Mapping regulatory DNA that governs Drosophila eggshell patterning

by

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ABSTRACT OF THE THESIS

MAPPING REGULATORY DNA DURING DROSPHILA EGGSHELL PATTERNING

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Organogenesis relies on extensive tissue patterning by regulating the expression of genes in a spatiotemporal manner. During oogenesis, the follicle cells, a monolayer of epithelial cells surrounding the developing oocyte, are patterned to drive the formation of the *Drosophila* eggshell; an organ that shelters the developing embryo. While follicle cells' patterning has been vastly documented, the regulatory domains that govern tissue patterning are mostly unknown. To find regulatory domains, we cross-listed 81 genes known to be expressed during oogenesis with the large collection of the Gerald M. Rubin (GMR) lines containing noncoding DNA fragments, and we found 19 common genes. These genes are represented by 223 GMR lines. Of great advantage, all GMR lines are driving the expression of a GAL4 thus providing an opportunity to screen these lines by crossing them to a GFP reporter gene. Of the tested GMR lines, 55 lines, (25%), express GFP during oogenesis, and 18 lines (33%), recapitulate the partial or full endogenous pattern of their corresponding genes. We found that regulatory information is enriched in certain positions of the genes' locus. We also demonstrated the use of the new driver lines to disrupt morphologies of the eggshell and other tissues. Our comprehensive screen identified multiple regulatory DNA fragments that govern eggshell patterning.

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1. INTRODUCTION

Organs are derived from flat layers of epithelial cells. These cells are instructed in a robust manner to form functional organs. This process, known as organogenesis, relies on differential gene expression within the tissues, known as tissue patterning (Dobens and Raftery, 2000, Horne-Badovinoc and Bilder, 2005). *Drosophila* oogenesis, an established model system for studying tissue patterning, is used to understand the dynamics of this process (Horn-Badovinoc and Bilder, 2005, Berg, 2005). Specifically, the follicle cells (FCs), a monolayer of epithelial cells engulfing the developing oocyte, are extensively patterned before forming the future eggshell (Berg, 2005).

The eggshell is an intricate structure surrounding the developing embryo that acts as a barrier between the embryo and its environment (Hinton, 1969). During oogenesis, the egg chamber, the precursor to the eggshell, undergoes fourteen morphologically defined developmental stages before maturity (King, 1970, Spradling, 1993) (Fig 1A). Oogenesis occurs within the *Drosophila* ovary. Each ovary has 14-16 ovarioles, with a production line of developing egg chambers (Cavaliere *et al*., 2008). Each ovariole houses the germarium, consisting of somatic and germline cells, and a proximal region consisting of egg chambers that are developing to maturity (Dobens and Raftery, 2000, Cavaliere *et al.*, 2008). There are 16 germline cells in each egg chamber; one of which, the posterior-most, will become the oocyte. The remaining 15 nurse cells support the oocyte as it continues to grow throughout development. Around mid-oogenesis, the egg chamber possesses distinct cell types including the stretch cells (SC), nurse cells (NC), and FCs (Fig 1A) (Dobens and Raftery, 2000). Throughout this study we will be looking at all of the developmental stages of oogenesis. A great advantage of the system is that all stages of development are present within each ovary collected, thus allowing us to easily observe patterning dynamics in in a single vial.

During egg development, the FCs receive signals from morphogens and consequently express genes (Berg, 2005). Two main signaling pathways, the epidermal growth factor receptor (EGFR) and bone morphogenetic protein (BMP), pattern the FCs along the axes of the egg chamber (Nueman-Silberberg and Schupbach, 1993, Twombly *et al*, 1996, Peri and Roth, 2000, Berg, 2005). Signaling through EGFR begins when the transforming growth factor α (TGF-α)-like ligand, Gurken (Grk), is secreted from around the oocyte nucleus and and activates a uniformly expressed EGF receptor in the overlying FCs (Ray and Schupbach, 1996, Berg, 2005). Grk is required to establish the anterior-posterior axis during early oogenesis when the oocyte is positioned at the posterior of the egg chamber (González-Reyes et al., 1995, Ray and Schupbach, 1996, Peri and Roth, 2000). During mid-oogenesis, Grk will establish dorsal follicle cell fates when the oocyte nucleus becomes positioned to the dorsal-anterior of the oocyte (Nueman-Silberberg and Schupbach, 1993, González-Reyes et al., 1995, Berg, 2005). Decapentaplegic (dpp) is the Drosophila ortholog of the mammalian BMP 2/4 ligand. BMP signaling begins when dpp is secreted from the anterior SCs and centripetally migrating FCs (Berg, 2005, Dobens and Raftery, 2000). Dpp interacts with the type-I receptor, *thickveins* (*tkv*) and type II receptor *wishful thinking* (*wit*) establishing an anterior-posterior gradient (Twombly *et al.*, 1996, Dobens and Raftery, 2000. Lembong *et al.*, 2008, Yakoby *et al*., 2008b, Marmion *et al*., 2012).

Signaling pathways activate genes through transcription factor (TF) interacting with *cis*-regulatory modules on the DNA (for review, Levine and Tjian, 2003). These modules are enhancer regions that interact with different TFs and consequently govern spatiotemporal expression of genes (Bonn and Furlong, 2008). During oogenesis, gene expression is governed by interactions between the two signaling pathways (Yakoby et al., 2008, Yakoby et al., 2008b, Berg, 2005). In the EGF signaling pathway, Grk binds to the DER receptor,

torpedo, activating the RAS-RAF-MAPK signaling cascade, which induces dorsoventral polarity and activates a second cascade involving three EGFR ligands (Brand and Perrimon, 1994, Berg, 2005). The BMP ligand, dpp, binds to the type I-type-II receptor complex and subsequently triggers the type II kinase to phosphorylate the type I receptor (Shi and Massaqué, 2003, Parker *et al*., 2003). The phosphorylated *tkv* phosphorylates the R-Smad, *mothers against dpp* (*mad*), activating the P-Mad-Med transcription factor complex (Shi and Massaqué, 2003, Parker *et al*., 2003). While it is known that tissue patterning is governed by the two pathways; the underlying molecular mechanism is mostly unknown.

Previously, the expression dynamics of 81 genes in the FCs of *Drosophila melanogaster* were documented (Yakoby *et al.,* 2008). The complete collection of gene expression patterns during several stages of oogenesis exceeds 250. To reduce the complexity of this dataset, a combinatorial code was developed to describe the assembly of complex expression patterns (Yakoby *et al.,* 2008). The code was able to describe complex gene patterns by using six primitive domains. The primitives are Dorsal (D), which reflects the shape of the intermediate levels of EGFR activation. Midline (M) reflects the high levels of EGFR activation. Anterior (A), reflects the anterior domain of BMP signaling. Roof (R) and Floor (F) reflect two groups of cells that fold into the two dorsal appendages. Uniform (U) applies to genes that are expressed throughout the FCs. Using this code, the authors successfully described the complete collection of gene patterns during oogenesis.

The code takes into consideration the signaling inputs that regulate the formation of functional domains. For instance, the dorsal appendages (DAs) are derived as the roof and floor cellular groups (Fig 1B). Roof (R) is marked by the zinc-finger transcription factor, Broad (BR). BR is expressed in two dorsolateral patches that form the roof of the dorsal appendages (Fig 1C) (Deng and Bownes, 1998, Ward and Berg , 2005, Niepielko *et al.,*

2012). Rhomboid (Rho), a protease in the EGFR pathway, is expressed in two "L-shaped" stripes adjacent to the BR domain. These cells form the future bottom of the dorsal appendages, known as the floor cells (Ruohola-Baker *et al*., 1993). While the code successfully described all expressed patterns in the FCs so far; the regulatory mechanisms from which these patterns are derived remain largely unknown.

To identify the mechanisms governing tissue patterning, we took advantage of a library of DNA fragments that were generated by the Rubin Lab from non-regulatory DNA, and thus potentially contain gene regulatory domains (Pfeiffer *et al.*, 2008). Specifically, the Gerald M. Rubin (GMR) collection is comprised of fly stocks containing fragments of flanking intronic and noncoding DNA sequences from genes expressed in the fly brain (Fig. 2A). These lines are a part of a collective library that allow for a potential regulatory fragment to express a reporter gene that identifies the spatial domains controlled by a particular enhancer. The GMR fragments are inserted into the genome locus at a specific *attP2* landing site in PhiC31recombinase (Pfeiffer *et al*., 2008, Manning *et al.*, 2012) (Fig. 2B). The GMR lines control the expression of a GAL4 transcriptional activator that will be used to identify transcription binding sites. Using the GAL4/UAS system (Brand and Perrimon, 1993, Duffy, 2002) (Fig. 3) we aim to drive the expression of a reporter gene, GFP, to detect the spatial and temporal regions controlled by these fragments using immunohistochemistry.

The dynamics of the 6650 GAL4 lines have been documented in the fly brain, embryo, and third instar imaginal discs and are available to the public online (Jenett *et al.*, 2012, Manning *et al*., 2012, and Jory *et al.*, 2012). There are no reports of these lines during oogenesis. The lines screened in other tissues were successfully able to show enhancer

activity, with only 10% not showing any enhancer activity in the fly brain, embryo, or third instar larva (Jenett *et al.*, 2012, Manning *et al*., 2012, and Jory *et al*., 2012).

We used the collection of flies consisting of 925 genes that were initially developed to study CRM expression in the fly brain (Pfeiffer *et al.*, 2008). To focus our screen we cross listed these 925 genes to the 81 genes expressed during oogenesis (Yakoby *et al*., 2008). There are 19 common genes that are represented by 223 lines with potential regulatory DNA fragments (Fig. 4). Since we already know the patterns and dynamics of these 19 genes, we used these flies to identify regulatory regions that correspond to the endogenous gene pattern. Using the selected GMR-GAL4 lines, we developed a reliable screen to determine their spatial and temporal regulation. Our data provides the tools to ask fundamental questions about gene regulation. Since the sequence of all fragments is known, our activities identified regulatory DNA sequences that control gene expression. Furthermore, we analyzed the distribution of the regulatory fragments to determine whether regulatory domains are enriched in the location of the respective genes. This analysis provides a useful reference to the location of potential regulatory domains within the genes' model for genes not included in this analysis. These lines drive the expression of the transcription factor GAL4, thus, these lines can be used as a valuable resource to manipulate genes using the GAL4-UAS system.

