MODELING A CELLULAR RESPONSE TO A GRADIENT

Mathematics and Molecular Biology Inform a Mechanistic Understanding

xuan ge, david stonko

Xuan Ge is a biology major with a minor in psychology who will be graduating in the spring of 2012. She is a member of the Honors College as well as a recipient of the UMBC Premier Award. She plans to continue on to medical school after UMBC. David Stonko is a junior math and biology double major who hopes to continue his education at medical school after graduation from UMBC in spring 2013. David is a member of the Undergraduate Training Program in Biology and Mathematics (UBM) and various other student organizations. Xuan and David would like to thank their research mentors, Dr. Michelle Starz-Gaiano and Dr. Bradford Peercy, for their continued guidance, patience, and encouragement throughout the course of this project. They would also like to thank everyone in the UBM program and their friends, family, and teachers who have supported them.

We began our research after we were selected to participate in the Undergraduate Training Program in Biology and Mathematics (UBM) supported by the National Science Foundation. This program was created to promote the integration of mathematics in biology and to foster an appreciation of interdisciplinary research. Our project is concerned with proposing and testing a new biological hypothesis while building our mathematical model. The entire research process has been an eye opening experience and we believe everyone in this program, as well as other undergraduates in research, can agree that it has helped to better shape our understanding and goals here at UMBC.

INTRODUCTION

Cell migration is prevalent in normal development of all animals, and pathological conditions like birth defects or metastatic cancers can arise when this process goes awry. Understanding the phenomenon of cells undergoing the transition from a stationary state to a migratory stage is of broad interest. Such a complex problem can be more easily studied in a simple organism, such as the fruit fly *Drosophila melanogaster* (Naora and Montell, 2005). *Drosophila* is one of the most studied organisms in biological research. Its short generation time, high fecundity, visible congenital markers, and well-characterized genome are just a few of the many traits that make it an ideal organism for genetic studies. In addition, about eighty percent of disease genes in humans encode proteins that are conserved in flies (Reiter et al., 2001). Thus, because the underlying molecular signaling mechanisms are well conserved, insight into cell migration gleaned from *Drosophila* will likely be broadly applicable.

We are interested in how a set of cells in flies, called the border cells, becomes motile. The border cells arise as part of the follicular epithelium during oogenesis, but change character from epithelial to migratory and travel between other cells to the oocyte, where they are required for embryo development (Fig 1A) (Jang et al., 2007; Montell, 2008). A single egg chamber of *Drosophila* consists of an oocyte accompanied by fifteen nurse cells, and together, they are surrounded by an epithelium of follicle cells. Early in the development of the egg chamber, a pair of cells on each end specializes into the polar cells. In mid-oogenesis, the polar cells at the anterior end release molecular signals that induce six to eight neighboring follicle cells to become border cells (Fig 1A-E). A few hours later, the border cells will withdraw from the epithelium and escort the polar cells a distance of about 20 cell diameters to the edge of the oocyte by stage ten (Fig 1D-E). The follicle cells' transformation into mobile border cells requires changes in gene expression that allow their detachment from neighboring cells and production of cytoskeletal changes that promote migration. Because a similar epithelial to mesenchymal transition underlies tumor metastasis, the analysis of *Drosophila* border cells not only allows us to expand our current knowledge of this particular molecular signaling system, but may also yield insight into mechanisms of human disease progression.

A CASCADE OF MOLECULAR SIGNALS ACTIVATES CELL MOTILITY

The Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) signaling pathway has been shown by previous studies to be intimately involved in the border cells' acquisition of mobility (Arbouzova and Zeidler, 2006; Beccari et al., 2002; Ghiglione et al., 2002; Silver and Montell, 2001; Xi et al., 2003). The anterior polar cells of the *Drosophila* egg chamber secrete the cytokine Unpaired, which acts as the ligand for the transmembrane Domeless receptor in neighboring follicle cells. The binding of Unpaired to Domeless activates JAK which leads to its phosphorylation. Once phosphorylated, the activated JAK/receptor complex recruits and phosphorylates STAT. The phosphorylated STAT is then able to dimerize, move to the nucleus, and act as a transcription factor to turn on specific target genes. As indicated by Fig 1, the released signal, Unpaired, diffuses outwards from the polar cells and forms a gradient across the neighboring epithelium. This ensures that the cells in closer proximity to the polar cells experience a higher level of the ligand and turn on a higher level of STAT activity, which promotes their transformation into mobile border cells. The gradient of STAT activity was directly observed using fluorescent proteins in the egg chambers and standard optical sectioning (Fig 1F), and is essential for the specification of the right number of motile cells.

