

Tissue-specific steroid hormone-regulated secretion of proteins in *Drosophila melanogaster* salivary glands – molecular and genetic aspects: A review

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Larval salivary glands (SGs) of *Drosophila melanogaster* (further *Drosophila*) are classical and well-known system, in which steroid hormone ecdysone controlling metamorphosis activates sequence of genes or transcriptional cascade that leads to formation distinct groups of puffs in polytene chromosomes and shortly prior to pupariation to release of unique glue that affix freshly formed puparium to the substrate. Although this system was studied since 1950s and most intensely during 1970s and 1980s, there are still several less known and unappreciated aspects, which deserve special attention. Some functional links between visible puffing and other active genes that do not form puffs are still missing. Production and formation of large secretory granules that are supposed to contain so-called Sgs-material that is secreted upon sturdy increase in ecdysone pulse require plethora of additional and supportive activities of SG cells that were not previously considered to be under direct hormonal control or considered at all. Although some of these physiological processes may appear to be hormone-independent and takes place because all necessary components of particular machinery are already present, there are several other processes that need be coordinated with early, mid or later phase of the ecdysone response and therefore they deserve special attention to fill the gaps. After detailed description of well studies model of Sgs-genes and their hormonal regulation, we tried to integrate this knowledge to a broader context of more advanced and current studies in *Drosophila* organism or cell lines using whole-genomic approach provided that functional and physiological information is conceptually included. After general introduction to the system, this review is committed to sketch functional and logical connections to processes that are conditionally required to fulfill SG organ-specific function but so far were out of the major research focus.

Keywords: hormone ecdysone, *Drosophila*, salivary glands, glue secretion, gene expression, transcriptional control

The steroid hormone ecdysone or 20-hydroxyecdysone (20HE) serves as a crucial signaling molecule during all stages of insect development (Riddiford 1993; King-Jones and Thummel 2005). One of the most complex developmental processes directed by this hormone is insect metamorphosis from the larval to the adult form. 20HE is derived from

ecdysone, a precursor of lower biological activity produced by the ring gland in the higher diptera. The action of 20HE on gene activity at the onset of metamorphosis can be analyzed by following the formation and regression of puffs on the polytene chromosomes of larval and prepupal salivary glands (SGs), which made them extremely attractive model

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to study not only molecular mechanisms of steroid hormone action in general, but also developmentally-linked control of gene expression. One of them is a control of massive secretion of so-called Sgs-proteins (for more see below) the production, transport and release, of which in *Drosophila* SGs is under guidance of 20HE. Before we proceed towards description of individual regulatory processes, we need to introduce most important features of the model system in its biological complexity. The larval SGs of *Drosophila* serve as a unique model organ (Figure 1). These glands are exemplary tubular and single-layered paired epithelial exocrine organs of ectodermal origin (Skaer 1993; Farkas 2016; Loganathan et al. 2021; Kim et al. 2026). Historically, these glands are best known for their giant polytene chromosomes. The detailed study of these chromosomes led to several important advances including creation of the first cytogenetical maps and the elaboration of chromomere theory (Painter 1933, Bridges 1935; Ashburner 1970; Lefevre 1976; Sorsa 1989; Lindsley and Zimm 1992). Likewise, the high degree of polyteny of SG chromosomes has made it possible to study the puffs, which represent particular active gene sites. These puffs can be easily distinguished from inactive sites by localized expansion of the normally banded structure of the chromosome into a diffuse structure called puff (Beermann 1956) that form local extensions of euchromatin. Detailed observations showed that the formation and regression of puffs follow a certain sequence and at least two major peaks of overall puffing activity were noted and three categories of puffs recognized (interecdysial, early, and late puffs). The entire sequence of 196 puffs,

which are induced and regressed in wild type during last 10 h of fully grown third instar larvae and 16 h after puparium formation, is known as the puffing pattern (Ashburner 1970, 1972, 1990; Ashburner et al. 1974; Ashburner and Berendes 1978). The induction of this pattern is the consequence of slight followed by abrupt increase in the hemolymph titer of the steroid hormone ecdysone released from the brain-controlled ring gland (Becker 1962; Borst et al. 1974; Ou et al. 2016).

For more than 70 years, the main function of the larval SG was believed to be associated only with the production of a mucinous cement, which serves as an adhesive during puparium formation (Fraenkel and Brookes 1953). In anticipation of this function, globular, highly refractile PAS-positive granules appear in the cytoplasm of SG cells around the middle of the third-instar (Bodenstein 1950; Kolesnikov and Zhimulev 1975). The hormone ecdysone plays a dual role in this process, by both initiating and then terminating a specific developmental program of protein synthesis required for the production of these secretory granules. When the ecdysone level increases a few hours prior to pupariation, the content of the granules is released into the SG lumen by exocytosis and then expelled by expectoration during puparium formation (Lane et al. 1972; von Gaudecker 1972; Farkas and Sutakova 1998; Beno et al. 2024) where it serves as a glue to attach the puparial case to a solid substrate (e.g. vial glass wall). These granules constitute the components of the salivary glue secretion (Sgs). The Sgs is a highly specialized and unique extracellular composite bioadhesive matrix the individual protein

components, of which are encoded by series of eight Sgs genes (Korge 1975, 1977a,b; Lehmann 1996) that are known to form a set of so-called interecdysial chromosomal puffs. Individual Sgs-genes at their cytogenetical bands were subsequently cloned and molecularly characterized (Muskavitch and Hogness 1980, 1982; Meyerowitz and Hogness 1982; Guild and Shore 1984; Wright et al. 1996; Roth et al. 1999; Da Lage et al. 2019). The Sgs genes encode eight unique proteins ranging in size from 7 to 135 kDa. The largest among them, Sgs-1 and Sgs-3, are heavily O-glycosylated (Ten Hagen et al. 2003; Tian et al. 2012; Farkas 2016). The smallest proteins, Sgs-7

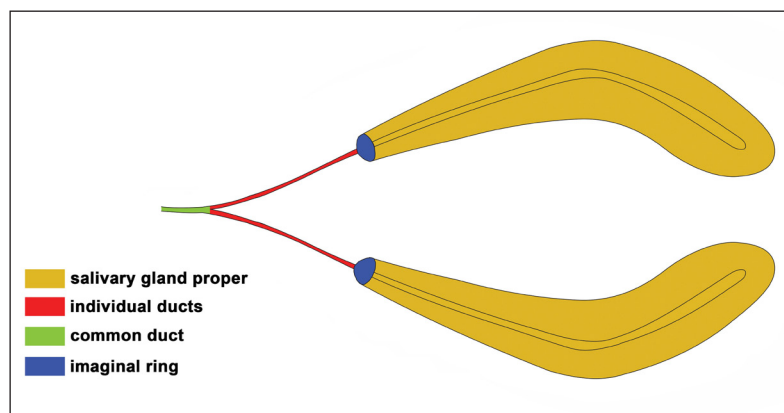


Figure 1. Schematic depiction of pair of salivary glands from 3rd instar larva of *Drosophila* showing salivary gland proper with polytene secretory cells and centrally located lumen, individual ducts connected to common duct. In the front of the salivary gland proper are diploid cells of imaginal discs.

and Sgs-8, lack glycosylation sites, while the medium-sized Sgs-4, Sgs-5, Sgs-5bis and Eig78Ee proteins have a few *N*- and *O*-glycosylation positions (Farkas 2016).