2. MATERIALS AND METHODS

Fly stocks

The GMR fly stocks were obtained through Bloomington *Drosophila* stock center, Indiana University. All GMR lines were generated at the Gerald M. Rubin lab as described (Pfeiffer *et al*., 2008). Select stocks used as listed (see attached). Flies were grown on cornmeal agar. Crosses were performed at 23°C. Crosses were performed using GAL4/UAS system (Duffy, 2002). All GAL4 fly lines were crossed to P{UAS-Stinger}GFP:NLS (Barolo *et al.*, 2000) females to detect enhancers. Addition fly stocks used in this study are UAS-λtop-4.2 (Queenan *et al.*, 1997), UAS-Dad (Tsuneizumi K, et al., 1997) and UAS-dpp (a gift from T. Schüpbach).

Immunofluorescence and Microscopy

Immunoassays were performed as previously described (Yakoby *et al.*, 2008b). Specifically, flies 3-7 days old were put on yeast and dissected into ice cold Grace's insect medium, fixed in 4% paraformaldehyde, washed several times, permeabilized (PBS and 1% Triton X-100), and blocked for 1 hour (0.2% Triton X-100 and 1% BSA). Ovaries were then incubated over night at 4°C with primary antibody diluted in PBST solution (0.2% Triton X-100). After washing three times with PBST (0.2% Triton X-100), ovaries were again incubated in secondary antibodies for 1h at room temperature in PBST (0.2% Triton, 1% BSA, and DAPI). After incubation ovaries were washed three times and mounted in Flouromount-G from Southern Biotech. Primary antibodies used were sheep anti-GFP (1:5000, Biogenesis) and mouse anti-broad (1:400, Hybridoma Bank). Secondary antibodies used were: Alexa Fluor (1:2000, Molecular Probes). Nuclear staining was performed using DAPI (1:10000). The pattern of BR was used as a spatial reference to characterize the dorsal side of the egg

chamber. An internal positive control was added to each immunohistochemistry performed. To do this, we used the rho38A01 (Fig S11) line that is robustly expressed. We added to ovaries to each dissecting tube. All immunoflourescent images were captured with a Leica DM3000 Compound Microscope.

SEM Imaging

Eggshells for SEM imaging were collected from agar plates and mounted onto standard SEM stubs using double-sided carbon tape. Next, eggs were coated with gold palladium for 60s. Scanning electron microscopy was conducted with a Leo 1450EP. Images were processed ImageJ (Rasband, 1997-2009).

RNA-seq analysis

Proper isoforms of each of the 19 genes were identified using RNA-seq analysis. The analysis was performed at the core facility at Princeton University. Three samples were analyzed; the first sample was for developmental stages younger than stage 9, the second sample was for stages 10A and 10B, and the third sample from stages larger than 11. The samples were sequenced for 72 cycles on flowcell H06T7ADXX within run 130131_SN387_0264_AH06T7ADXX using a SN387 TruSeq Rapid SBS Kit and run using an Illumina HiSeq 2000. RNA-seq data was viewed using IGV software (Thorvaldsdóttir *et al.*, 2012 and Robinson *et al.*, 2011).

Mapping distribution of GAL4 fragments

Fragments of DNA were divided into three bins: those upstream of the transcription start site (TSS) were categorized into Bin1, fragments within the first intron were added to Bin2, and those downstream of the second exon were added to Bin3. Introns shorter than 300 bp were

not included in this analysis due to not having any GMR lines as described in (Pfeiffer *et al*., 2008). Statistical analysis was performed using a Chi-square test.

3. RESULTS

i. Screening Gerald M. Rubin (GMR) GAL4 fly lines for regulatory DNA sequences.

The purpose of this screen was to identify DNA fragments that contain regulatory sequences that guide tissue patterning. The GMR GAL4 construct lines drive the expression of the transcription factor, GAL4, providing the opportunity to use a reporter UAS-GFP line to monitor their ability to drive GFP expression. We aimed to find the spatial and temporal information coded by these sequences. Enhancer DNA sequences have been shown to span only a few hundred base pairs (Levine and Tjian, 2003). The GAL4 lines have an average size of 3kb ranging between 1kb and 5kb (Pfeiffer *et al.,* 2008). Intergenic regions larger than 4 kb were separated into smaller fragments with an overlap of 500bp to 1500bp (Pfeiffer *et al.,* 2008). Based on the size of the DNA fragment, it is possible that each line contains more than a single *cis*-regulatory module (CRM). Each GAL4 line was crossed to a UAS-GFP:NLS (nuclear localized GFP). We co-stained for the transcription factor, BR, as a spatial reference (Deng and Bownes, 1998, Ward and Berg , 2005, Niepielko *et al.,* 2012). BR is expressed in two dorsolateral patches on either side of the dorsal midline. The co-staining was necessary for determining the relative spatial location of GFP. As a positive control, we added ovaries from a fly line specific to the border cells (Fig. S11) to each tube to be sure that the immunoassay was successful.

Out of 223 GAL4 lines; 55 of them expressed GFP (Fig. 4). Of these 55 lines, 18 lines (S1-11 for all 18 lines) showed GFP expression related to their endogenous gene (Fig. 5A-E, S1-11) The two drivers, *dad*^{44C10}-GAL4 and *dpp*^{18E05}-GAL4 were both expressed in the centripetally migrating FCs and SCs (Fig. 5B,B', E, E'). We were able to predict the anterior expression of the Dad^{44C10}-GAL4 driver based on the conserved SMAD binding site

(Weiss *et al*., 2010). Two drivers of the dad gene locus expressed partial anterior patterns in the SC and BC (Fig. S3D,E) Interestingly, the *dpp*^{18E05}-GAL4 driver was also expressed in the first intron (Fig. 5I). The *in situ* hybridization image of a S9 egg chamber stained for *br* shows a uniform distribution (Fig.D.). The *in situ* hybridization of late *br* shows the two dorsolateral Roof patches (Fig.C). The two regulatory fragments for *broad* were able to recapitulate the uniform and roof patterns of *broad* (br), respectively (Fig. C',D') (Deng and Bownes, 1997, Tzolovsky *et al*., 1999). The two dorsolateral Roof patches that are expressed by the regulatory fragment within the first intron recapitulate the expression of late *br*. This is in agreement with the late enhancer of *br* (Fuchs *et al*., 2012). The uniform pattern expressed by Br^{69B10}-GAL4 reflects the uniform pattern of *br* expressed in earlier stages of oogenesis shown in the *in situ* hybridization image (Fig.5D, D').

ii. Mapping the distribution of enhancer fragments

The gene fragment lines tested span the length of the entire gene in overlapping fragments, which provided the opportunity to map the fragments that drive GFP expression to the genes' model. We aimed to identify whether these fragments favor any location in the genes' locus. Under the assumption that the distribution of the GAL4 lines is random, we mapped all 223 fragments to their corresponding genes. The fragments were placed into three categories based on the relative position in the gene model (Fig. 6A). All DNA fragments that are upstream of the first exon were classified as Proximal (Bin 1). All fragments that were downstream of the first exon and within the first intron were placed in Bin 2, and all other downstream fragments were placed in Bin 3. Additional characterized enhancers such as: *rhomboid* (Nakamura *et al.,* 2007), *wit* (Marmion *et al*., 2012), *Vm32E* (Andrenacci *et al*., 2000), *pipe* (Technau *et al*., 2011, Fuchs *et al*., 2012), and the early *br* enhancer (Fuchs *et al.*, 2012) were included in our analysis.

One problem that we encountered with this analysis was that certain genes express multiple isoforms that start from different exons. For example, the *dpp* locus has four predicted isoforms with a different first exon. To find the proper isoform to use for our analysis, we had to run an RNA-seq analysis of RNA samples from the ovary to properly classify the regulatory sequences. Three specimens were collected during different developmental stages (Sample 1: Stages≤9, Sample 2: Stages 10A-B, Sample 3: Stages≥11). The stage-specific analysis enabled us to determine the correct isoforms expressed during oogenesis at the appropriate developmental stages. Our analysis was able to detect exons expressed during oogenesis, which enabled us to identify the correct isoform. For example, we found that *dpp* has only a single isoform expressed during oogenesis (Fig. 6B). Of importance, while the level of transcripts was different among developmental stages, the same isoforms were expressed throughout oogenesis. This procedure eliminated discrepancies between transcripts for 10 of our genes.

After binning; we tested the distribution of the regulatory gene fragments in the genes' model. The null hypothesis is that the distribution of the fragments in the bins would be random. We tested this hypothesis by applying a Chi square test. If the distribution was at random we would expect that the number of lines expressing GFP in each bin to be equal to the overall number of GFP-expressing lines (55) and the number of known lines (6) over the total number of fragments (GMR lines: 223, published lines: 6=229), or 26.64%. The first bin has a total of 117 lines. The calculated expected value of this bin size is 26.64% of 117 (31 lines expected to express GFP). The observed number of GFP expressing lines is 26, or 16% less than the expected value. The second bin, or intron 1, is comprised of 72 gene fragment lines. The expected value of a bin this size is 26.64% of 72 (19 lines). The observed number of GFP expressing lines is 28, or 47% more than the expected value. The

third bin is comprised of 40 lines. The expected expression of GFP was 26.64% of 40, or 11 lines. The observed number of GFP expressing lines is 28, or 36% less than the expected value.

Applying the Chi-square test with two degrees of freedom, we had a *p*-value= 0.038 which allows us to reject the null hypothesis showing that the data is not randomly distributed. Furthermore, we were able to show that the regulatory fragments were enriched in Bin 1, which is the first intron (Fig. 7). Our analysis is in agreement with a STARR-seq analysis that also uncovered enhancer enrichment in the first intron. STARR-seq uses random DNA sequences inserted downstream from a promoter region to self-transcribe if any enhancer activity is present (Arnold *et al.,* 2013). This study detected 55.6% of the enhancers found to be located in introns. Specifically, 37.2% were located within the first intron in *Drosophila* S2 and ovarian somatic cells (OSC) (Arnold *et al.,* 2013). Our analysis is in agreement with the STARR-seq analysis of enhancer activity in the *Drosophila* ovary being enriched in the first intron.

iii. Genetic manipulations using GMR-GAL4 drivers.

