Experiment 3C: Genetic Rescue with the GAL4/UAS System in *Drosophila**

***Note:** This lab was created by Dr. Haley Brown, a former graduate student at Indiana University, Bloomington, IN.

Introduction:

Thus far in the semester we have used EMS mutagenesis and CRISPR in yeast as an example of forward and reverse genetics, respectively. Here, we will continue this theme in *Drosophila* by manipulating gene expression in a specific set of cells. There are a variety of tools available to genetically manipulate flies in this manner – for this experiment, we will focus on the *GAL4/UAS system*, which was originally adapted from yeast.

GAL4/UAS in yeast

Similar to the bacterial lac operon, the yeast GAL regulon consists of seven main GAL genes that are required for yeast growth on galactose. These GAL genes act either structurally to transport galactose into the cell and move the glycolytic pathway along (*GAL1*, *GAL10*, *GAL2*, and *GAL7*) or regulators, controlling the transcription of the structural GAL genes (*GAL4*, *GAL80*, and *GAL3*) (Hopper *et al.* 1978). In an environment without galactose, GAL80 binds to the activation domain of GAL4 (Carrozza *et al.* 2002) (Figure 1A). This allows GAL4 to still bind DNA but prevents it from recruiting the necessary machinery to initiate transcription (i.e. RNA Pol II). However, when galactose is present, GAL3 competitively inhibits GAL80 (Peng and Hopper, 2000) thereby freeing the GAL4 activation domain to initiate transcription of *GAL1* (*galactokinase*), *GAL10* (*UDP-glucose 4-epimerase*), and *GAL7* (*galactose-1-phosphate uridylyltransferase*) genes (Figure 1B).

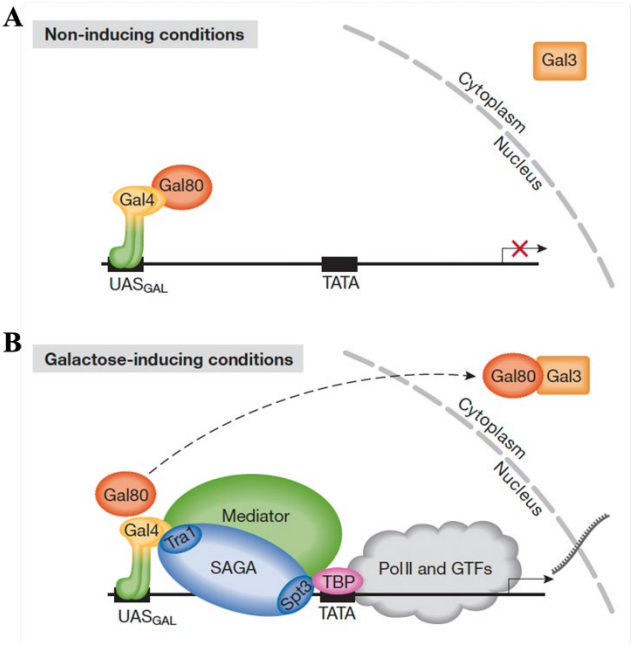

Figure 1: Transcriptional regulation by GAL4 in *Saccharomyces cerevisiae*. (A) When galactose is absent, GAL80 binds GAL4 and prevents transcriptional activation. (B) When galactose is present, GAL80 is removed and the GAL4 activation domain can recruit transcriptional machinery downstream of the UAS consensus. Figure adapted from Traven *et al.* 2006.

GAL4 was initially found to bind to four 17-bp sites located between *GAL1* and *GAL10* genes (Giniger *et al.* 1985). Using this consensus sequence, approximately 300 GAL4-binding sites have been identified throughout the yeast genome (Traven *et al.* 2006). These sites are canonically referred to as Upstream Activator Sequences (UAS) and are sufficient to drive transcription when bound by GAL4. This transcriptional activation occurs in a similar manner to how enhancer elements function in multicellular eukaryotes.

First adapted into flies

Just as CRISPR/Cas9 (an endogenous bacterial system) was quickly adapted to a myriad of other model organisms (see Experiment 2), the GAL4/UAS system was adapted to flies (Brand *et al.* 1994) approximately two decades after the GAL4 regulon's discovery in yeast.