Ultrastructural studies using transmission electron microscopy (TEM) have confirmed that Sgs-secretory granules display three different components: a fine particulate dense component, a filamentous-like paracrystalline component, and a foamy electron-transparent component (Lane et al. 1972; von Gaudecker 1972; Farkas and Sutakova 1998). Each corpuscular secretory SG cell contains 3000 to 3500 of these secretory granules (Farkas and Sutakova 1999), which increase in size through the continuous fusion of individual vesicles. The three different granular components may represent distinct stages of granule maturation and densification (Farkas and Sutakova 1998; Farkas 2016). At the moment of exocytosis, the secretory material appears as an amorphous or a homogenous electron-dense matrix with scattered lighter (electron lucent) rosettes (Poels et al. 1971; Lane et al. 1972; von Gaudecker 1972; Thomopoulos 1987; Farkas and Sutakova 1998). Exocytosis is a well-defined process where vesicles formed in the *trans*-Golgi are transported to targeted sites on the plasma membrane. After undergoing nucleation, zippering, budding, and priming, these vesicles fuse with the membrane to release the Sgs-glue into the extracellular space (for more details see Rouso et al. 2016; Kamalesh et al. 2024). Nevertheless, it is still unclear whether the Sgs-proteins are present in an equimolar ratio within the secretory granules or inside the expectorated secretory glue, so it is not possible to evaluate how their relative molar ratios are related to the overall function of the glue. Although Ji et al. (2018) and Syed et al. (2022) attempted to describe bundles of parallel filaments thought to be composed of Sgs-3 protein, along with electron-lucent discs associated with organized Sgs-1 protein in *Drosophila* SG granules, there is no information on the role of each individual Sgs-protein in granule formation and maturation or densification, or in the subsequent hydration of the glue after it is exocytosed into the lumen.

Recently, we observed that exocytosed Sgs-glue inside the lumen where it adopts a highly fibrous sponge-like structure filled with numerous small cavities. Upon expectoration and solidification, the outside surface of glue forms a smooth layer, whereas the interior structure consists of a multidirectional trabecular arrangement of thick micellar fibers of varied density. We also found, that besides its cementing properties the glue is highly effective at glueing and trapping microorganisms suggesting

that it may play a significant immune and defense role (Benova-Liszekova et al. 2019). It is clear that SGs in late larval stages as they approach hormonally-controlled metamorphosis, perform numerous assignments to fulfill their function related to the production, release, and expectoration of secretory glue to affix freshly forming puparium to the substrate (Benova et al. 2024). This involves a complex set of tasks that must be coordinated to ensure the synchronized functioning of numerous independent machineries or pathways. Specifically, the production of Sgs secretory proteins and formation of Sgs-granules as well their secretion are multistep processes that require a high level of coordination between the transcription of multiple genes and various cellular pathways. The control of transcription appears to be a primary level where at least some tasks need be tightly regulated to achieve temporally precise control over these processes.

This review summarizes the current understanding and perspectives on the role of ecdysone signaling as a major temporal factor coordinating various tasks during this critical developmental transition and will summarize potential connections with other pathways that are simultaneously active, but have not yet been the focus of mainstream research.

Control of Sgs genes transcription by steroid hormone 20HE

The Sgs genes show a salivary gland-specific expression, which is restricted to the second half of the last larval instar prior to ecdysis (metamorphosis). The series of intermolt puffs was first described by Becker (1959, 1962) followed by more detailed study of Ashburner (1970, 1972). The interecdysial puffs have been linked to production of the Sgs-proteins (Korge 1975, 1977a,b; Lehmann 1996). These cytogenetic and genetic studies were shortly followed by the cloning of individual Sgs-genes from their cytological locations by using so-called molecular walking technique: the puff 68C encodes the Sgs-3, Sgs-7 and Sgs-8 genes (Meyerowitz and Hogness 1982), puff 3C encodes Sgs-4 (Muskavitch and Hogness 1980), puff 95B encodes Sgs-5 (Guild and Shore 1984), and puff 25B2-3 encodes Sgs-1 (Roth et al. 1999). Later, the previously overlooked gene *Eig71Ee* co-expressed temporally- and tissue-specifically with other Sgs genes (Restifo and Guild 1986; Wright et al. 1996) was characterized as one of the Sgs genes. Very recently, a new eighth gene *Sgs-5bis* was identified at the same location as Sgs-5, a 95B band (Da Lage et al. 2019).

Among these, the regulation of two of the genes, *Sgs-3* and *Sgs-4*, residing in the intermoult puffs at 68C and 3C11-12, respectively, has been studied in considerable detail. Both genes are located in close proximity to other genes. The *Sgs-3* is clustered with two other glue genes, *Sgs-7* and *Sgs-8*, at 68C, whereas *Sgs-4* shares a common upstream region with *pre-intermoult 1* (*Pig-1*), a gene of poorly understood function. Like *Sgs-4*, *Pig-1* exhibits salivary gland-specific expression, but the two divergently transcribed genes differ in their temporal pattern of expression. *Pig-1* is expressed in the early larval stages under complete absence of 20HE titer. Expression then changes from *Pig-1* to *Sgs-4* toward the middle of the last larval instar when 20HE titer begins to rise and is maintained at a low steady level for almost 24 h. A first hint regarding the hormonal regulation of the *Sgs* genes came from the observation of Ashburner (1972) that some intermoult puffs regress more rapidly under *in vitro* culture in the presence of exogenously supplemented 20HE. Along with the temporal correlation between the cessation of *Sgs* gene transcription and the rise in hormone levels during the late third instar. This suggests that the high-level surge of 20HE might be responsible for the repression of *Sgs* genes.

A significant tool to assess and evaluate the role of ecdysone signaling in the control of developmental transitions including puff induction/regression and the expression of *Sgs*-genes were temperature-sensitive ecdysone-deficient mutations *l(3)ecd^{lts}* (= *ecdysoneless^l*) or *ecd^l* (Garen et al. 1977), and *suppressor of forked^{ts67g}* (*su(f)^{ts67g}*) (Snyder and Smith 1976; Klose et al. 1980), which when exposed to restrictive temperature, 29°C, stop producing 20HE. Hence, further evidence supporting this model came from observations using the ecdysone-deficient mutations *su(f)^{ts67g}*, which has shown that the induction of *Sgs* genes depends on 20HE, as neither *Sgs-4* nor the *Sgs* genes clustered at 68C were transcribed under ecdysteroid-deficient conditions (Hansson and Lambertsson 1983). Additionally, feeding the mutant larvae with 20HE which they were still exposed to non-permissive temperature, resulted in a restoration of glue gene transcription (Hansson and Lambertsson 1983). Strikingly, very similar results have been observed in the hormonal control of *Drosophila* malate dehydrogenase (MDH) by sesquiterpenoid juvenile hormone (JH), which also required the presence of 20HE (Farkas and Knopp 1997, 1998; Farkas et al. 2002). Yet another example came from observations of Richards (1976), who studied the course of puffing pattern in homozygotes

of *lethal (2) giant larvae* tumor suppressor (*l(2)gl*) gene. These larvae became ecdysone-deficient by the end of the third larval instar. Partial recovery of the puffing pattern and the non-pupariating phenotype was achieved by injecting 20HE or by feeding it starting from the middle of the last instar.