The GAL4/UAS system is a universal tool to manipulate genes in *D. melanogaster* (Brand and Perrimon, 1993). Using the identified GAL4-drivers, we were able to ectopically express genes in specific subsets of cells using the GAL4-UAS system. In oogenesis, genetic manipulations are limited due to the restricted availability of GAL4 lines that were expressed in subsets of cells. Most GAL4 lines are expressed uniformly throughout all FCs. Using the posterior driver, pnt^{43H01}-GAL4, we were able to induce ectopic expression of the BMP ligand, *decapentaplegic* (*dpp*), in the posterior (Fig. 7B) as shown by the P-Mad staining in the posterior end. The wild-type egg chamber only shows an anterior band of P-Mad

extending approximately five cells wide at S10B (Fig. 7A) (Yakoby *et al*., 2008b). In addition to regulatory expression in the posterior, this particular driver contains multiple enhancers, especially in the anterior SCs and BCs (Fig. 5A'). Expression of a constitutively active EGF receptor (λ-top, Queenan *et al.*, 1997) using the *pnt*^{43H01}-GAL4 driver allows us to alter cell fates in a very specific subset of cells. The anterior components of this driver cause eggshell deformities such as an enlarged midline that extends beyond the posterior base of the DAs (Fig. 8D, D') compared to the wild type eggshell (Fig. 8C, C') (penetrance=59%). Severe phenotypes affecting the length of the dorsal appendages were also observed (Fig. 8E, E') (penetrance=8%).

The most common drivers used in oogenesis are CY2-GAL4 and GR1-GAL4 (Queenan *et al.*, 1997). These drivers are uniformly expressed throughout the FCs. The Br^{69B10}-GAL4 driver is a new uniform driver that can also be used for genetic perturbations. When overexpressing the BMP inhibitor Dad, the size of the eggshell collar drastically reduced when driven by the uniform Br^{69B10}-GAL4 driver (8F, F'). The collar serves as an anterior-most structure that adjoins the operculum to the ventral eggshell and is controlled by BMP signaling (Twombly *et al.*, 1996, Dobens *et al.*, 2000). During oogenesis, BMP signaling regulates development of the eggshell anterior (Twombly *et al.*, 1996). Development of the collar and anterior-most operculum has been shown to be regulated by BMP signaling (Shravage and Roth, 2007). It is no surprise that reduction of the collar size would be a result of overexpressing an ectopic BMP inhibitor.

iv. Identified GAL4 drivers affect other tissues

Perturbations with these drivers affect tissues other than the fly ovary. Wing phenotypes were observed in numerous GAL4 drivers. The wild type wing possesses five wing veins,

and two crossveins between veins L3 and L4, and also between L4 and L5 (for review, Raftery and Umulis, 2012). The phenotypes in the wing ranged from moderate to severe depending upon the driver. The *Eip75B*^{44D06}-GAL4 driver expressed extra crossvein and vein material when crossed to λ-top (Fig. 10B, S.6C). Over expression of λ-top using the *br*^{69B08}-GAL4 driver (Fig. 5C') caused severe wing defects, including excess vein and wing material (Fig. 10C) compared to the wild type (Fig. 10A). In this F1 generation, we also observed a rough-eyed phenotype as compared to the wild type phenotype of the *br*^{69B08}-GAL4 driver. These results are consistent with results obtained by Queenan *et al.,* 1997, who observed a similar phenotype using a uniform T155-GAL4 driver.

4. DISCUSSION

i. GAL4 drivers express GFP in domains related to the endogenous gene

The goal of screening the GMR lines was to identify the location of the enhancers responsible for the expression patterns of 19 genes known to be expressed during oogenesis. These 19 genes were designed and synthesized from a collection of genes specific to the fly brain. Of the 7000 gene fragments, 95% drove expression in the fly brain (Jenett *et al.*, 2012). The fragments in the imaginal discs expressed 19% expression in the wing, haltere, and eye (Jory *et al*., 2012). The lines that were screened for patterns in the embryo reported GFP expression in 5000 of 5500 lines, or 90% (Manning *et al.*, 2012). Similarly, we selected 19 genes whose patterns and dynamics were known during oogenesis (Yakoby *et al.*, 2008), and found 25% of the related lines to express GFP.

Patterning of the *Drosophila* eggshell is characterized by nonuniform expression of genes. The domains known are related to the six primitive patterns of Dorsal (D), Midline (M), Anterior (A), Uniform (U), Roof (R), and Floor (F). This screen found three of these domains: anterior, uniform, and roof. These 18 lines displayed the endogenous gene- related primitives of uniform, anterior, and roof (Yakoby *et al.,* 2008) and additional patterns were found in the posterior, stretched cells, border cells, and stalk cells (Fig S1-S11).

Screening for the enhancers that govern these patterns gave us new insights to the complexity of these "primitives". We found that the anterior pattern is regulated by many enhancers in full or partial domains (Fig. 10). Anterior patterns were observed in the stretch cells; full or partial, and centripetally migrating cells. The binding sites that regulate these enhancers are not the same either. The SMAD binding site that regulates the anterior pattern of *dad* was not found in the *dpp* fragment (Weiss *et al.,* 2010). Furthermore, *dally* has a SMAD binding

site in two of the GMR lines, 71E04 and 71D04 (Weiss *et al.*, 2010); surprisingly, both of them showed no expression. It is possible that the same mechanism that regulates the anterior of *dally* is not the same mechanism as *dad*.

Pnt, however, is more complex because it has two isoforms that have individual dynamics of gene expression in oogenesis. The first smaller isoform, *pnt-P1*, is expressed in the posterior and midline (Klämbt, 1993, Morimoto *et al*., 1996). We were able to find three enhancers for *pnt* in the posterior, stretched cells and border cells. Two overlapping fragments shared expression in the posterior and border cells (Fig. 5F, S10). The longer of the two fragments also has GFP in the stretch cells (Fig 5F). From this we can deduce that the enhancer regulating the posterior is a smaller overlapping domain within the two fragments.

We did not find the floor and midline enhancers as were expected for *pnt-P1*. This is consistent with all of the 9 genes that were reported to be expressed floor and midline domains (Yakoby *et al.*, 2008). We reason that the fragments were not large enough for the complexity of these domains. Fragments such as midline and floor require both activation and repression. For instance the Floor pattern detected for *rhomboid* requires a repression input from Broad (Yakoby *et al.*, 2008b). *broad* itself expresses a complex pattern that is regulated by two enhancer elements (Fuch *et al.*, 2012). One enhancer is expressed uniformly with repression of the dorsal and midline regions of high EGFR activation (Fuchs *et al.*, 2012). The second enhancer expresses the two dorsolateral roof patches found in a similar domain as the br^{69B08}-GAL4 roof driver (Fig. 5D'). It was found that the dynamic pattern of broad requires multiple inputs from the transcription factors, Capicua (CIC), Mirror (MIRR), and Pointed (PNT) (Fuchs et al., 2012). In addition, the roof pattern of *rho* depends on the BMP, Notch, and Wg signaling pathways (Ward and Berg, 2005, Jordan *et al*., 2005, Ward *et al*, 2006)

ii. Regulatory sequence distribution during oogenesis is not random.

Every gene contains several regulatory enhancers in the flanking untranslated regions and introns (Levine and Tjian, 2003, Pfeiffer et al., 2008). The advantage of this screen is that all fragments driving GFP can be mapped to the genes' model. We found that the distribution of the regulatory enhancers is not random. Specifically, enhancers are enriched within the first intron. Our results are in agreement with Arnold *et al.*, 2013. We next tested whether specific expression domains are also differentially distributed in the genes' model. After testing this hypothesis, we found that the distribution of these DNA fragments appeared to be random across the three bins. However, we only studied 19 of the 81 oogenesis genes. Perhaps a larger pool of regulatory enhancers could be tested to show otherwise.

The RNA-seq analysis enabled us to accurately identify the various isoforms expressed in the selected 19 genes. Since we collected the egg chambers at different developmental stages, we could identify various isoforms at different developmental stages. While the levels of transcripts were different among the developmental stages, the same isoforms were represented throughout oogenesis. RNA-seq analysis is an accurate method for distinguishing differences among multiple isoforms simultaneously (Drewe *et al*., 2013). Thus we conclude that the same repertoire of genes is expressed at different levels and cellular compartments during oogenesis.

iii. GAL4 drivers can be used to manipulate specific subsets of cells.

The GAL4 drivers identified in this study are useful because they are expressed in restricted cellular domains. These drivers are useful tools for the GAL4/UAS system. Until now, very few of such tools have been available for studying oogenesis. Current drivers activate UAS uniformly throughout the FCs (Ward *et al.,* 2002, Queenan *et al.,* 1997). The *dad*^{44C10}-GAL4

and *dpp*^{18E05}-GAL4 are expressed in an anterior domain restricted to the centripetally migrating cells. We also found a driver restricted to the dorsolateral patches (roof domain). These drivers are valuable tools to manipulate subdomains of the FCs (Fig. 8).

The posterior *pnt*^{43H01}-GAL4 driver is expressed in posterior end, stretch cells, and border cells, and thus can manipulate signaling pathways in the posterior and anterior ends. We showed the anterior effects of the driver by expressing the constitutively active EGF receptor, *torpedo* (λtop), expanded the midline of the mutated egg shell and caused defects to the dorsal appendages. The variety of phenotypes could be due to the two anterior components of the *pnt*^{43H01}-GAL4 driver, stretch cells (SC) and border cells (BC). We were able to repeat these phenotypes when expressing λtop and individual drivers specific to the stretch cells or border cells (Fig. S12).