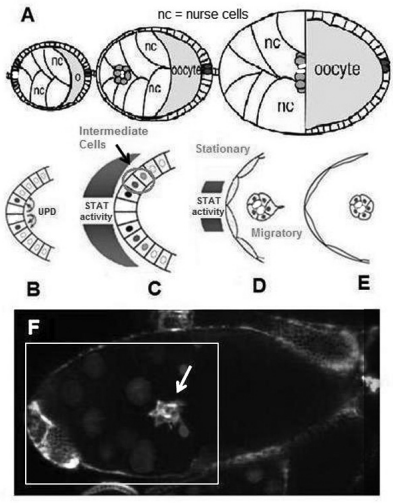

FIG.1 Egg development and the migration of border cells. Panel A is a cartoon showing the maturation process of a *Drosophila* egg chamber and the relative movement of the border cells between the nurse cells (nc). Panels B through E are a magnification of the anterior quarter of an egg chamber during normal development, in which the signaling molecule Unpaired is secreted from the polar cells (B) and induces a gradient of STAT activity across the anterior epithelium at stage 8 (C). By late stage 9, STAT activity is limited to the follicle cells very close to the polar cells, which assume the identity of border cells, become motile (D), and migrate towards the oocyte (E). Panel F is a Fluorescent Protein-STAT reporter (from (Baeg et al., 2005)) staining of a wild type egg chamber, which demonstrates that STAT transcriptional activity is highest in the migrating border cell cluster (arrow). Panel E corresponds to the boxed region of panel F.

Studies by several labs (Beccari et al., 2002; Ghiglione et al., 2002; Silver and Montell, 2001), showed that the loss of STAT signaling in the follicle epithelium produced no migratory cells, and that a higher STAT signaling in the follicle cells induced more cells to migrate. In 2005, Silver et al showed that the proper level of STAT signaling was required during the cell movements as well as in the initial specification (Silver et al., 2005). These findings suggest that precise regulation of JAK/STAT signaling is necessary to generate the migratory behavior in the correct number border cells and that it can induce motile behavior in other follicle cells.

Interestingly, the normal decision of border cells to migrate, while other cells in the egg chamber do not, cannot be controlled by Unpaired and STAT alone. In fact, there are other signaling molecules, discovered through genetic analysis, such as SLBO and Apontic, involved in the JAK/STAT signaling pathway that also play integral roles in defining the trajectory of border cell development (Starz-Gaiano et al., 2008).

TRIGGER MOLECULE FOR MIGRATION: SLBO

Slow Border Cells, or SLBO, is an important downstream transcriptional target of activated STAT and promotes the migratory behavior. Montell, Rorth, and colleagues (1992) demonstrated that insufficient levels of SLBO suppress border cell migration and therefore prevent the formation of viable eggs (Montell et al., 1992). The *slbo* gene contains several binding sites of varying affinity for STAT, which causes SLBO expression to follow the same graded pattern as JAK/STAT in normal development, with the highest concentrations in cells adjacent to the polar cells early (as indicated by Green Fluorescent Protein (GFP) reporters (Starz-Gaiano et al., 2008).). A few hours later, however, STAT and SLBO are expressed in a step-wise manner with high levels in cells that become migratory and none in other follicle cells. This observation suggests that high levels of SLBO are positively correlated with high expression of STAT, promoting the migratory pathway, and that this signal must be shut off in non-motile cells.

INHIBITOR MOLECULE FOR MIGRATION: APONTIC

Apontic is a transcription factor that opposes the function of JAK/STAT and SLBO, and functions to inhibit migration (Fig A: Starz-Gaiano et al., 2008.) It is also a downstream target of STAT transcriptional activity, but is additionally turned on by another transcription factor; thus, it is more evenly expressed than SLBO within the domain of the STAT activity gradient. The apontic mutant phenotype in egg chambers is characterized by the migration of additional cells trailing behind the main mobile group, as demonstrated in Fig 2B. This loss-of-function phenotype closely resembles the overstimulation of JAK/STAT, which strongly suggests that normally, Apontic has antagonizing effects on JAK/STAT activity. Prior work shows that Apontic acts as a feedback inhibitor on the JAK/STAT pathway, and that this process is mediated by Apontic's activation of microRNAs that reduce STAT protein (Yoon et al., 2011).

It is clear that Apontic and SLBO inhibit the expression of one another (Starz-Gaiano et al., 2008). Unlike the wild type, apontic mutants maintained high levels of SLBO in follicle cells farther away from the polar cells, and therefore induced the migration of additional cells. In wild-type egg chambers, all eight migrating border cells express SLBO, but when Apontic was overexpressed SLBO protein was undetectable. Additional experiments showed that APT can directly repress slbo transcription. SLBO inhibits the expression of Apontic also, but to a lesser degree, since SLBO overexpression only decreases the levels of Apontic instead of blocking it completely, and expression from the *apt* locus is unchanged. The mechanism for SLBO inhibition of APT is unknown.