Temporally precise activation of glue gene expression: interplay of multiple transcription factors

The *cis*-acting regulatory sequences of *Sgs-3* and *Sgs-4* have mainly been identified through the use of P-element-mediated germ line transformation. Early research on the regulation of *Sgs-3* has been summarized by Meyerowitz et al. (1987) and Martin et al. (1989). The expression of *Sgs-3* is regulated by at least three distinct regions. The two of these regions, the proximal element (Roark et al. 1990), and the distal element (Romain et al. 1988), are each capable and sufficient of directing the stage- and tissue-specific expression of *Sgs-3* although at lower level (Roark et al. 1990). However, when combined, these two regions act synergistically to produce high level expression. A wild-type or high expression level, however, is only achieved when additional sequences located further upstream are also actively involved (Giangrande et al. 1987). The proximal element has been divided into two small regions of 18 bp and 22 bp, each of which, when combined with distal element, is sufficient to direct high level expression (Martin et al. 1989). A mutational analysis identified six key positions within these two subregions, which are likely involved in the binding of yet-unknown transcription factors (Todo et al. 1990). The distal element is marked by a stage- and tissue-specific DNase I hypersensitive site (DHS) at -600 bp (Romain et al. 1986). Elimination of a 63-base pair sequence from this region results in reduced *Sgs-3* expression, while the removal of a 20-base pair sequence, which includes a 10-base pair tandem repeat, leads to a less significant decrease (Romain et al. 1988). A factor termed glue enhancer binding factor I (GEBF-I) has been identified that binds to the -600 region and may be involved in the regulation of *Sgs-3* (Georgel et al. 1991). Further research has shown that the induction of the *Sgs-4* gene is regulated by the coordinated action of the ecdysone receptor and secretion enhancer binding protein 3 (SEBP 3). The EcR binds to two sites, element I and element II, within the regulatory region of *Sgs-4*. Element II appears to have limited importance for *Sgs-4* expression, whereas element I is an ecdysone response element

(EcRE) that is necessary, but not sufficient, for the induction of *Sgs-4*. Close to elements I and II, two binding sites for SEBP3 were identified. The complex hormone response unit also contains binding sites for a third factor, SEBP2, which overlap with binding sites for the Broad-Complex (BR-C) (Lehmann and Korge 1995). Authors also found that SEBP2 binds to multiple sites in the upstream region of *Sgs-4* and these sites are essential for full transcriptional activation of *Sgs-4*. Additionally, transcription factor encoded by the region-specific homeotic gene *fork head (fkh)* has two binding sites within an ecdysone response unit that controls the tissue- and stage-specific responses of *Sgs-4* to the 20HE. These binding sites are important for the transcriptional activation of *Sgs-4* and are occupied by native Fkh *in vivo* (Lehmann and Korge 1996).

A region critical for the regulation of *Sgs-4* was identified early on through studies of four naturally occurring *Sgs-4* underproducer strains *Berlin-1*, *Hikone R*, *Kochi R* and *Seto* (Muskavitch and Hogness 1980, 1982). Defective expression of these *Sgs-4* alleles was attributed to small deletions in the upstream region encompassing one or more of three tissue-specific DHSs (Shermoen and Beckendorf 1982). Later, P-element transformation studies (McNabb and Beckendorf 1986; Jongens *et al.* 1988; Mougneau *et al.* 1993) confirmed that these deletions lie within a larger region that is crucial for the regulation of both *Sgs-4* and *Pig-1*. This region contains three regulatory elements with different functional roles (Jongens *et al.* 1988; Mougneau *et al.* 1993). Element III acts as an enhancer capable of directing the stage- and tissue-specific expression of *Sgs-4* on its own. It is also necessary for *Pig-1* expression. Element II is proposed to guide element III to the *Pig-1* promoter at early developmental stages, while element I acts as a switch that represses element II during mid-third instar, thereby enabling the activation of the *Sgs-4* promoter by element III. Since 840 bp and even 2.6 kb upstream of *Sgs-4* are not sufficient for wild-type expression levels, as in the case of *Sgs-3*, it is likely that additional regulatory elements are located more distally upstream or downstream of the transcription unit. This is similar to the situation observed in the 20HE-inducibility of the *Eip28/29* gene in the Kc cell line, which contains several EcREs, one of which is located far downstream (Cherbas *et al.* 1991; Andres and Cherbas 1992).

The expression of *Sgs-3* and *Sgs-4* depends on different degrees on the products of the *Broad-Complex* (BR-C), which are encoded in the 2B5 early puff. The BR-C is a complex gene (DiBello *et al.*

1991; von Kalm *et al.* 1994) that directs the synthesis of at least six different mRNAs through the use of multiple promoters and alternative splicing. These mRNAs encode a family of related proteins. Some of these mRNAs, whose identity is still unknown, have features of Class I or intermoult transcripts, which means they are detectable early in the third larval stage (Bayer *et al.* 1997). The BR-C proteins can be classified into four groups based on the presence of unique pairs of C2H2 zinc fingers (Z1, Z2, Z3, and Z4), which are fused to a common core region. The core region contains the conserved PO2/BTB domain, which has been shown to reduce DNA binding and to be involved in various protein-protein interactions (Bardwell and Treisman 1994). The BR-C locus was initially identified through mutations that can be classified into at least three complementation groups (*broad (br)*, *reduced bristles on the palpus (rbp)* and *l(1)2Bc*) and a group of *non-pupariating (npri)* alleles that do not complement any of these functions. An analysis of *Sgs* gene expression in different BR-C mutants showed that *Sgs-4* can, in principle, be expressed independently of BR-C, although the timing and level of expression are affected by BR-C. Conversely, *Sgs-3* expression is entirely dependent on BR-C (Crowley *et al.* 1984). The effects of the BR-C seem to be specifically mediated by the *rbp1* and *2Bc⁺* functions (Guay and Guild 1991; Karim *et al.* 1993; von Kalm *et al.* 1994), which have been tentatively linked to the BR-C protein isoforms Z1 and Z3, respectively (DiBello *et al.* 1991; Emery *et al.* 1994). The expression patterns of Z1 and Z3 align with their proposed role in regulating *Sgs* gene expression (Bayer *et al.* 1997; Mugat *et al.* 2000). The ectopic expression of Z1 protein isoform under the control of a heat shock promoter is sufficient to rescue *Sgs-4* expression in *rbp5* mutation (von Kalm *et al.* 1994).

The question remains how do the BR-C proteins exert their effect on *Sgs* gene expression? The presence of zinc finger motifs suggests that they function as DNA-binding transcription factors. This assumption is supported by the fact that bacterially produced BR-C proteins can bind to numerous sites in the *Sgs-4* upstream region with at least two of these sites being important for *Sgs-4* expression in living organisms (Lehmann and Korge 1995). Interestingly, these sites are also recognized by a secretion enhancer binding protein 2 (SEBP2), which is independent of BR-C and is detectable in nuclear extracts from third instar SGs (Lehmann and Korge 1996). It should be considered that *in vivo* BR-C proteins may only recognize a subset of their potential binding sites by interacting with other DNA-bound proteins *via* their POZ

domain, thereby reducing the inhibitory effect of this domain on DNA binding. Furthermore, studies by Kucharova-Mahmood et al. (2002) and Farkas et al. (2011) have suggested that BR-C can also act as chromatin remodeling factors, thus having a broader influence on gene expression control. Transcriptional regulation of genes from 3C11 and 68C puffs is depicted in Figures 2 and 3.

Higher level of ecdysone-response complexity - genomic studies

As mentioned above, the ecdysone-initiated puffing pattern in the polytene chromosomes of wild type *Drosophila* includes 196 identified loci (Ashburner 1970, 1972). A single puff-forming locus can contain several transcription units or even multiple genes in a cluster. For instance, the 68C cluster includes the *Sgs-3*, *Sgs-7*, and *Sgs-8* genes, while the late 71DE puff harbors at least six genes, *Eig71Ea* to *Eig71Ef* (Wright et al. 1996) just to give a few examples. Moreover, a great number of puffs have not been molecularly characterized despite the fact that the *Drosophila* genome has been completely sequenced

(Adams et al. 2000, 2003; Celniker and Rubin 2003; Stark et al. 2007; Xu et al. 2008). Therefore, it is clear that many other ecdysone-responsive puffs might contain multiple genes and it should be emphasized that a significantly larger number of active genes responding to 20HE may not form puffs. Hence, the total number of ecdysone-responsive genes in any single tissue could be considerably higher than what is suggested by traditional puff studies. Several chromosomal proteins such as PEP, Df31, and HP1 have been found associated with hundreds of active puffs and unpuffed loci in polytene chromosomes, indicating actively transcribed genes (Mayfield et al. 1978; Amero et al. 1991, 1993; Hamann and Stratling 1998; Piacentini et al. 2003) and they could still represent a fraction of the truly active genes. For clear reasons, further analysis of puffing could offer some additional insights, but with few exceptions, it would not provide versatile information about the role of individual puffs. Many of the early, late, and mid puffs cannot be clearly related to a specific cellular process (Ashburner et al. 1974).