Experimentation with these drivers will give us more new and exciting results. A newly described driver, *dally*^{71D08}-GAL4, shows a limited GFP expression that is scattered over the columnar FCs (S4.C). This driver will be an interesting comparison to the uniform *br*^{69B10}-GAL4 considering that it only affects the columnar follicle cells and does not extend into the anterior stretch cells.

iv. GAL4 expression in many tissues

Our finding of phenotypes in the eye or wing is not surprising, considering that less than 10% of the 6650 GAL4 lines tested did not show expression in the fly brain, embryo, and third instar larva imaginal discs (Jenett *et al.*, 2012, Manning *et al*., 2012, Jory *et al,* 2012). Of the 18 lines recapitulating expression patterns, we found that 13 were present in the brain and 15 were present in the embryo. Only 2 of the 18 lines showed expression of GFP in the imaginal discs according to the public online database (janelia.org). Based on our results, we

know that at least three of the lines show mutant phenotypes in the wing and must therefore have some effect on the imaginal disc tissue.

We also know that these drivers must be expressed in other tissues based on the lethality of certain perturbations. Several crosses to UAS lines were performed to drivers that did not give live progeny or UAS-expressing flies. This lethality can be attributed to 15 of these lines having effects on the embryo (Manning *et al.*, 2012). The function and importance of the protein in the system can be determined by the developmental stage at which the lethality occurred (Berg, 2002).

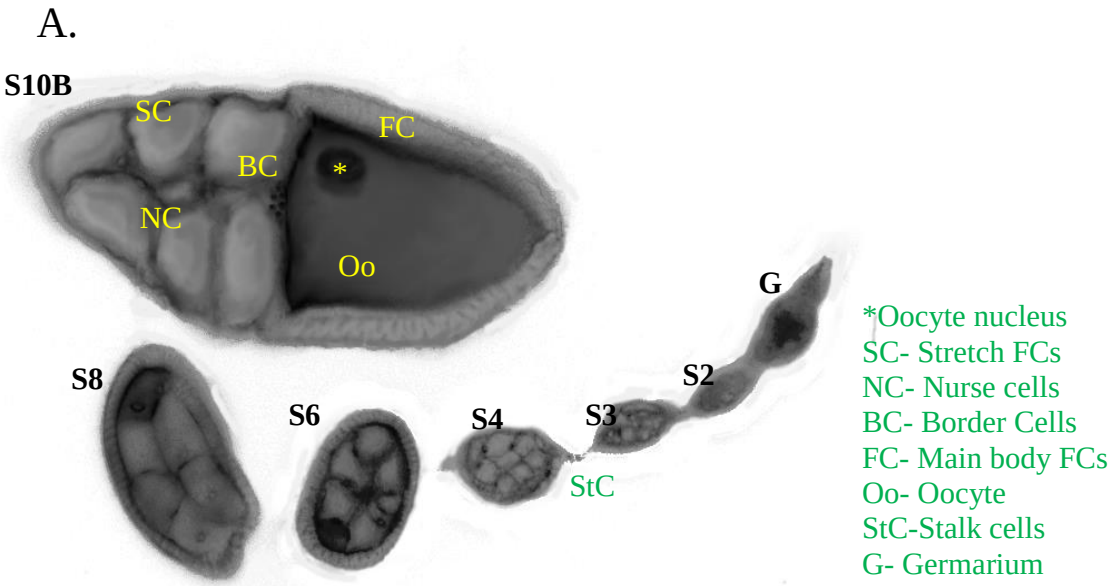
5. FUTURE DIRECTION

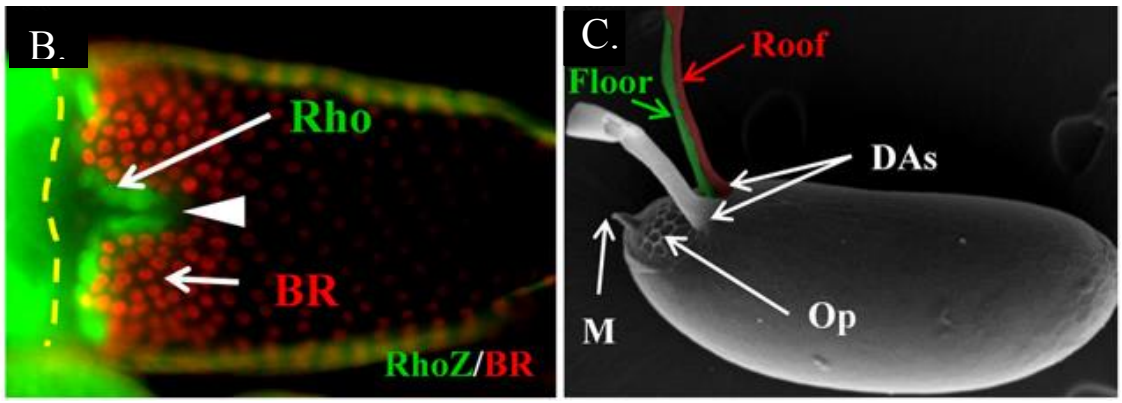
We located several regions of DNA that are controlling gene expression. These fragments focus the search after regulatory domains. The size of each GAL4 fragment ranges between 1kb and 5kb (Pfeiffer *et al.,* 2008). The size of the actual enhancer is expected to be a few hundred base pairs in length (Levine and Tjian, 2003). In the future, it will be interesting to identify the CRMs controlling specific domains. Furthermore, it will be interesting to test whether similar patterns, including the anterior domains, have a similar regulation.

The next step will be to find common transcription factor binding sites within the enhancer domains that have been found. Enrichments for *dpp* activation sites have been described in several anterior genes (Weiss *et al*., 2010). However, the regulatory element that activates *dpp* is still not known. The next goal will be to try identifying transcription factor binding sites by using the enhancers that we found. By doing so, we can try understanding the underlying mechanisms of gene regulation.

6. FIGURES

Fig. 1.



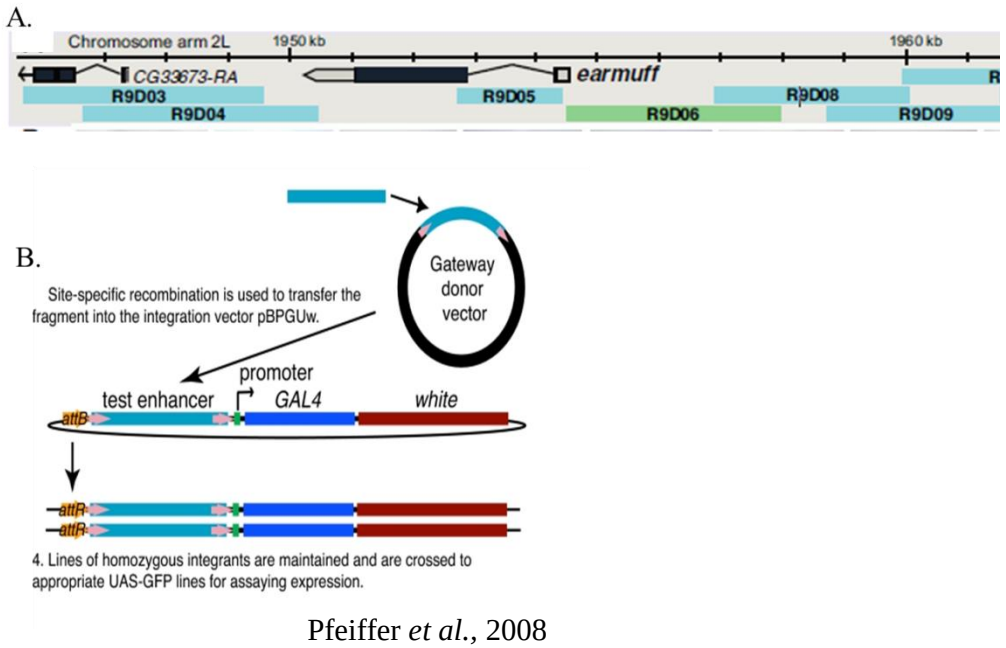


Niepielko *et al.*, 2012

Fig 1.

Drosophila oogenesis and eggshell patterning (A). The egg chamber is the precursor to the mature egg. It has well morphologically defined compartments, including the nurse cells (NC), stretch cells (SC), border cells (BC), oocyte (Oo), and follicle cells (FCs). (B) Two genes mark the future dorsal appendage primordial. Broad (BR) is a zinc-finger transcription factor expressed in two dorsolateral patches on either side of the dorsal midline. These cells will form the roof of the DAs (Deng and Bownes, 1998; Ward and Berg , 2005) marked by red in (C). Rhomboid (Rho) is a protease in the EGFR signaling expressed. It is expressed in two "L-shaped" stripes adjacent to BR cells. These cells will form the floor of the DAs (Ruohola-Baker *et al.*,1993) marked by green in (C). (C) A mature eggshell showing the roof (red) and floor (green) domains of the dorsal appendages. Arrowhead denotes the dorsal midline in B.

Fig. 2.



Pfeiffer *et al.*, 2008

Fig. 2. Synthesis of the GMR-GAL4 fly lines.

GMR-GAL4 lines were constructed from fragments of DNA averaging 3kb (Pfeiffer et al., 2008). The GMR fragments are inserted into the genome locus at a specific *attP2* landing site in PhiC31 recombinase to insert fragment in the same location (Pfeiffer et al., 2008). The test enhancer is placed upstream of a GAL4, which upon activation will synthesize the transcription factor.

Fig. 3.