One of the first ways this system was adapted to flies was to inject a plasmid with *either* the GAL4 transcriptional activator fused to a heat-shock promoter (Struhl *et al.* 1985) *or* a UAS fused to the target gene of interest into early embryos to create two distinct transgenic fly lines. The target gene (UAS) and transcriptional activator (GAL4) are kept separate in stable stocks to ensure the parental flies are viable. It is only when those two flies are crossed together that the progeny will possess both the UAS and GAL4 (Figure 2A). Furthermore, as the GAL4 is tied to a heat shock promoter, your gene of interest will only be expressed when the progeny are exposed to heat (37°C).

Only when these conditions are met can you observe the phenotypic consequences of your expressing your target gene in cells where it is not usually found. This system can be fine-tuned by altering the degree and duration of heat shock to regulate the level of gene expression (Manoukian and Krause, 1992). This was a clever way to induce gene expression throughout the entire organism at a specific point in development in order to elucidate the hierarchy of gene networks.

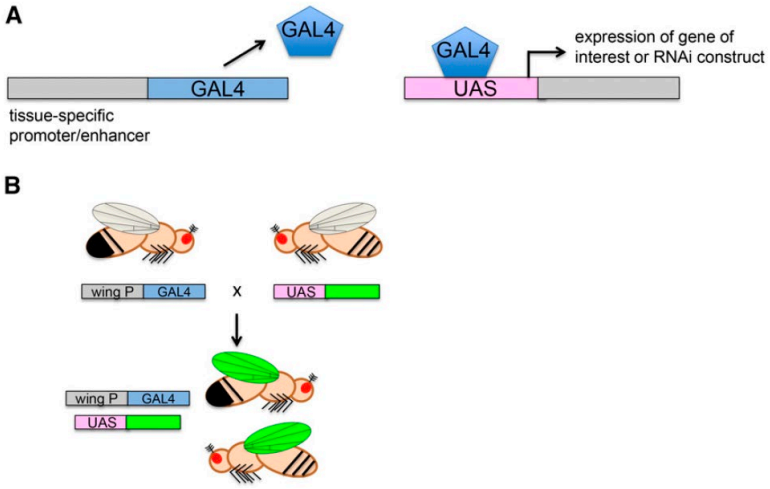

Figure 2: GAL4/UAS system in *Drosophila*. (A) Parental flies will contain *either* a GAL4 transcriptional activator tied to a tissue-specific promoter *or* an Upstream Activating Sequence (UAS) linked to your gene of interest. (B) When the parental flies are crossed together, the progeny will express your gene of interest (green) in a tissue-specific manner. Here, a wing promoter drives expression of the green gene of interest only in the wings of the progeny. Figure adapted from Hales *et al.* 2015.

However, when addressing questions of cell fate or gene function, it is more prudent to target gene expression in a specific set of cells rather than the whole fly. To do this, the GAL4/UAS system was tweaked to fuse GAL4 to enhancer sequences of defined gene promoters (Parkhurst and Ish-Horowicz, 1991) rather than a general heat shock promoter. In this modification progeny expressing GAL4 in specific cells (driven by the enhancer) will bind to UAS and activate the target gene. Cells that do not express GAL4 will not bind to UAS and drive expression of the target gene (Figure 2B). The use of tissue-specific promoters also alleviates potential lethality often associated with either the heat shock step in general or the consequences of overexpressing a target organism-wide. Directing gene expression in this manner has been used to investigate where genes fall in signaling cascades, how genes function outside of their typical cell/tissue, as well as how genes direct development of specific cell types. Since its inception, fly geneticists have used many iterations of this system to selectively overexpress genes, knock down targets by expressing a short hairpin RNA (RNAi – which you will learn more about in Experiment 5A), and even drive expression of a fluorescent reporter (i.e. GFP) to determine exactly what cells/tissue are expressing our promoter. The *Drosophila* community has generated a list of GAL4 drivers and their expression patterns that can be found on FlyBase (https://flybase.org/ under 'GAL4 etc'). Here you can filter to find a list of GAL4 lines that will be expressed in your tissue and life stage of interest and then order them directly from the Bloomington *Drosophila* Stock Center (BDSC).

The GAL4/UAS system is a widely used genetic tool that is critical for investigating how spatial and temporal control of gene expression can determine the identity of a cell. For instance, if you were to overexpress a gene not normally present in a cell, could it change its fate? What if you induce it earlier or later in development than normal? Could you *rescue* a phenotype by overexpressing a gene in a fly that is genetically mutant for that gene? Experiment 3C will address this last example – genetic rescue.

Classically, a *genetic rescue* is an experiment that attempts to connect a gene to the loss of its function, as demonstrated by restoring a mutant phenotype (such as the loss of a structure or process) to wild type. This is typically done by expressing a wild-type version of whatever mutated gene resulted in the phenotype.