BUILDING A MATHEMATICAL MODEL OF A MOLECULAR NETWORK THAT GOVERNS TWO CELL FATES

A simple circuit demonstrates the positive and negative regulatory relationships among all the factors involved in the JAK/STAT signaling system as well as the fates they promote, based on genetic and biochemical data (Fig 2A). However, this representation does not explain well how some cells with STAT activity promoted sig-

naling through APT to remain stationary while others reinforced SLBO activation and became motile.

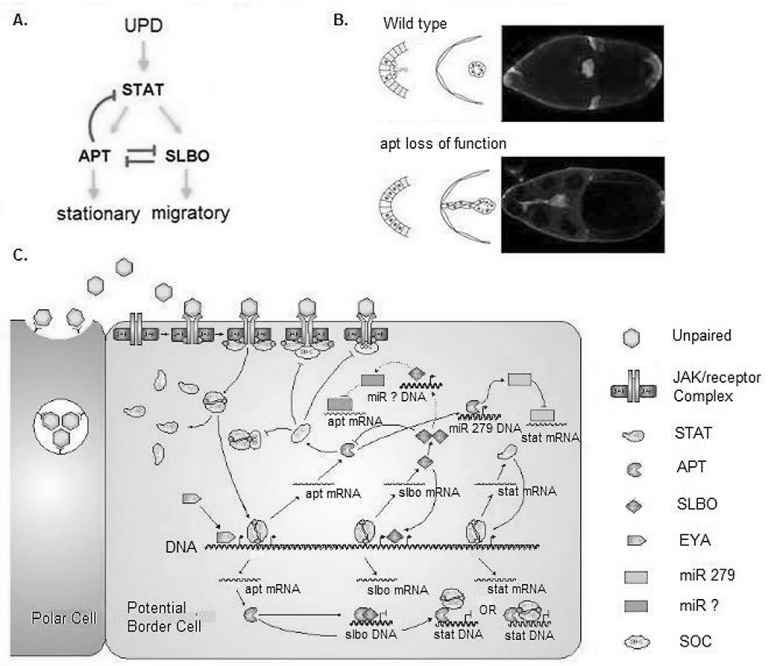

FIG. 2 Genetic components that regulate migratory cell fate**.** Panel A shows the initially proposed biological circuit demonstrating the stimulatory and inhibitory relationships among STAT, SLBO, and Apontic, along with the outcomes they induce (Starz-Gaiano et al., 2008). Apontic inhibits, but is also stimulated by STAT. The high expression of Apontic contributes to the stationary phenotype and inhibits SLBO expression as well. STAT upregulates SLBO, which in turn promotes the migratory behavior. High SLBO represses Apontic expression. The top figures of Panel B illustrate the migration of wild type border cells out of the epithelium. The bottom figures of Panel B show a mutant phenotype, in which a loss of function mutation in Apontic induces the migration of additional cells. The top figures demonstrate the migration of wild type border cells, and they can be found by the edge of oocyte by early stage 10. In comparison, the bottom figures of Panel B show the trailing of additional cells behind the migrating cluster due to the loss of function of Apontic (Starz-Gaiano et al., 2009; Starz-Gaiano et al., 2008). Panel C shows the more comprehensive biological schematic we build upon the simple circuit in Panel A, and was informed by multiple research articles. This schematic attempts to address all the molecular interactions among the factors involved as well as the stimulatory and inhibitory interchanges between them.

How do cells set a threshold to determine the level of signal that is sufficient to promote movement? We observed that initially STAT-positive cells could acquire either motile or non-motile states, and this led us to consider which known components were essential to act as a switch. The simple biological circuit previously described (Starz-

Gaiano et al., 2009; Starz-Gaiano et al., 2008) did not describe all the possible molecular interactions among the components. Similarly, even though the previous mathematical model qualitatively captures the behaviors of the parameters involved, it is a heuristic model that cannot be used to understand or predict the molecular interactions of the system and direct future biological study. To circumvent these problems, we first developed a more comprehensive biological schematic (Fig 2C), and then, developed a mechanistic mathematical model that builds on the elementary reactions, which can be used to determine which components may be more important for understanding the cell fate switching behavior.

UNDERSTANDING THE THRESHOLD LEVEL OF MOLECULAR SIGNALING ESSENTIAL FOR MOTILITY

All follicle cells that receive extracellular Unpaired activate STAT, but cells that are closest to the polar cells (the source of UPD) have high levels of STAT activity and cells that are far away have low STAT activity. It is the cells at an intermediate distance from the polar cells that are of greater interest to us because these are the cells in which cell fate determination becomes crucial. Mutant analysis has shown that disruption of STAT activity in these cells results in poorly specified, poorly motile cells, as is shown in Fig 2B. At this distance the cells turn on an intermediate level of STAT activity, which could either fall above or below the threshold for transition, which is a precise level of STAT activation. Cells that do not surpass the threshold will eventually turn off all STAT activity and remain stationary, whereas cells that do meet the requirement will amplify the signal to become migratory. Because these intermediate follicle cells have the potential to achieve two different fates, we can treat their transition as a bistable system.