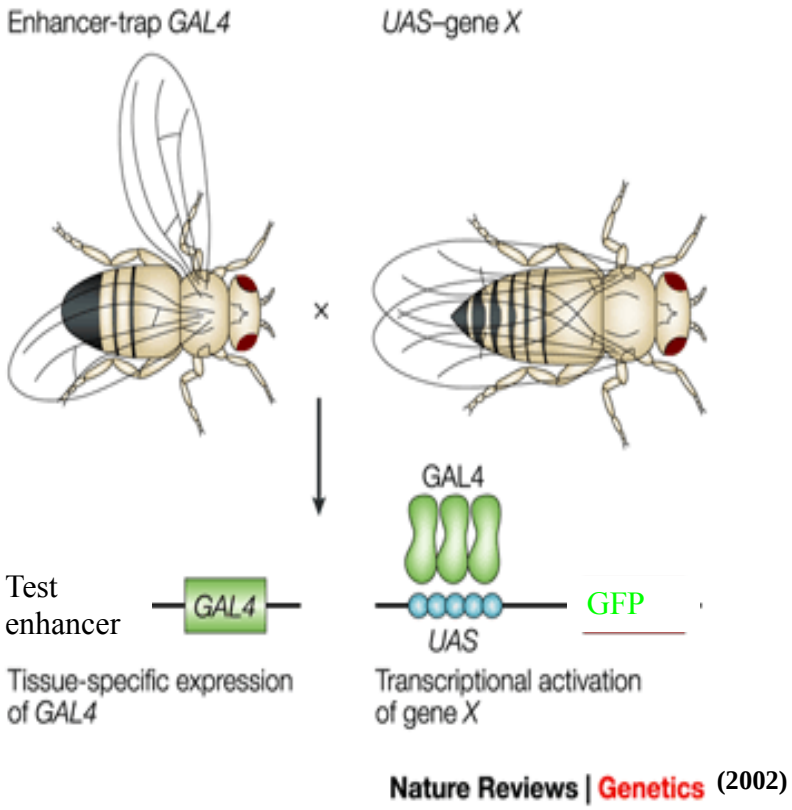


Fig. 3. The GAL4-UAS system.

Each test enhancer is integrated into a GAL4 driving fly line. The GAL4 driver is crossed to a UAS fly. The GAL4 driver is then ectopically express the reporter gene (GFP), or any other gene or RNAi. We can then evaluate the spatial and temporal expression of GFP being activated by the test enhancer.

Fig. 4.

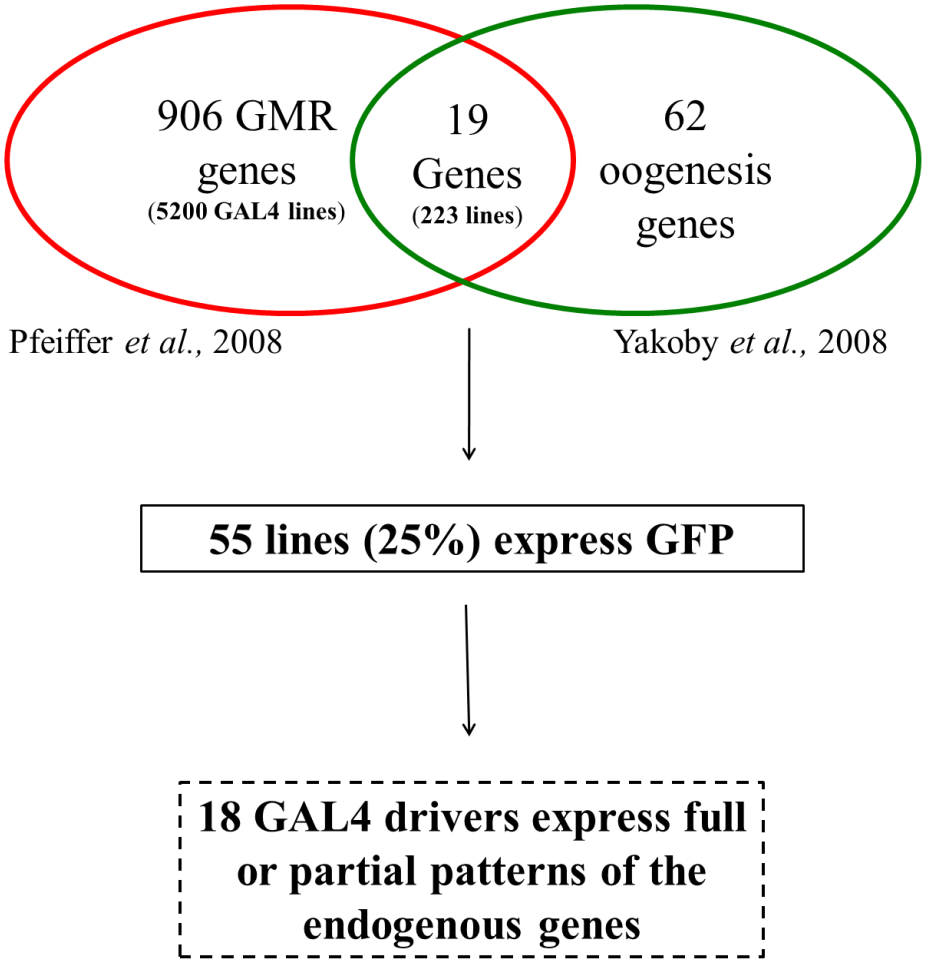
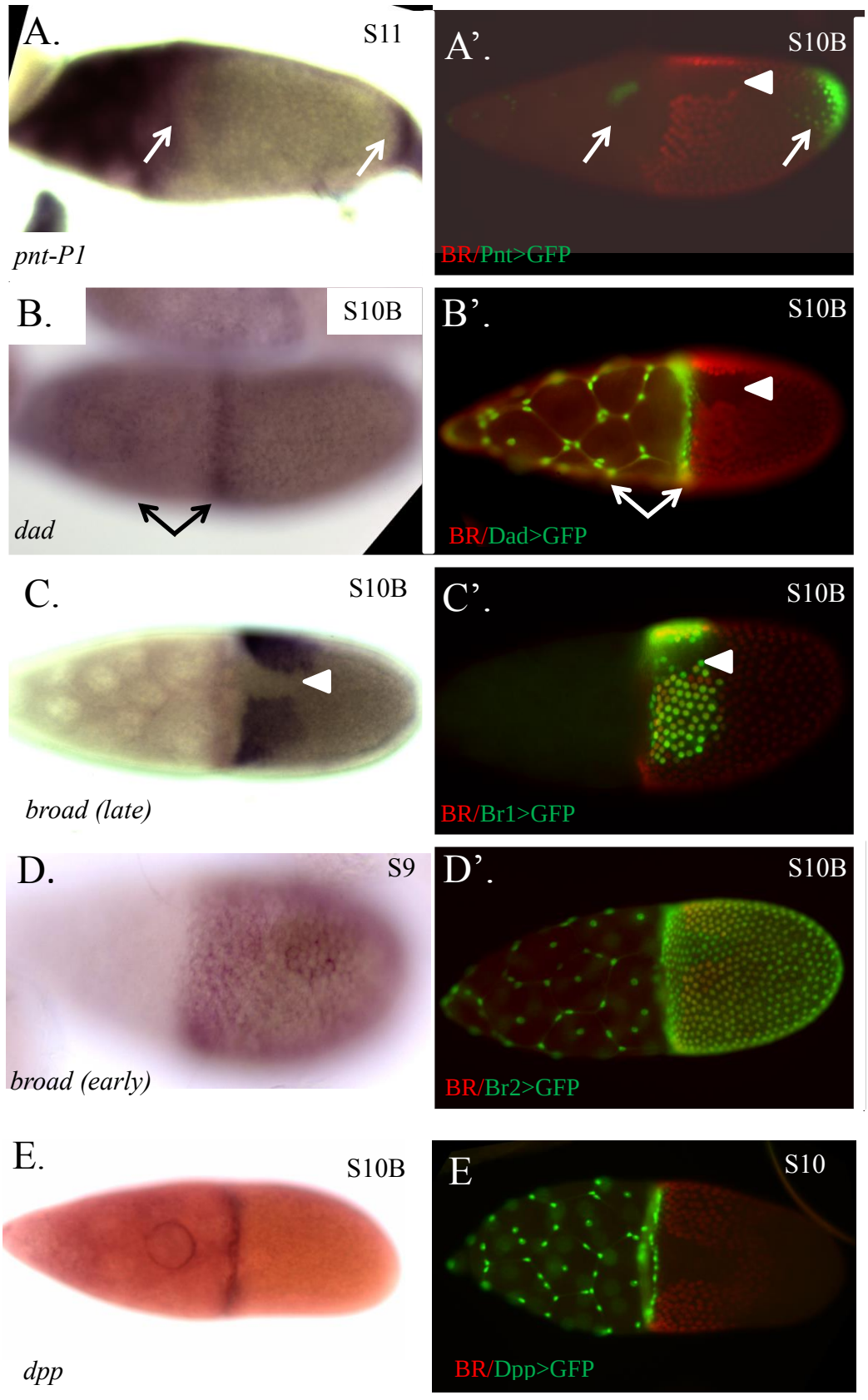


Fig. 4. Selecting the experimental system.

To test for regulatory sequences, we cross listed the collection of 5200 GAL4 drivers designed to characterize regulatory enhancers of 925 genes. There are 223 common GAL4 line representing 19 genes. All GAL4 lines were crossed to a UAS-GFP and progeny were screened for GFP expression in the ovaries.

Fig. 5.