There have been pioneering efforts to obtain high-throughput data related to gene expression profiling

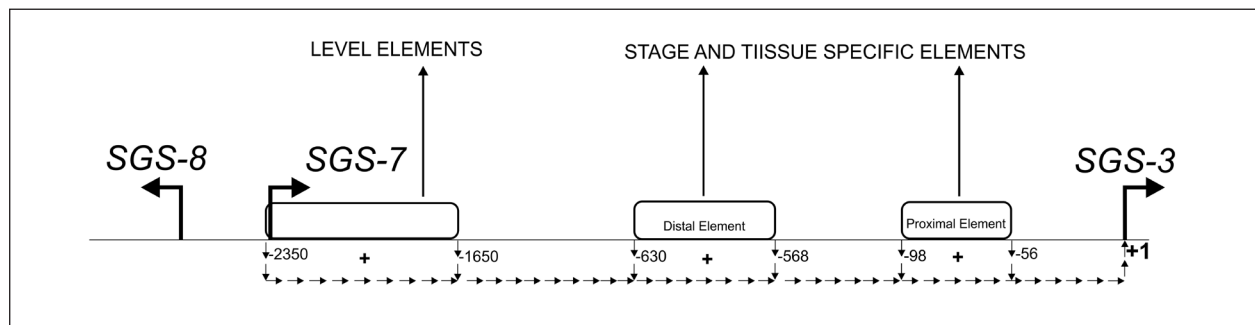


Figure 2. Promoter of *Sgs-3* gene and 2350 bp long intergenic region between *Sgs-3* and *Sgs-7* and *Sgs-8* genes where stage- (distal element) and tissues-specific (proximal element) expression is regulated by ecdysone and other factors. Interecdysial activation of this expression cause formation of 68C puff. (Adopted from Lehmann 1996).

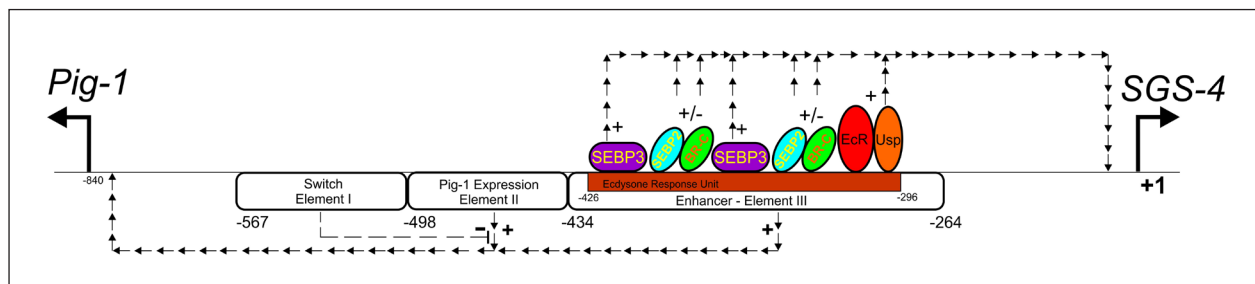


Figure 3. Promoter and intergenic region of *Sgs-4* gene and *Pig-1* gene from 3C11 locus on X chromosome. About 450 bp upstream of *Sgs-4* contains enhancer element necessary to bind not only ecdysone receptor (EcR/Usp dimer), but also SEBP2 and SEBP3 transcription factors in combination with chromatin remodeling factor Broad-Complex (BR-C) where all of them are required to initiate *Sgs-4* expression and intermolt puff formation and downregulation of *Pig-1* gene expression. (Adopted from Lehmann 1996).

during metamorphosis using whole-genomic approaches at both organismal and tissue-specific levels. White et al. (1999) and Li and White (2003) used high-density DNA microarrays to analyze gene expression profiles in five tissues during metamorphosis. Through a computational approach, it was possible to identify regulatory motifs and a cognate transcription factor involved in the regulation of a set of co-expressed genes in the SGs. The SGs, along with other tissues, show the activation of factors from the hedgehog, Notch, EGF, dpp, and wingless pathways, as well as cell cycle genes, upon ecdysone exposure. Mutation of the ecdysone receptor abolishes their induction. These data provide insights into the multiple interactions between the 20HE cascade and spatially-acting regulatory networks.

A new approach to understanding how the 20HE gene is controlled was introduced by Davis and Li (2013). Their research showed that the hormone and its receptor can control specific genes in addition to the standard direct target genes. The study used a broad genome analysis that included not only the normal version of the gene but also a temperature-sensitive version and a version without the receptor. When the metamorphosis started, about 12% of the genome responded to the 20HE signal and more than half of these responses were not dependent on the receptor. Interestingly, a significant number of genes regulated by the receptor showed different levels of expression depending on whether the receptor was bound or not. The genes found to respond to 20HE included proteins from many different groups, such as enzymes, proteins involved in breaking down large molecules, post-translational changes, chaperones, immune responses, metabolism, and other cellular processes. These results go beyond the data obtained from traditional methods such as cloning, transgene monitoring, and Northern blotting (Palanker et al. 2006). It was surprising to find that more than 50% of the genes affected by the metamorphic pulse of 20HE do not use the classic receptor pathway meaning they respond in a non-genomic way. Of these, more than 60% were turned down and the rest were turned upregulated.

Although cell lines grown in the lab cannot provide the same complex reactions as living tissues or organs and they are not very helpful for understanding physiological or development processes like puff formation, Sgs-glue production, secretion, granule formation and transport, postpupal vacuolation, and programmed cell death, they still offer some useful insights, especially when combined with strong genomic techniques. Different cell types in

Drosophila show different physical reactions to 20HE. In nature, there are various analogs of 20HE and one of these, ponasterone A (PoA), is the most effective. Using genome-wide microarray analysis, Gonsalves et al. (2011) have found that more genes are regulated by PoA than by 20HE and the gene responses to the two hormones are largely different. They also found that many genes controlled by 20HE in Kc₁₆₇ cells are not controlled in SGs of third-instar larvae and the levels of different EcR isoforms vary between Kc cells and SGs, suggesting a possible reason for the differences in gene responses between the cell types. In several studies, it has been shown that PoA can be harmful to various insect groups, and one reason for this might be that in these insects, PoA regulates a different set of genes compared to 20E.

Stoiber et al. (2016) have mapped the 20HE signaling network in a group of 41 cell lines creating the largest collection of a steroid hormone transcriptional response yet. First, it is important to note that cell lines have different transcription profiles (Cherbas et al. 2011). Over 5,840 genes are constantly expressed in all cell lines, while 1,459 are expressed in just one. The number of transcription factors each cell line has ranges from 406 to 450, and 595 of all the transcription factors in flies are expressed in at least one cell line. The early transcriptional response to 20HE shows how different the cell lines are in terms of their biology and origins. In this early stage, genes respond in specific ways depending on the expression of many transcription factors. A small group of genes is affected regardless of the initial cell state. A total of 1,645 genes showed a significant transcriptional response to 20HE in at least one cell line. About 60 transcription factors were induced by 20HE, and 35 of these were affected in five or fewer cell lines. Some of these are known to respond to ecdysone, while others are newly identified and show new hormone-related pathways. The 100 genes that showed widespread responses included 68 that were turned on and 32 that were turned off in more than half of the cell lines. Five genes, *DHR4*, *DHR46*, *Eip75B*, *CG44004*, and *bip1*, were activated in all 41 cell lines. All five have been previously identified in other genome-wide surveys of 20HE exposure response (Beckstead et al. 2005; Gauhar et al. 2009; Gonsalves et al. 2011; Shlyueva et al. 2014). There are no genes that are consistently turned off across all cell lines, with *fruitless (fru)* being the most common one (turned off in 33 out of 41 cell lines). More broadly, the genes that are widely turned on are significantly linked to biological processes such as metamorphosis, SG cell death, and steroid hormone signaling.

as most of these genes have been previously identified in other studies on 20HE responses.