Let's use the axon guidance receptor, *roundabout1* (*robo1*), to better illustrate this concept. Robo1 contributes to axon guidance by preventing axons from inappropriately crossing the midline to innervate the opposite side of the body (Evans and Bashaw, 2010). When functioning properly, this regulation results in three distinct longitudinal pathways on either side of the midline (Figure 3A). However, in *robo1* loss-of-function animals, axons attempt to cross and recross the midline forming tracks that look similar to a roundabout (Figure 3B). This prevents proper innervation and results in lethality. Using the GAL4/UAS system you can attempt to rescue both lethality and the mutant phenotype by specifically driving wild-type Robo1 (*UAS-Robo1*) in all neurons with the *embryonic lethal abnormal vision* (*elav*) promoter fused to GAL4 (*elav-GAL4*) of *robo1* mutant animals. To obtain progeny which are homozygous for *robo1*, as well as contain copies of both *UAS-Robo1* and *elav-GAL4*, both parents must be *robo1* mutants and carry either the driver (GAL4) or responder (UAS). But remember that *robo1* homozygous animals are embryonic lethal – *so how can we get around this parental lethality to achieve the desired genetic background in the F1 progeny?* This is where balancers (see Experiment 3A week 2) come in handy. To alleviate lethality, you must have *robo1* over a balancer on the same chromosome – such as curly wings (*CyO*) – in the parents to then get homozygotes in the F1 offspring (i.e. cross *robo1*/*CyO; UAS-Robo1* male flies to *robo1*/*CyO; elav-GAL4* female flies). These animals develop longitudinal axons that no longer inappropriately cross the midline (Figure 3C) and alleviate lethality.

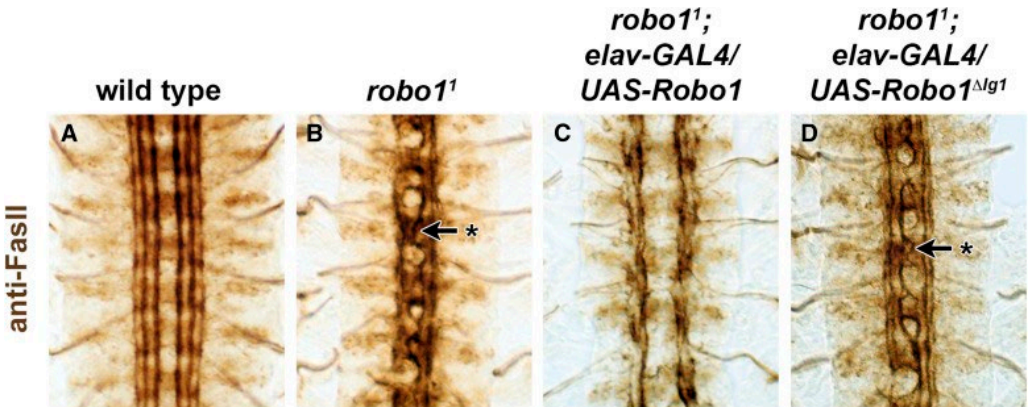

Figure 3: Genetic rescue in the ventral nerve cord (VNC) of *Drosophila* embryos. (A) Wild-type VNC with three distinct longitudinal tracks on either side of the midline. (B) robo1 loss-of-function embryos, where axons cross the midline to form characteristic 'roundabouts'. (C) When Robo1 is driven in all neurons, axons no longer cross the midline. (D) Driving Robo1 without its Ig1 domain in all neurons fails to rescue midline repulsion. Figure adapted from Brown *et al.* 2015.

This can even be taken a step further to test the different functional domains of your protein of interest. Robo1 has five immunoglobulin-like (Ig) domains and three fibronectin repeats (FN) (Kidd *et al.* 1998). The first Ig domain (Ig1) has long been proposed to be paramount to Robo1's midline repulsive function, but how can we test this? Well, using the GAL4/UAS system you can drive a version of Robo1 that is only missing Ig1 in a *robo1* loss-of-function background and determine whether this version of the protein can rescue midline repulsion. What you find is that these embryos show levels of midline crossing that phenocopies the mutant *robo1* embryos (Figure 3D). *In vivo* structure-function assays like this are quite common to determine what role each domain of a protein plays in its function (*something to remember as you develop your Experiment 5B proposal later in the semester*).