A.
S11
pnt-P1
A'.
S10B
BR/Pnt>GFP
B.
S10B
dad
B'.
S10B
BR/Dad>GFP
C.
S10B
broad (late)
C'.
S10B
BR/Br1>GFP
D.
S9
broad (early)
D'.
S10B
BR/Br2>GFP
E.
S10B
dpp
E
S10
BR/Dpp>GFP

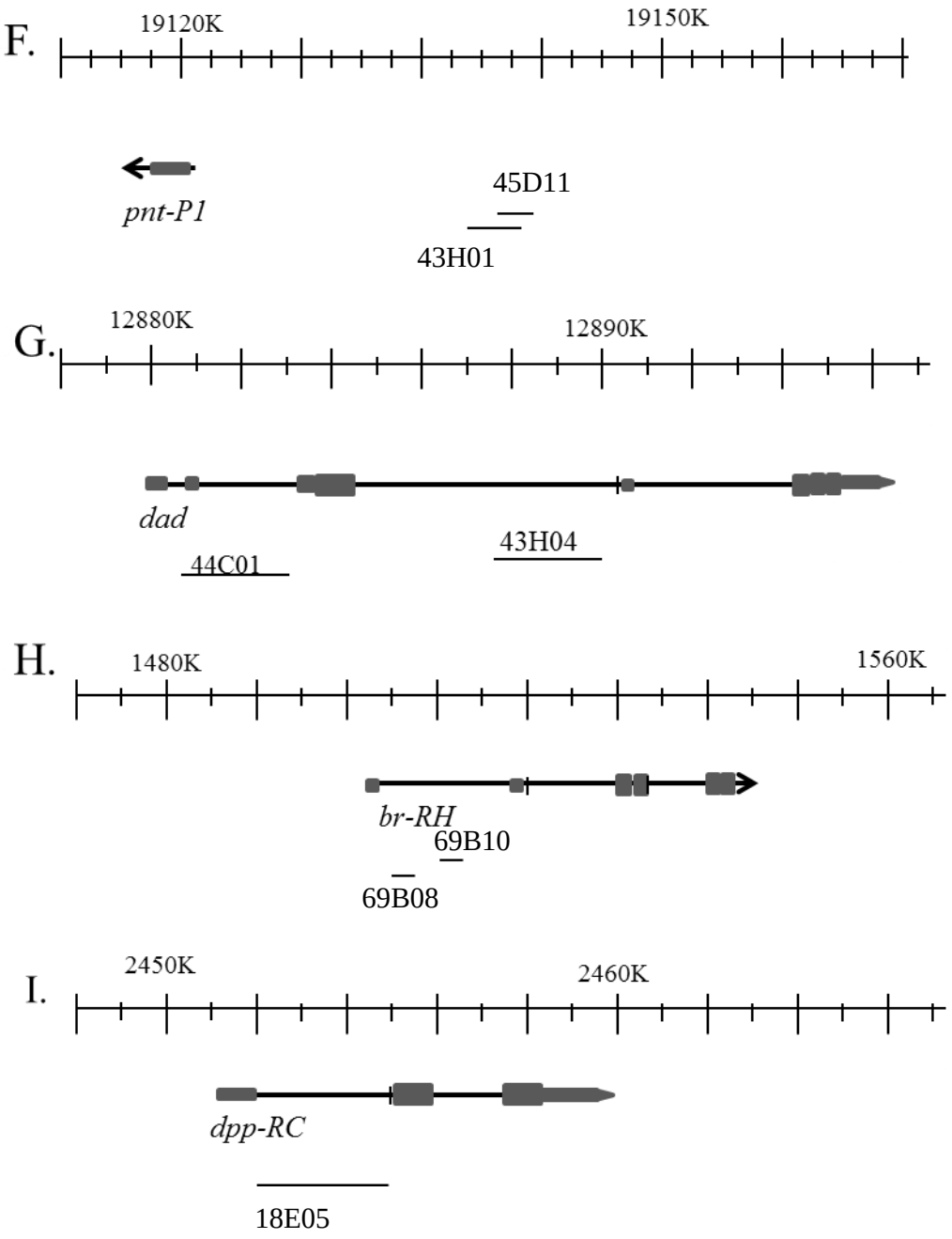
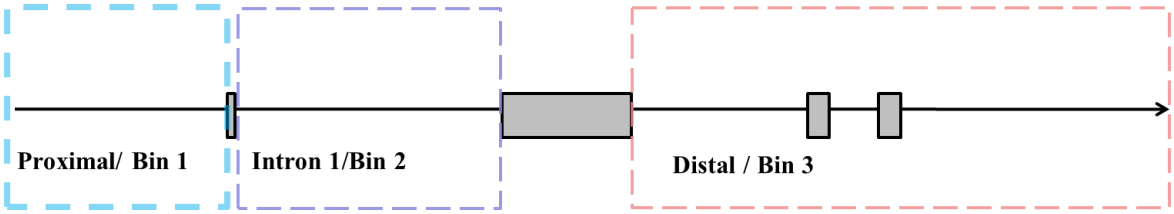

F.
19120K
19150K
pnt-P1
45D11
43H01
G.
12880K
12890K
dad
43H04
44C01
H.
1480K
1560K
br-RH
69B10
69B08
I.
2450K
2460K
dpp-RC
18E05

Fig. 5. GAL4 regulatory sequences recapitulate patterns of their endogenous gene.

In situ hybridization images of endogenous gene patterns (A-E). Total or partial patterns of the endogenous gene expressed by nuclear localized GFP under fluorescence microscopy (A'-E'). Enhancer fragments mapped to their corresponding gene model (F-I). *pnt*^{45D11}-GAL4 and *pnt*^{43H01}-GAL4 enhancer fragments relative to the *pnt-P1* locus (F). *dad*^{44C01}-GAL4(A) and *dad*^{43H04}-GAL4(SC) enhancer fragments relative to the *dad* gene locus (G). *br*^{69B08}-GAL4 (R) and *br*^{69B10}-GAL4 (U) enhancer fragments relative to the *br-RH* gene locus (H). *dpp*^{18E05}-GAL4 enhancer fragments relative to the *dpp* gene locus (I). Anterior is to the left in all images. Dorsal midline is marked by an arrowhead. Arrows point at the actual pattens.

Fig. 6.

A.


Proximal/ Bin 1
Intron 1/Bin 2
Distal / Bin 3

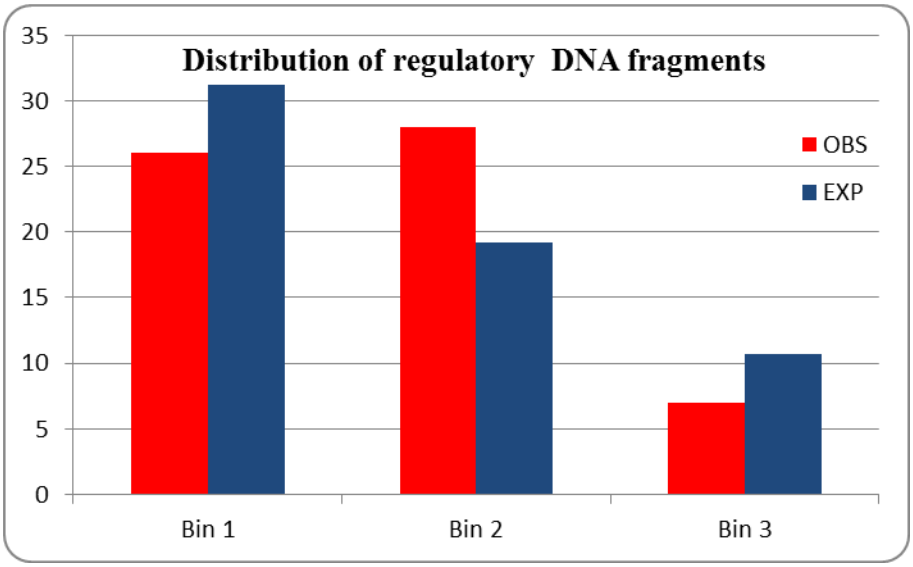
B.


p-4g
p-3g
p-1g
dpp
dpp
dpp
dpp
dpp

Fig. 6. Identifying distribution of regulatory enhancers within the gene's model.

The individual fragments were mapped to their corresponding gene and placed into three categories, (bin 1 to 3), depending on their position. (A) A representative cartoon of the gene model. Individual bins are represented by dotted lines. The gray boxes represent exons. The black represents intergenic and intronic DNA. (B) An example of RNA-seq results of *dpp*. The absence of scale bars indicates inactive exons during oogenesis. The results indicate that only a single isoform (the 4th from the top) is expressed during oogenesis.

Fig.7.



p=0.038

Fig. 7. Distribution of regulatory DNA fragments using Chi square test.

Fragments were sorted as according to the criteria in Fig. 6. Expected values (EXP) were calculated based on ratio between the total number of lines with expression patterns and the total number of lines tested (61/229 or 26.64%). This value was applied to the number of lines tested per bin. Observed values (OBS) were then compared using Chi-square test. It was found that the GAL4 fragments are not distributed at random (p=0.038) with enrichment in the first intron.

Fig. 8.