An accompanying problem with many of these studies is that very few hormone-dependent enhancers have been well characterized and important aspects of their structure, cell-type-specific functions, and the role of their chromatin structure are still unclear. In fact, some of these conclusions could have been drawn from detailed studies in *Sgs-3*, *Sgs-4* and *Pig-1* promoters mentioned earlier. Shlyueva et al. (2014) used STARR-seq along with 20HE signaling in two different *Drosophila* cell lines, S2 and Kc₁₆₇, to identify genome-wide activity of hormone-dependent enhancers. Enhancer activation was found to depend on combinations of cis-regulatory motifs that vary between cell lines and can predict cell-type-specific ecdysone targeting. Activated enhancers are often not accessible before they are induced. Enhancer repression after hormone treatment appears to be independent of receptor motifs and the binding of the receptor to the enhancer, but instead depends on motifs for other factors, including the 20HE-inducible E74. Overall, 20HE-induced enhancers have a unique cis-regulatory signature that is predictive and includes EcR motifs and motifs for partner transcription factors. The signature of enhancer sequences and EcR binding patterns suggests that the repression of many gene targets by 20HE might occur indirectly. The genes responsive to 20HE tend to be organized into directional, same-stranded units. Half of the EcR heterodimer was identified as the main limiting factor in the response, and the initial levels of the receptor isoform influence the group of activated transcription factors.

Synopsis - challenges and future directions

The above discussion reveals how a single steroid hormone-driven physiological process involves distinct cellular actions leading to the secretion of tissue-specific proteins that are components of the extracellular glue matrix. This document highlights the coexistence of detailed information on some aspects of its regulation and numerous missing links that hinder a more comprehensive understanding of the phenomenon. On one hand, we have a relatively large amount of data on cis-regulatory elements in the *Sgs-3*, *Sgs-4* and *Sgs-7/8* genes, but we have very limited data on the hormonal regulation of other *Sgs* genes and no data on additional genes required for the completion and/or support of the associated process, such as numerous post-translational modifications, secretory vesicle formation, vesicle trafficking and

fusion, cargo maturation, membrane targeting and docking, and exocytosis. Even though it is clear that some *Sgs* proteins are heavily O-glycosylated and some are also N-glycosylated, studies on the hormonal control of the transcription of individual components of this glycosylation machinery are still lacking. On the other hand, omics-based studies in individual tissues or even established *Drosophila* cell lines show that the genomic response to ecdysone treatment is far more complex involving hundreds or even thousands of genes that represent a wide range of signaling pathways and various Gene Ontology categories. Although data from cell lines or other tissues cannot be directly applied or transformed to the salivary gland-*Sgs* model system, it is evident that the period of a few hours before pupariation, when SGs respond to hormonal stimuli, cannot be limited to genes responsible for intermolt and early puffs. Although some steps in the mentioned cellular processes – from the activation of *Sgs* gene expression through post-translational modifications, secretory vesicle formation up to exocytosis – could be independent of hormone action or transcriptional control and occur autonomously, many of them require at least some level of coordination and synchronization.

Recently, we published a study on the physico-chemical properties of *Sgs-glue* in *Drosophila* wild-type animals, which revealed the unexpected complexity of factors involved in the successful adhesion of this glue to the substrate (Benó et al. 2024). These factors include triboelectric charge or topography of the substrate, stiffness, hardness, and elasticity of the glue plaque, pH of the expectorated glue, the extreme speed of glue solidification or curing (within seconds), and many others, all of which have biological determinants in the glue components. If the steroid hormone ecdysone acts as a unifying coordinator and/or synchronization factor for this complex process, many more proteins (and their corresponding genes) must be involved than previously anticipated.

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References

- Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, George RA, Lewis SE, Richards S, Ashburner M, Henderson SN, Sutton GG, Wortman JR, Yandell MD, Zhang Q, Chen LX, Brandon RC, Rogers YH, Blazej RG, Champe M, Pfeiffer BD, Wan KH, Doyle C, Baxter EG, Helt G, Nelson CR, Gabor Miklos GL, Abril JF, Agbayani A, An HJ, Andrews-Pfannkoch C, Baldwin D, Ballew RM, Basu A, Baxendale J, Bayraktaroglu L, Beasley EM, Beeson KY, Benos PV, Berman BP, Bhandari D, Bolshakov S, Borkova D, Botchan MR, Bouck J, Brokstein P, Brottier P, Burtis KC, Busam DA, Butler H, Cadieu E, Center A, Chandra I, Cherry JM, Cawley S, Dahlke C, Davenport LB, Davies P, de Pablos B, Delcher A, Deng Z, Mays AD, Dew I, Dietz SM, Dodson K, Doup LE, Downes M, Dugan-Rocha S, Dunkov BC, Dunn P, Durbin KJ, Evangelista CC, Ferraz C, Ferreira S, Fleischmann W, Fosler C, Gabrielian AE, Garg NS, Gelbart WM, Glasser K, Glodek A, Gong F, Gorrell JH, Gu Z, Guan P, Harris M, Harris NL, Harvey D, Heiman TJ, Hernandez JR, Houck J, Hostin D, Houston KA, Howland TJ, Wei MH, Ibegwam C, Jalali M, Kalush F, Karpen GH, Ke Z, Kennison JA, Ketchum KA, Kimmel BE, Kodira CD, Kraft C, Kravitz S, Kulp D, Lai Z, Lasko P, Lei Y, Levitsky AA, Li J, Li Z, Liang Y, Lin X, Liu X, Mattei B, McIntosh TC, McLeod MP, McPherson D, Merkulov G, Milshina NV, Mobarry C, Morris J, Moshrefi A, Mount SM, Moy M, Murphy B, Murphy L, Muzny DM, Nelson DL, Nelson DR, Nelson KA, Nixon K, Nusskern DR, Pacleb JM, Palazzolo M, Pittman GS, Pan S, Pollard J, Puri V, Reese MG, Reinert K, Remington K, Saunders RD, Scheeler F, Shen H, Shue BC, Siden-Kiamos I, Simpson M, Skupski MP, Smith T, Spier E, Spradling AC, Stapleton M, Strong R, Sun E, Svirskas R, Tector C, Turner R, Venter E, Wang AH, Wang X, Wang ZY, Wassarman DA, Weinstock GM, Weissenbach J, Williams SM, Woodage T, Worley KC, Wu D, Yang S, Yao QA, Ye J, Yeh RF, Zaveri JS, Zhan M, Zhang G, Zhao Q, Zheng L, Zheng XH, Zhong FN, Zhong W, Zhou X, Zhu S, Zhu X, Smith HO, Gibbs RA, Myers EW, Rubin GM, Venter JC. The genome sequence of *Drosophila melanogaster*. *Science* 287, 2185–2195, 2000.
- Adams MD, Sutton GG, Smith HO, Myers EW, Venter JC. The independence of our genome assemblies. *Proc Natl Acad Sci USA* 100, 3025–3026, 2003.
- Amero SA, Elgin SC, Beyer AL. A unique zinc finger protein is associated preferentially with active ecdysone-responsive loci in *Drosophila*. *Genes Dev* 5, 188–200, 1991.
- Amero SA, Matunis MJ, Matunis EL, Hockensmith JW, Raychaudhuri G, Beyer AL. A unique ribonucleoprotein complexes assembles preferentially on ecdysone-responsive sites in *Drosophila melanogaster*. *Mol Cell Biol* 13, 5323–5330, 1993.
- Andres AJ, Cherbas P. Tissue-specific ecdysone responses: regulation of the *Drosophila* genes *Eip28/29* and *Eip40* during larval development. *Development* 116, 865–876, 1992.
- Ashburner M. The genetic analysis of puffing in polytene chromosomes of *Drosophila*. *Proc R Soc Lond B Biol Sci* 176, 319–327, 1970.
- Ashburner M. Puffing patterns in *Drosophila melanogaster* and related species. In: *Developmental Studies on Giant Chromosomes*. (Ed. W Beerman), pp. 101–151, Springer-Verlag, Berlin, Heidelberg, New York, 1972.
- Ashburner M, Chihara C, Meltzer P, Richards G. Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harb Symp Quant Biol* 38, 655–662, 1974.
- Ashburner M, Berendes HD. Puffing of polytene chromosomes. In: *The Genetics and Biology of Drosophila* vol 2b (Eds M Ashburner, TRF Wright), pp. 315–395, Academic Press, London, New York, 1978.
- Ashburner M. Puffs, genes, and hormones revisited. *Cell* 61, 1–3, 1990.
- Bardwell VJ, Treisman R. The POZ domain: A conserved protein-protein interaction motif. *Genes Dev* 8, 1664–1677, 1994.
- Bayer C, von Kalm L, Fristrom JW. Relationship between protein isoforms and genetic functions demonstrate functional redundancy at the *Broad-Complex* during *Drosophila* metamorphosis. *Dev Biol* 187, 267–282, 1997.
- Becker HJ. Die Puffs der Speicheldrussenchromosomen von *Drosophila melanogaster*. I. Beobachtungen zum Verhalten des Puffmuster im Normalstamm und bei zwei Mutanten, *giant* und *lethal-giant-larvae*. *Chromosoma* 10, 654–678, 1959.
- Becker HJ. Die Puffs der Speicheldrussenchromosomen von *Drosophila melanogaster*. II. Die Auslösung der Puffbildung, ihre Spezifität und ihre beziehung zur Funktion der Rindgruse. *Chromosoma* 13, 341–384, 1962.
- Beckstead RB, Lam G, Thummel CS. The genomic response to 20-hydroxyecdysone at the onset of *Drosophila* metamorphosis. *Genome Biol* 6, R99, 2005.
- Beermann W. Nuclear differentiation and functional morphology of chromosomes. *Cold Spring Harb Symp Quant Biol* 21, 217–232, 1956.

