermo Fisher Scientific (accession nos. BC015781 and BC110813, respectively, from the Mammalian Gene Collection), and the

full-length and truncated versions (Creb3L1, amino acids 1–375; Creb3L2, amino acids 1–379) were cloned into pCDNA3.1/Hygro (Invitrogen) for transient transfection. DNA (1 µg of Creb3Lx with 0.1 µg of the cotransfection marker pEGFP-C1; Invitrogen) was transfected into cells using the Fugene6 transfection reagent according to the manufacturer's instructions (Roche). Immunofluorescent staining was performed using established protocols (Sbodio and Machamer, 2007). Primary antibodies for Creb3L1 (NCBI Protein database accession no. NP_443086) and Creb3L2 (accession no. NP_919047) were obtained from Aviva Systems Biology, and used at a dilution of 1:500. Fluorescent secondary antibodies (Invitrogen) were used at a dilution of 1:500. Co-staining with an ER marker (antibody DM286; a gift from C. Nicchitta, Duke University, Durham, NC) and the nuclear marker DAPI confirmed both the ER localization and nuclear localization of the full-length and truncated Creb3L proteins, respectively.

Cell sorting, RNA extraction, and microarray analysis from HeLa cells

HeLa cells were cotransfected with both Creb3L1 T and EGFP ~20 h before sorting (see the previous paragraph). As a control, HeLa cells were mock transfected with Fugene6 reagent, and sorted using identical conditions. Cells were sorted on a FACSaria flow cytometer, and at least 200,000 cells were isolated. RNA was extracted using the RNeasy kit (QIAGEN). Total RNA (100 ng) was labeled and hybridized to the Affymetrix Human Gene 1.0 array according to standard Affymetrix protocols. After scanning, raw data were normalized by RMA (Partek Inc.; Irizarry et al., 2003a,b), and statistical analysis was performed using Spotfire software (TIBCO).

Accession nos.

All microarray data has been deposited in Gene Expression Omnibus (GEO), accession no. GSE23349.

Online supplemental material

Fig. S1 shows the additional EMSAs that were performed for all putative CrebA binding sites within the SPCG enhancers. Table S1 shows the results of the GO cluster analysis using DAVID. Table S2 contains the GO cluster analysis for the HeLa cell array data. Table S3 is a list of all 383 CrebA target genes with functional annotation and fold change values. Table S4 contains the list of genes up-regulated when active Creb3L1 is expressed in HeLa cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201004062/DC1.

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