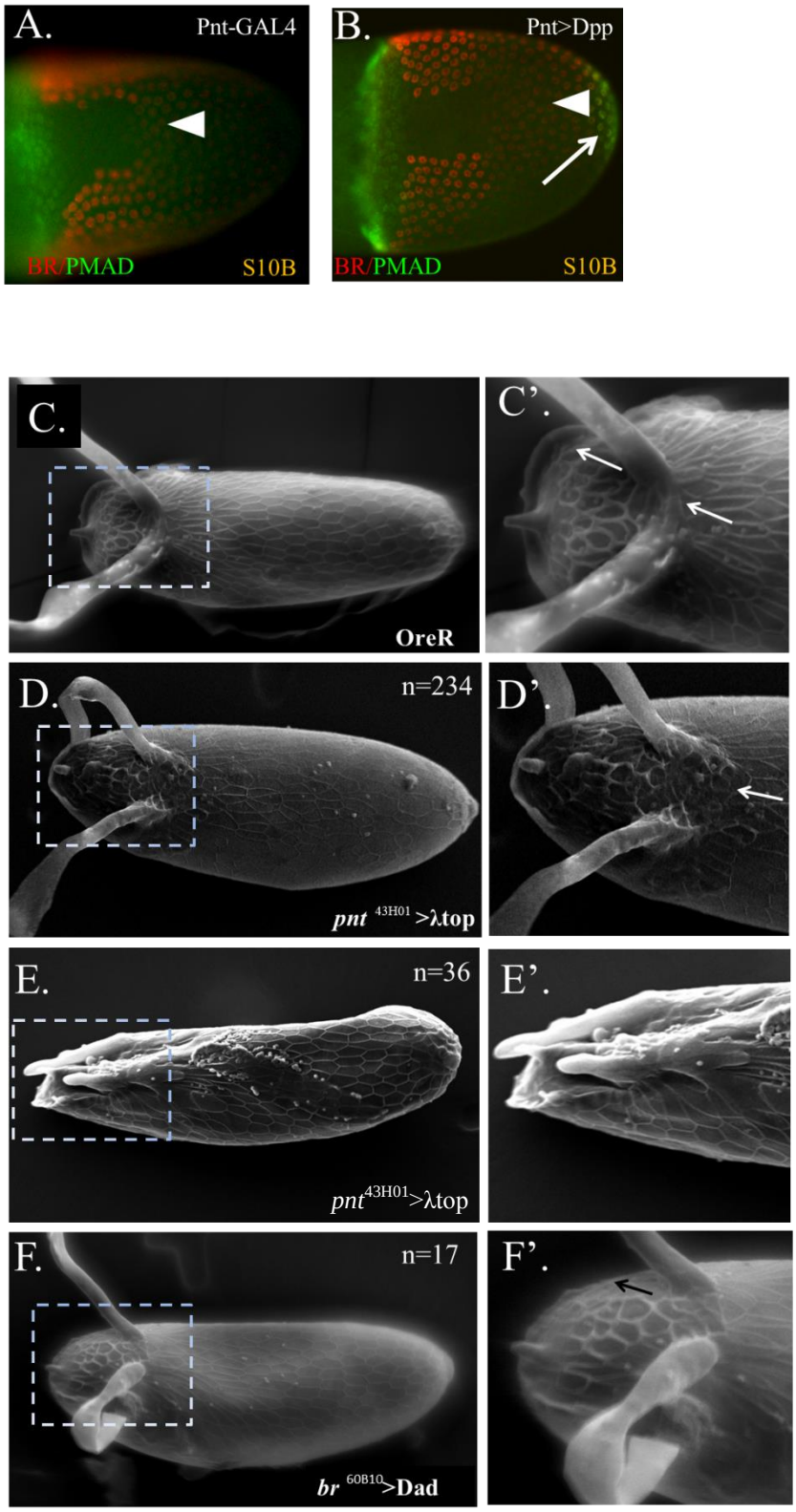

A.
Pnt-GAL4
BR/PMAD
S10B
B.
Pnt>Dpp
BR/PMAD
S10B
C.
OreR
C'.
D.
n=234
pnt 43H01>λtop
D'.
E.
n=36
pnt43H01>λtop
E'.
F.
n=17
br 60B10>Dad
F'.

Fig.8. GAL4 drivers express ectopic protein in specific cellular domains.

pnt^{43H01}-GAL4 driver stained for Broad (red) and P-Mad (green) (A). Dorsal is indicated by arrowhead and the anterior is left. *pnt*^{43H01}-GAL4 drives *decapentaplegic (dpp)* in the posterior end (B). The consequent induction of P-Mad is marked by an arrow. Wild-type eggshell (C, C'). Over expression of a constitutively active EGFR receptor (λtop) using the *pnt*^{43H01}-GAL4 causes an expansion of the the operculum between the two dorsal appendages (Penetrance=59%) (D,D'). *pnt*^{43H01}-GAL4 driver causes severe defects to the DAs when overexpressing a constitutively active EGFR receptor (Penetrance=8%) (E,E'). Uniform GAL4 driver (*br*^{69B10}-GAL4) induces expression of Dad, a BMP inhibitor, causing reduction in collar size (Penetrance=85%) (F, F').

Fig. 9.

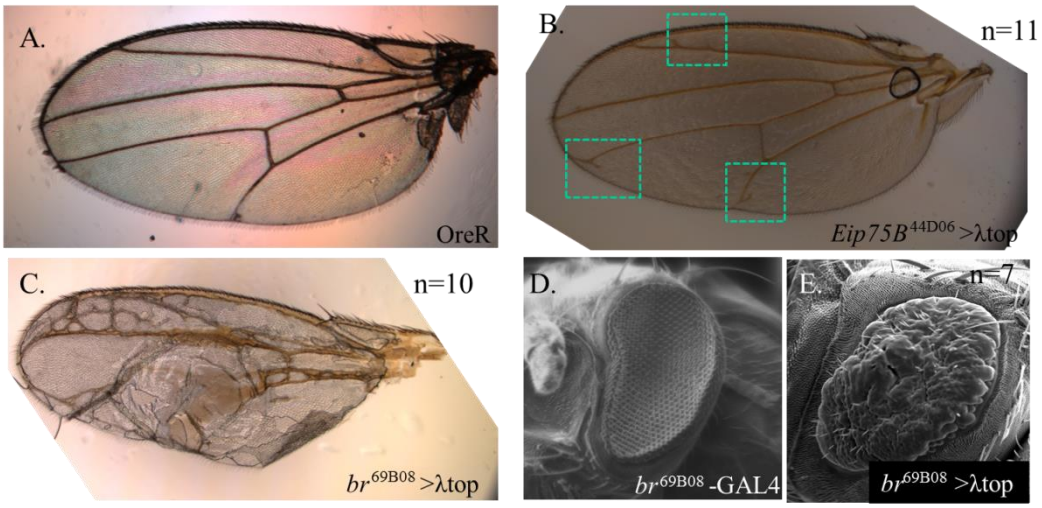


Fig. 9. Identified GAL4 lines can also perturb other tissues.

Wild type wing (A). Wing defects including mild excess vein material (B) (penetrance=100%) and severe defects (C) (penetrance=100%) in two different GAL4 lines compared to wild type (A). SEM images of wild type-like eye of the GAL4 driver (D) compared to same driver expressing over activated EGF receptor.

Fig. 10.

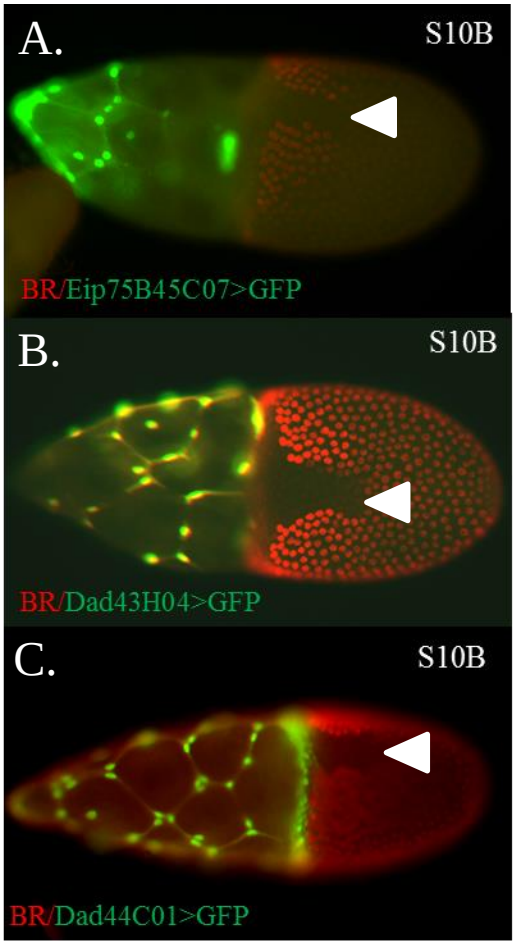


Fig. 10.

Full and partial patterns of the anterior. (A) GFP expression in partial stretch cells. (B) GFP expression in all stretch cells. (C) GFP expression in full anterior pattern, stretch cells and centripetally migrating cells. In all images, anterior is to the left and dorsal is marked by the white arrow.

7. TABLES

Table 1.

Domain	Expression
Anterior (10)	Dad-43H04 Kay-42E11 Dad-44C01 Eip75B-45A08 Eip75B-44D06 Dpp-18E05 Kay-39H03 Eip75B-46E12 Eip75B-45C07 Pnt-45C12
Uniform (6)	Jim-80D10 Br-69B10 Dally-71D08 Ana-23E11 Kay-41G10 Rho-37F01
Posterior (4)	Argos-24G11 Pnt- 45D11 Pnt 43H01 Kay-41G05
Border cells (10)	Rho-38A01 Lin29-40G09 Dpp-17G08 Dad-45C11 Dally-71E07 Eip75B-45B06 Jar-45F03 Jar-44B06 Dally-56H02 Jim-80E01
Roof (1)	Br-69B08
Early Oogenesis (≤S8) (6)	Lin29-40C06 Emc-10B05 Jim-80D09 Dally-60D01 Eip78c-35D08 Emc-10D04
Late Oogenesis (S13-14) (18)	Dpp-19B04 Mirr-33B03 Mirr-34C01 Mirr-34B11 Lin29-40G01 Pnt-43E07 Pnt-44B07 Pnt-45E10 Eip75B-44A12 Argos-25C01 Eip75B-45H09 Eip78c-36C01 Dpp-17E04 Dpp-19E02 Kay-39F12 Kay-41B11 Kay-42A05 Ras85D-57A08

Table 1. Expression of GAL4 drivers

8. REFERENCES

Andrenacci, D., Cavaliere, V., Cernilogar, F.M., Gargiulo, Giuseppe. 2000. Spatial Activation and Repression of the *Drosophila* Vitelline Membrane Gene *VM32E* Are Switched by a Complex *cis*-Regulatory System. Developmental Dynamics. 218:499-506.

Arnold, C.D., Gerlach, D., Stelzer, C., Boryń ŁM, Rath,M., Stark,A. 2013. Genome-wide quantitative enhancer activity maps identified by STARR-seq. 2013. Science. 339(6123):1074-7.

Barolo, S., Carver, L.A., Posakony, J.W. 2000. GFP and b-Galactosidase Transformation Vectors for Promoter/Enhancer Analysis in *Drosophila*. BioTechniques. 29:726-732.

Berg, C.A. 2005. The *Drosophila* shell game: patterning genes and morphological change. Trends Genet. 21: 346–355.

Brand, A.H., Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 118(2): 401-415.

Brand, A.H., Perrimon, N. 1994. Raf acts downstream of the EGF receptor to determine dorsoventral polarity during Drosophila oogenesis. Genes Dev. 8(5): 629-639.

Bonn S, Furlong EE. 2008. *cis*-Regulatory networks during development: a view of *Drosophila*. CurrOpinGenet Dev. 18:513–520.

Cavaliere,V., Bernardi, F., Romani, P., Duchi, S., Gargiulo, G. 2008. Building Up the *Drosophila* Eggshell: First of All the Eggshell Genes Must Be Transcribed. Dev Dyn. 237:2061–2072

Deng,W.M., Bownes, M. 1997. Two signalling pathways specify localised expression of the broad-complex in *Drosophila* eggshell patterning and morphogenesis. Development. 124: 4639–4647.

Dobens, L.L. and Raftery, L.A. 2000. Integration of epithelial patterning and morphogenesis in *Drosophila* ovarian follicle cells. Developmental Dynamics. 218:80-93.