Our goal in Experiment 3C is to use the *Drosophila* eye as a model to observe genetic rescue. Like many tissues, the *Drosophila* eye-antennal disc – which will give rise to the eye, antenna, and head – is fated and patterned by expressing a series of transcription factors in a specific manner throughout embryogenesis and larval development (Kumar, 2018). This network of transcription factors is canonically referred to as the Retinal Determination Network (RDN). When temporal or spatial patterning of these factors is lost, the fly will fail to develop eyes during pupation. Here we will focus on one transcriptional activator in the RDN – *eyes absent* (*eya*). Eya loss-of-function mutants (eya^2) are particularly strong and consistently result in no-eyed flies (Weasner and Kumar, 2022). *How can we use the GAL4/UAS system to restore eye development to* eya^2 *mutant flies?*

Experimental Protocol for Experiment 3C

Experimental Organism:
We will use the GAL4/UAS system to 'rescue' eye development in an *eya*^{2} null mutant background (eyeless flies). To do this we will set up a cross where the *responder* (*UAS-eya*) will specifically be *driven* in cells that are meant to express *eya* (*eya-GAL4*) or a subset of cells that are meant to express *eya* (*dpp*^{blk}*-GAL4*) at 25°C. Your task is to measure the degree to which the eye is rescued in the resulting progeny.

Week 1:

1. Set up *cross 1*: You will receive two vials of parental flies: (1) *eya*^{2}*; UAS-eya* males and (2) *eya*^{2}*; eya-GAL4* females. Set up the cross by tapping the females into the male vial. Label this vial 'eya^{2}; eya-GAL4 > eya^{2}; UAS-eya'.
2. Rubberband your labeled cross vial and empty female vial together and turn in up front.
3. Set up *cross 2*: Receive two vials of parental flies: (1) *eya*^{2}*; UAS-eya* males and (2) *eya*^{2}*; dpp*^{blk}*-GAL4* females. Set up the cross exactly as described above. Label this vial 'eya^{2}; dpp^{blk}-GAL4 > eya^{2}; UAS-eya'.
4. Rubber band your labeled cross vial and empty female vial together and turn in up front.

Thought Question 1: What is the point of driving *UAS-eya* with two different GAL4 lines? Based on their expression patterns, which do you hypothesize will best rescue eye development?

Thought Question 2: What does using the GAL4/UAS system in this capacity tell us genetically and molecularly? What other variations to this experiment could we conduct to determine more about the retinal determination network?

Week 2:

1. Examine the "empty" female vial for signs of larvae. If larvae are present, discard the cross and obtain a backup from your AI. If no larvae are present, discard vial.
2. Examine the parental cross for signs of larvae & pupae. If both are present, morgue the parental flies in ethanol and turn vial in up front. If no larvae are present, discard vial and obtain a new cross from your AI.
3. Obtain vials of (1) *eya*^{2}*; eya-GAL4* and (2) *eya*^{2}*; dpp*^{blk}*-GAL4* transgenic flies. Image and record your observations (focus on the eye, ocelli, and head) in Table 1.

Thought Question 3: What is meant by the term 'wild-type'? In Experiments 3A & 3B, we used the traditional, red-eyed fly line *Oregon R* as our wild-type. Why do we instead use the respective drivers (in an *eya*^{2} mutant background) as our wild-type comparison here?

Week 3: This is the end of Experiment 3C.

1. Examine the F1 progeny in both crosses.
2. Image and record the phenotypes (and the number of flies with each phenotype) in Table 1.
3. When finished, discard all vials.

Thought Question 4: What phenotypic variation did you observe in the F1 progeny of both crosses? What could account for this variation? Which driver was best able to rescue eye development?

Include your observations in your lab notebook. In your discussion of your data include an explanation of what we can learn from using *genetic rescue* in general and what our experiment in particular shows us. Suggest another experiment using GAL4/UAS to further probe the *eya* gene's role in eye development.

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	CROSS 1		CROSS 2	
	'Wild-type': *eya^2; eya-GAL4*	**Rescue:** *eya^2; eya-GAL4 > eya^2; UAS-eya*	**'Wild-type':** *eya^2; dpp^{blk}-GAL4*	**Rescue:** *eya^2; dpp^{blk}-GAL4 > eya^2; UAS-eya*
No eye	n =	n =	n =	n =
Small eye	n =	n =	n =	n =
WT eye	n =	n =	n =	n =

Table 1. Number of flies observed with no eyes, small eyes, or wild-type (WT) eyes for each genotype.