- Beno M, Benova-Liszekova D, Kostic I, Sery M, Mentelova L, Prochazka M, Soltys J, Trusinova L, Ritomsky M, Orovčík L, Jerigo M, Velic D, Machata P, Omastova M, Chase BA, Farkas R. Gross morphology and adhesion-associated physical properties of *Drosophila* larval salivary gland glue secretion. *Sci Rep*, 14, 9779, 2024.
- Benova-Liszekova D, Beno M, Farkas R. Fine infrastructure of released and solidified *Drosophila* larval salivary secretory glue using SEM. *Bioinspir Biomim*, 14, 55002, 2019.
- Bodenstein D. The postembryonic development of *Drosophila*. In: *Biology of Drosophila* (Ed. M Demerec), pp 1–35, Wiley and Sons Inc, New York, 1950.
- Borst DW, Bollenbacher E, O'Connor JD, King DS, Fristrom JW. Ecdysone levels during metamorphosis of *Drosophila melanogaster*. *Dev Biol* 39, 308–316, 1974.
- Bridges CB. Salivary chromosome maps with a key to the banding of the chromosomes of *Drosophila melanogaster*. *J Hered* 26, 60–64, 1935.
- Celniker SE, Rubin GM. The *Drosophila melanogaster* genome. *Annu Rev Genomics Hum Genet* 4, 89–117, 2003.
- Cherbas L, Lee K, Cherbas P. Identification of ecdysone response elements by analysis of the *Drosophila Eip28/29* gene. *Genes Dev* 5, 120–131, 1991.
- Cherbas L, Willingham A, Zhang D, Yang L, Zou Y, Eads BD, Carlson JW, Landolin JM, Kapranov P, Dumais J, Samsonova A, Choi JH, Roberts J, Davis CA, Tang H, van Baren MJ, Ghosh S, Dobin A, Bell K, Lin W, Langton L, Duff MO, Tenney AE, Zaleski C, Brent MR, Hoskins RA, Kaufman TC, Andrews J, Graveley BR, Perrimon N, Celniker SE, Gingeras TR, Cherbas P. The transcriptional diversity of 25 *Drosophila* cell lines. *Genome Res* 21, 301–314, 2011.
- Crowley TE, Mathers PH, Meyerowitz EM. A trans-acting regulatory product necessary for expression of the *Drosophila melanogaster* 68C glue gene cluster. *Cell* 39, 149–156, 1984.
- Da Lage JL, Thomas GWC, Bonneau M, Courtier-Orgogozo V. Evolution of salivary glue genes in *Drosophila* species. *BMC Evol Biol* 19, 36, 2019.
- Davis MB, Li T. Genomic analysis of the ecdysone steroid signal at metamorphosis onset using *ecdysoneless* and *EcRnull* *Drosophila melanogaster* mutants. *Genes Genomics* 35, 21–46, 2013.
- DiBello PR, Withers DA, Bayer CA, Fristrom JW, Guild GM. The *Drosophila* Broad-Complex encodes a family of related proteins containing zinc fingers. *Genetics* 129, 385–397, 1991.
- Emery IF, Bedian V, Guild GM. Differential expression of *Broad-Complex* transcription factors may forecast distinct developmental tissue fates during *Drosophila* metamorphosis. *Development* 120, 3275–3287, 1994.
- Farkas R, Knopp J. Ecdysone-modulated response of *Drosophila* cytosolic malate dehydrogenase to juvenile hormone. *Arch Insect Biochem Physiol* 35, 71–83, 1997.
- Farkas R, Knopp J. Genetic and hormonal control of cytosolic malate dehydrogenase activity in *Drosophila melanogaster*. *Gen Physiol Biophys* 17, 37–50, 1998.
- Farkas R, Sutakova G. Ultrastructural changes of *Drosophila* larval and prepupal salivary glands cultured *in vitro* with ecdysone. *In Vitro Cell Dev Biol Anim* 34, 813–823, 1998.
- Farkas R, Sutakova G. Developmental regulation of granule size and numbers in larval salivary glands of *Drosophila* by steroid hormone ecdysone. *Cell Biol Int* 23, 671–676, 1999.
- Farkas R, Danis P, Medvedova L, Mechler BM, Knopp J. Regulation of cytosolic malate dehydrogenase by juvenile hormone in *Drosophila melanogaster*. *Cell Biochem Biophys* 37, 37–52, 2002.
- Farkas R, Kucharova-Mahmood S, Mentelova L, Juda P, Raska I, Mechler BM. Cytoskeletal proteins regulate chromatin access of BR-C transcription factor and Rpd3- Sin3A histone deacetylase complex in *Drosophila* salivary glands. *Nucleus* 2, 489–499, 2011.
- Farkas R. The complex secretions of the salivary glands of *Drosophila melanogaster*, a model system. In: *Extracellular composite matrices in Arthropods* (Ed. E Cohen, B Moussian), pp 557–599, Springer International Publishing, Switzerland, 2016.
- Fraenkel G, Brookes VJ. The process by which the puparia of many species of flies become fixed to a substrate. *Biol Bull Mar Lab Woods Hole* 105, 442–449, 1953.
- Garen A, Kauver L, Lepesant JA. Roles of ecdysone in *Drosophila* development. *Proc Natl Acad Sci USA* 74, 5099–5103, 1977.
- Gauhar Z, Sun LV, Hua S, Mason CE, Fuchs F, Li TR, Boutros M, Kevin P. White KP. Genomic mapping of binding regions for the Ecdysone receptor protein complex. *Genome Res* 19, 1006–1013, 2009.
- Georgel P, Romain P, Giangrande A, Dretzen G, Richards G, Bellard M. Sgs-3 chromatin structure and trans-activators: developmental and ecdysone induction of a glue enhancer-binding factor, GEBF-I, in *Drosophila* larvae. *Mol Cell Biol* 11, 523–532, 1991.