Dobens, L.L., Peterson, J.S., Treisman, J., Raftery, L.A. 2000. Drosophila bunched integrates opposing DPP and EGF signals to set the operculum boundary. Development. 127: 745-754

Duffy J. B. 2002. GAL4 System in *Drosophila: A* Fly Geneticist's Swiss Army Knife. Genesis. 34:1–15.

Drewe, P., Stegle, O., Hartmann, L., Kahles , A., Bohnert, R., Wachter, A., Borgwardt, K., Rätsch, G. 2013. Accurate detection of differential RNA processing. Nucleic Acids Res. [Epub ahead of print].

Fuchsa, A., Cheungb, L.S., Charbonniera, E., Stanislav Y. Shvartsmanb, S.Y., Pyrowolakisa,G. 2012. Transcriptional interpretation of the EGF receptor signaling gradient. PNAS. 109(5):1572-7.

González-Reyes, A., Elliott, H. and St Johnston, D. 1995. Polarization of both major body axes in *Drosophila* by *gurken-torpedo* signalling. *Nature* 375: 654-658.

Horne-Badovinac S., Bilder D. 2005. Mass Transit: Epithelial Morphogenesis in the *Drosophila Egg Chamber.* 2005. Developmental Dynamics. 232:559–74. Review.

Hinton, H.E. 1969. Respiratory Systems of Insect Egg Shells. Annual Review of Entomology. 14: 343-368

Jenett, A., Rubin, J.A.,G.M., Ngo, T-TB, Shepherd, D., Murphy, C., Dionne, H., Pfeiffer, B.D., Hibbard, K.L., Cavallaro, A., Hall, D. 2012. A GAL4-Driver Line Resource for Drosophila Neurobiology. 4:991-1001.

Jordan, K.C., Hatfield, S.D., Tworoger, M., Ward, E.J., Fischer, K.A., Bowers, S., and Ruohola-Baker, H. 2005. Genome wide analysis of transcript levels after perturbation of the EGFR pathway in the Drosophila ovary. Dev. Dyn. 232: 709–724.

Jory, A., Estella, C., Giorgianni, M.W., Slattery, M., Laverty, T.R., Rubin, G.M., Mann, R.S. 2012. A survey of 6300 genomic fragments for *cis*-regulatory activity in the imaginal discs of *Drosophila melanogaster*. Cell Rep. 2(4): 1014-1024.

King, R.C. 1970. Ovarian development in *Drosophila* melanogaster. New York: Academic Press.

Lembong, J., Yakoby, N., Shvartsman, S.Y. 2008. Spatial regulation of BMP signaling by patterned receptor expression. Tissue Eng. A 14: 1469–1477.

Levine, M., Tjian, R. 2003. Transcription regulation and animal diversity. Nature. 10:424(6945):147-51.

Luan, H., Lemon, W.C., Peabody, N.C., Pohl, J.B., Zelensky, P.K., Wang, D., Nitabach, M.N., Holmes, T.C., White, B.H. 2006. Functional dissection of a neuronal network required for cuticle tanning and wing expansion in *Drosophila*. The Journal of neuroscience : the official journal of the Society for Neuroscience. 26:573–584.

Manning, L., Purice, M.D., Roberts, J., Pollard, J.L., Bennett, A.L., Kroll, J.R., Dyukareva, A.V., Doan, P.N., Lupton, J.R., Strader, M.E. 2012. Annotated embryonic CNS expression patterns of 5000 GMR Gal4 lines: a resource for manipulating gene expression and analyzing *cis*-regulatory motifs.

Marmion, R.A., Jevtic, M., Springhorn, A., Pyrowolakis, G., Yakoby, N. 2013. The *Drosophila* BMPRII, Wishful thinking, is required for eggshell patterning. Developmental Biology 375:45-53.

Morimoto, A.M., Jordan, K.C., Tietze, K., Britton, J.S., O'Neill, E.M., Ruohola-Baker, H. 1996. Pointed, an ETS domain transcription factor, negatively regulates the EGF receptor pathway in *Drosophila* oogenesis. Development. 122(12):3745-54.

Nakamura Y, Kagesawa T, Nishikawa M, Hayashi Y, Kobayashi S, Niimi T, Matsuno K. 2007. Soma-dependent modulations contribute to divergence of rhomboid expression during evolution of *Drosophila* eggshell morphology. Development. 134(8):1529-37.

Neuman-Silberberg, F.S., Schupbach, T. 1994. Dorsoventral axis formation in *Drosophila* depends on the correct dosage of the gene gurken. Development 120: 2457–2463.

Niepielko,M., Ip, K., Jitendra, K.S., Desmond, L.S., Yakoby, N. 2012. Evolution of BMP Signaling in *Drosophila* Oogenesis: A Receptor-Based Mechanism . Biophysical Journal. 102:1722-1730.

Parker, L., Stathakis, D.G., Arora, K. 2004. Regulation of BMP and Activin Signaling in Drosophila. Prog Mol Subcell Biol. 34:73-101.

Pfeiffer, B.D., Jenett, A., Hammonds, A.S., Ngo, T.B., Misra, S., Murphy, C., Scully, A., Carlson, J.W., Wan, K.H., Laverty, T.R., Mungall, C., Svirskas, R., Kadonaga,J.T., Doe, C.Q., Eisen, M.B., Celniker, S.E., Rubin, G.M. 2008. Tools for neuroanatomy and neurogenetics in *Drosophila*. PNAS. 105, 28: 9715-9720.

Pfeiffer BD, Ngo TT, Hibbard KL, Murphy C, Jenett A, Truman JW, Rubin GM. 2010. Refinement of tools for targeted gene expression in *Drosophila*. Genetics. 186:735–755.

Peri, F., Roth, S. 2000. Combined activities of Gurken and decapentaplegic specify dorsal chorion structures of the *Drosophila* egg. Development. 127: 841-850.

Queenan, A.M., Ghabrial, A., Schupbach, T. 1997. Ectopic activation of torpedo/Egfr, a *Drosophila* receptor tyrosine kinase, dorsalizes both the eggshell and the embryo. Development. 124: 3871–3880.

Raftery, L.A., Umulis, D.M. 2012. Regulation of BMP activity and range in *Drosophila* wing. Development. Curr Opin Cell Biol. 24(2): 158–165.

Rasband, W.S. ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA. 1997-2012. http://imagej.nih.gov/ij/. Robinson *et al.*, 2011

Ray, R. and Schupbach, T. 1996. Intercellular signaling and the polarization of the body axes during *Drosophila* oogenesis. Genes Dev. 10, 1711-1723.

Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G., Mesirov, J.P. 2011. Integrative Genomics Viewer. 2011. Nature Biotechnology 29, 24–26

Ruohola-Baker, H., Grell, E., Chou, T.B., Baker, D., Jan, L.Y., Jan, Y.N. 1993. Spatially localized rhomboid is required for establishment of the dorsal-ventral axis in *Drosophila* oogenesis. Cell. 73: 953–965.

Scholz, H., Deatrick, J., Klaes, A., Klambt, C. 1993. Genetic Dissection of Pointed, a *Drosophila* Gene Encoding Two Ets-Related Proteins. Genetics. 135(2): 455-468.

Shi, Y., Massaqué, J. 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell. 113(6):685-700.

Shravage, B.V., Altmann, G., Technau, M., Roth, S. 2007. The role of Dpp and its inhibitors during eggshell patterning in Drosophila. Development 134, 2261–2271.

Spradling, A.C. 1993. Developmental genetics of oogenesis. The Development of Drosophila melanogaster. Cold Spring Harbor Laboratory Press, Plainview.

Technau, M., Knispel, M., Roth, S. 2011. Molecular mechanisms of EGF signaling-Dependent regulation of pipe, a gene crucial for dorsoventral axis formation in *Drosophila*. Dev Genes Evol. 222:1–17.

Thorvaldsdóttir, H. Robinson, J.T., Mesirov, J.P. 2012. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Briefings in Bioinformatics.

Tsuneizumi K., Nakayama, T., Kamoshida, Y., Kornberg, T.B., Christian, J.L., Tabata, T. 1997. Daughters against dpp modulates dpp organizing activity in *Drosophila* wing development. Nature. 389:627–631.

Twombly, V., Blackman, R. K., Jin, H., Graff, J.M., Padgett, R.W., Gelbart, W.M. 1996. The TGF-b signaling pathway is essential for Drosophila oogenesis. Development. 122: 1555-65.

Tzolovsky G, Deng WM, Schlitt T, Bownes M. 1999. The function of the broad-complex during Drosophila melanogaster oogenesis. Genetics. 153(3):1371-83.

Ward, E.J., Thaipisuttikul, I., Terayama, M., R.L., Jackson, S.M., Cosand, K.A., Tobler, K.J., Dorman, J.B., Berg, C.A. 2002. GAL4 enhancer trap patterns during *Drosophila* development. Genesis. 34:46-50.

Ward, E.J., Berg, C.A. 2005. Juxtaposition between two cell types is necessary for dorsal appendage tube formation. Mechanisms of Development. 122: 241–255.

Ward, E.J., Zhou, X., Riddiford, L.M., Berg, C.A., Ruohola-Baker, H. 2006. Border of Notch activity establishes a boundary between the two dorsal appendage tube cell types. Dev Biol. 297 (2): 461-70

Weiss, A., Charbonnier, E., Ellertsdóttir1, E., Tsirigos, A., Wolf, C., Schuh, R., Pyrowolakis, G., Affolter., M. 2010. A conserved activation element in BMP signaling during *Drosophila* development. Nat Struct Mol Biol. 17(1):69-76.

Yakoby, N., Bristow, C.A., Gong, D., Schafer, X., Lembong, J., Zartman, J.J., Halfon, M., Schupbach, T., and Shvartsman, S.Y. 2008a. A combinatorial code for pattern formation in *Drosophila* oogenesis. Developmental Cell. 15:725-737.

Yakoby, N., J. Lembong, ., S. Y. Shvartsman. 2008. Drosophila eggshell is patterned by sequential action of feedforward and feedback loops. Development. 135:343–351.