- Giangrande A, Mettling C, Richards GP. *Sgs-3* transcript levels are determined by multiple remote sequence elements. *EMBO J* 6, 3079–3084, 1987.
- Gonsalves SE, Neal SJ, Kehoe AS, Westwood JT. Genome-wide examination of the transcriptional response to ecdysteroids 20-hydroxyecdysone and ponasterone A in *Drosophila melanogaster*. *BMC Genomics* 12, 475, 2011.
- Guay PS, Guild GM. The ecdysone-induced puffing cascade in *Drosophila* salivary glands: a Broad-Complex early gene regulates intermolt and late gene transcription. *Genetics* 129, 169–175, 1991.
- Guild M, Shore EM. Larval salivary gland secretion proteins in *Drosophila*. Identification and characterization of the *Sgs-5* structural gene. *J Mol Biol* 179, 289–314, 1984.
- Hansson L, Lambertsson A. The role of *su(f)* gene function and ecdysterone in transcription of glue polypeptide mRNAs in *Drosophila melanogaster*. *Mol Gen Genet* 192, 395–401, 1983.
- Hamann S, Stratling WH. Specific binding of *Drosophila* nuclear protein PEP (protein on ecdysone puffs) to hsp70 DNA and RNA. *Nucleic Acids Res* 26, 4108–4115, 1998.
- Ji S, Samara NL, Revoredo L, Zhang L, Tran DT, Muirhead K, Tabak LA, Ten Hagen KG. A molecular switch orchestrates enzyme specificity and secretory granule morphology. *Nat Commun* 9, 3508, 2018.
- Jongens TA, Fowler T, Shermoen WA, Beckendorf SK. Functional redundancy in the tissue-specific enhancer of the *Drosophila Sgs-4* gene. *EMBO J* 7, 2559–2567, 1988.
- Kamalesh K, Segal D, Avinoam O, Schejter ED, Shilo BZ. Structured RhoGEF recruitment drives myosin II organization on large exocytic vesicles. *J Cell Sci* 137, jcs261944, 2024.
- Karim FD, Guild GM, Thummel CS. The *Drosophila* Broad-Complex plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis. *Development* 118, 977–988, 1993.
- Kim JH, Shoemaker AM, Hutchings KA, Shinde S, Andrew DJ. The *Drosophila* larval salivary gland, a simple and elegant model system to understand secretory organ development and function. *Curr Opin Insect Sci* 73, 101435, 2026.
- King-Jones, Thummel CS. Nuclear receptors - a perspective from *Drosophila*. *Nature Rev Genet* 6, 311–323, 2005.
- Klose W, Gateff E, Emmerich H, Beikirch H. Developmental studies on two ecdysone deficient mutants of *Drosophila melanogaster*. *Roux's Arch Devel Biol* 189, 57–67, 1980.
- Kolesnikov NN, Zhimulev IF. Synthesis of mucoprotein secretory substance in salivary glands of the *Drosophila melanogaster* 3rd instar larvae. *Ontogenez* 6, 171–182, 1975.
- Korge G. Chromosome puff activity and protein synthesis in larval salivary glands of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* 72, 4550–4554, 1975.
- Korge G. Larval saliva in *Drosophila melanogaster*: production, composition, and relationship to chromosome puffs. *Dev Biol* 58, 339–355, 1977a.
- Korge G. Direct correlation between a chromosome puff and the synthesis of a larval saliva protein in *Drosophila melanogaster*. *Chromosoma* 62, 155–174, 1977b.
- Kucharova-Mahmood S, Raska I, Mechler BM, Farkas R. Temporal regulation of *Drosophila* salivary gland degeneration by the Broad-Complex transcription factors. *J Struct Biol* 140, 67–78, 2002.
- Lane NJ, Carter YR, Ashburner M. Puffs and salivary gland function: the fine structure of the larval and prepupal salivary glands of *Drosophila melanogaster*. *Wilhelm Roux's Arch* 169, 216–238, 1972.
- Lefevre G. A photographic representation and interpretation of the polytene chromosomes of *Drosophila melanogaster* salivary glands. In: *The Genetics and Biology of Drosophila* (Eds. M Ashburner, E Novitski), pp. 31–66, Academic Press, London, New York, 1976.
- Lehmann M, Korge G. Ecdysone regulation of the *Drosophila Sgs-4* gene is mediated by the synergistic action of ecdysone receptor and SEBP 3. *EMBO J* 14, 716–726, 1995.
- Lehmann M. *Drosophila Sgs* genes: stage and tissue specificity of hormone responsiveness. *BioEssays* 18, 47–54, 1996.
- Lehmann M, Korge G. The fork head product directly specifies the tissue-specific hormone responsiveness of the *Drosophila Sgs-4* gene. *EMBO J* 15, 4825–4834, 1996.
- Li TR, White KP. Tissue-specific gene expression and ecdysone-regulated genomic networks in *Drosophila*. *Dev Cell* 5, 59–72, 2003.
- Lindsley DL, Zimm GG. *The Genome of Drosophila melanogaster*. Academic Press, San Diego, California, 1992.
- Loganathan R, Kim JH, Wells MB, Andrew DJ. Secrets of secretion-How studies of the *Drosophila* salivary gland have informed our understanding of the cellular networks underlying secretory organ form and function. *Curr Top Dev Biol* 143, 1–36, 2021.
- Martin M, Giangrande A, Ruiz C, Richards G. Induction and repression of the *Drosophila Sgs-3* glue gene are mediated by distinct sequences in the proximal promoter. *EMBO J* 8, 561–568, 1989.

- Mayfield JE, Serunian LA, Silver LM, Elgin SC. A protein released by DNAase I digestion of drosophila nuclei is preferentially associated with puffs. *Cell* 14, 539–544, 1978.
- McNabb SL, Beckendorf SK. Cis-acting sequences which regulate expression of the Sgs-4 glue protein gene of *Drosophila*. *EMBO J* 5, 2331–2340, 1986.
- Meyerowitz EM, Hogness DS. Molecular organization of a *Drosophila* puff site that responds to ecdysone. *Cell* 28, 165–176, 1982.
- Meyerowitz EM, Raghavan KV, Mathers PH, Roark M. How *Drosophila* larvae make glue: control of Sgs-3 gene expression. *Trends Genet* 3, 288–293, 1987.
- Mougneau E, von Seggern D, Fowler T, Rosenblatt J, Jongens T, Rogers B, Gietzen D, Beckendorf SK. A transcriptional switch between the Pig-1 and Sgs-4 genes of *Drosophila melanogaster*. *Mol Cell Biol* 13, 184–195, 1993.
- Mugat B, Brodu V, Kejzlarova-Lepesant J, Antoniewski C, Bayer CA, Fristrom JW, Lepesant JA. Dynamic expression of Broad-Complex isoforms mediates temporal control of an ecdysteroid target gene at the onset of *Drosophila* metamorphosis. *Dev Biol* 227, 104–117, 2000.
- Muskavitch MA, Hogness DS. Molecular analysis of a gene in a developmentally regulated puff of *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 77, 7362–7366, 1980.
- Muskavitch MA, Hogness DS. An expandable gene that encodes a *Drosophila* glue protein is not expressed in variants lacking remote upstream sequences. *Cell* 29, 1041–1051, 1982.
- Ou QX, Zeng J, Jamanaka N, Brakken-Thal C, O'Connor, MB, King-Jones K. The insect prothoracic gland as a model for steroid hormone biosynthesis and regulation. *Cell Rep* 16, 247–262, 2016.
- Painter TS. A new method for the study of chromosome rearrangements and the plotting of chromosome maps. *Science* 178, 585–586, 1933.
- Palanker L, Necakov AS, Sampson HM, Ni R, Hu C, Thummel CS, Krause HM. Dynamic regulation of *Drosophila* nuclear receptor activity *in vivo*. *Development* 133, 3549–3562, 2006.
- Piacentini L, Fanti L, Berloco M, Perrini B, Pimpinelli S. Heterochromatin protein 1 (HP1) is associated with induced gene expression in *Drosophila* euchromatin. *J Cell Biol* 161, 707–714, 2003.
- Poels CL, de Loof A, Berendes HD. Functional and structural changes in *Drosophila* salivary gland cells triggered by ecdysterone. *J Insect Physiol* 17, 1717–1729, 1971.
- Ramain P, Bourouis M, Dretzen G, Richards G, Sobkowiak A, Bellard M. Changes in the chromatin structure of *Drosophila* glue genes accompany developmental cessation of transcription in wild type and transformed strains. *Cell* 45, 545–553, 1986.
- Ramain P, Giangrande A, Richards G, Bellard M. Analysis of a DNase I-hypersensitive site in transgenic *Drosophila* reveals a key regulatory element of Sgs3. *Proc Natl Acad Sci U S A* 85, 2718–2722, 1988.
- Restifo LL, Guild GM. An ecdysterone-responsive puff site in *Drosophila* contains a cluster of seven differentially regulated genes. *J Mol Biol* 188, 517–528, 1986.
- Richards GP. The *in vitro* induction of puffing in salivary glands of the mutant *l(2)gl* of *Drosophila melanogaster* by ecdysone. *Wilhelm Roux's Arch* 179, 339–348, 1976.
- Riddiford LM. Hormones and *Drosophila* development. In: *The Development of Drosophila melanogaster*, vol. 2 (Eds. M Bate, A Martinez-Arias), pp. 899–939, Cold Spring Harbor Press, New York, 1993.
- Roark M, Raghavan KV, Todo T, Mayeda CA, Meyerowitz EM. Cooperative enhancement at the *Drosophila* Sgs-3 locus. *Dev Biol* 139, 121–133, 1990.
- Roth GE, Wattler S, Bornschein H, Lehmann M, Korge G. Structure and regulation of the salivary gland secretion protein gene *Sgs-1* of *Drosophila melanogaster*. *Genetics* 153, 753–762, 1999.
- Rousso T, Schejter ED, Shilo BZ. Orchestrated content release from *Drosophila* glue-protein vesicles by a contractile actomyosin network. *Nat Cell Biol* 18, 181–190, 2016.
- Shermoen AW, Beckendorf SK. A complex of interacting DNAase I-hypersensitive sites near the *Drosophila* glue protein gene, Sgs4. *Cell* 29, 601–607, 1982.
- Shlyueva D, Stelzer C, Gerlach D, Yanez-Cuna JO, Rath M, Boryn LM, Arnold CD, Stark A. Hormone-responsive enhancer-activity maps reveal predictive motifs, indirect repression, and targeting of closed chromatin. *Molec Cell* 54, 180–192, 2014.
- Skaer H. The alimentary canal. In: *The Development of Drosophila melanogaster*, vol. 2 (Eds. M Bate, A Martinez-Arias), pp. 941–1012, Cold Spring Harbor Press, New York, 1993.
- Snyder RD, Smith PD. The supressor of forked mutation in *Drosophila melanogaster*: interaction with lozenge gene. *Biochem Genet* 14, 611–617, 1976.
- Sorsa V. *Chromosome Maps of Drosophila melanogaster*. CRC Press Inc, Boca Raton, Florida, 1989.

- Stark A, Lin MF, Kheradpour P, Pedersen JS, Parts L, Carlson JW, Crosby MA, Rasmussen MD, Roy S, Deoras AN, Ruby JG, Brennecke J, Harvard FlyBase curators; Berkeley Drosophila Genome Project; Emily Hodges, Angie S Hinrichs, Anat Caspi, Benedict Paten, Seung-Won Park, Mira V Han, Morgan L Maeder, Benjamin J Polansky, Bryanne E Robson, Stein Aerts, Jacques van Helden, Bassem Hassan, Donald G Gilbert, Deborah A Eastman, Michael Rice, Michael Weir, Matthew W Hahn, Yongkyu Park, Colin N Dewey, Lior Pachter, W James Kent, David Haussler, Eric C Lai, David P Bartel, Gregory J Hannon, Thomas C Kaufman, Michael B Eisen, Andrew G Clark, Douglas Smith, Celniker SE, Gelbart WM, Kellis M. Discovery of functional elements in 12 *Drosophila* genomes using evolutionary signatures. *Nature* 450, 219–232, 2007.
- Stoiber M, Celniker S, Cherbas L, Brown B, Cherbas P. Diverse hormone response networks in 41 independent *Drosophila* cell lines. *G3 (Bethesda)* 6, 683–694, 2016.
- Syed ZA, Zhang L, Tran DT, Bleck CKE, Ten Hagen KG. Regulated restructuring of mucins during secretory granule maturation in vivo. *Proc Natl Acad Sci USA* 119, e2209750119, 2022.
- Ten Hagen KG, Tran DT, Gerken TA, Stein DS, Zhang Z. Functional characterization and expression analysis of members of the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase family from *Drosophila melanogaster*. *J Biol Chem* 278, 35039–35048, 2003.
- Thomopoulos GN. An ultrastructural and histochemical study of the secretory granules in the salivary gland cells of *Drosophila melanogaster* species subgroup: phylogenetic relationships and evolutionary aspect. *Can J Zool* 65, 950–957, 1987.
- Tian E, Hoffman MP, Ten Hagen KG. O-glycosylation modulates integrin and FGF signalling by influencing the secretion of basement membrane components. *Nat Commun* 3, 869, 2012.
- Todo T, Roark M, Raghavan KV, Mayeda C, Meyerowitz E. Fine-structure mutational analysis of a stage- and tissue-specific promoter element of the *Drosophila* glue gene Sgs-3. *Mol Cell Biol* 10, 5991–6002, 1990.
- von Gaudecker B: Der Strukturwandel der larvalen Speicheldrüse von *Drosophila melanogaster* Ein Beitrag zur Frage nach der steuernden Wirkung aktiver Gene auf das Cytoplasma. *Z Zellforsch* 127, 50–86, 1972.
- von Kalm L, Crossgrove K, Von Seggern D, Guild GM, Beckendorf SK. The *Broad-Complex* directly control a tissue-specific response to the steroid hormone ecdysone at the onset of *Drosophila* metamorphosis. *EMBO J* 13, 3505–3516, 1994.
- White KP, Rifkin SA, Hurban P, Hogness DS. Microarray analysis of *Drosophila* development during metamorphosis. *Science* 286, 2179–2184, 1999.
- Wright LG, Chen T, Thummel CS, Guild GM. Molecular characterization of the 71E late puff in *Drosophila melanogaster* reveals a family of novel genes. *J Mol Biol* 255, 387–400, 1996.
- Xu R, Deng K, Zhu Y, Wu Y, Ren J, Wan M, Zhao S, Wu X, Han M, Zhuang Y, Xu T. A large-scale functional approach to uncover human genes and pathways in *Drosophila*. *Cell Res* 18, 1114–1127, 2008.