The concept of a threshold and bistability allow us to develop a model that consists of two stable steady states for JAK/STAT activity. For our purposes, the rate at which Unpaired is degraded is kept at a constant due to the presumed similarity in metabolic rates of the cells under investigation. The cells in closer proximity to the polar cells are more likely to exceed the threshold of STAT activation than cells farther away, and therefore they transform into mobile border cells, which travel with the polar cells to the periphery of the egg

chamber. There are two stable steady states in JAK/STAT. The first steady state, due to the negligible concentration of Unpaired at time zero, represents an absence of JAK/STAT activity. The second steady state, due to the high concentration of Unpaired at some long time after its initial release, represents the amount of JAK/STAT activity to allow the migratory transition. This model is consistent with our understanding that any subthreshold signaling, as experienced by follicle cells far away from the polar cells, would cause the system to fall back to the first steady state and compel the follicle cells to remain epithelial. Any suprathreshold signaling, as experienced by cells immediately adjacent to the polar cells, would push the system to the second steady state, enabling enough JAK/STAT activity to initiate the migratory pathway and generate mobile border cells that carry the polar cells to the boundary of the oocyte. The details of this dynamic will be explained further in the sections below.

INTERMEDIATE FOLLICLE CELLS ATTENUATE STAT SIGNALING IN THE ABSENCE OF MIGRATION

In a wild type *Drosophila* egg chamber, STAT activity initially forms a gradient across the anterior epithelium (Starz-Gaiano et al., 2008; Xi et al., 2003). This graded pattern is mirrored closely by SLBO expression since STAT acts as a transcriptional activator to increase SLBO expression. Depending on whether or not cells reach the necessary high level of STAT activity, they may or may not prepare to make the transition into motile border cells. As described above, intermediate follicle cells (which do not migrate) initially have STAT activity, but then shut it off in an APT- dependent manner as the border cells move away.

Possibly, the down-regulation of STAT activity could merely be a result of the activator, UPD secreted from the polar cells, being removed as the border cells migrate. To test this idea, we examined carefully-staged egg chambers just before migration takes place, and assayed STAT activity by expression of SLBO. We found that only the rounded-up border cells, and not the intermediate cells, expressed SLBO (Fig 3), even when they were a similar distance from the polar cells. This supports the idea that the gradient of biological activator forms before migration takes place, that migration is not required for the establishment of two distinct cell fates (establishment of a

step-wise STAT activation), and that the intermediate cells have an intrinsic mechanism for down-regulating sub-threshold levels of STAT activity.

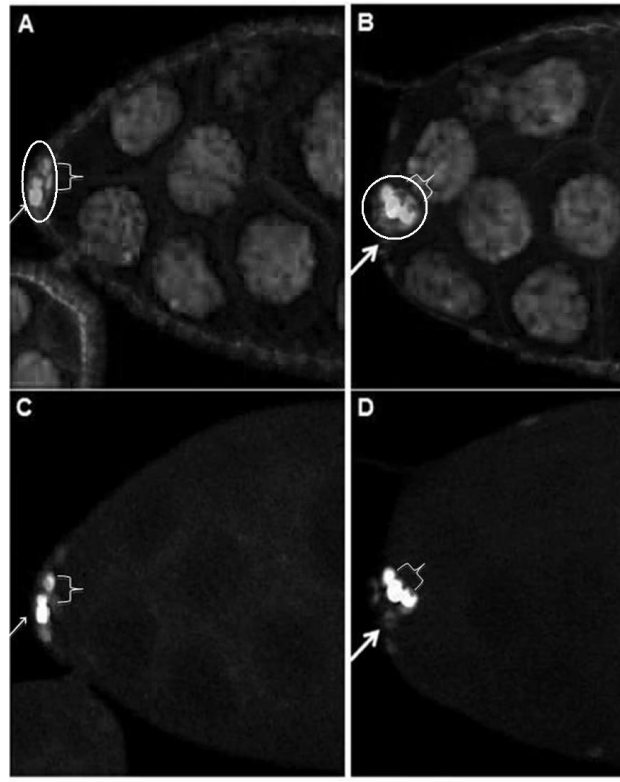

FIG. 3 Intermediate cells downregulate STAT prior to the activating signal being removed. Grayscale images of fluorescent antibodies that label the border cells. All cells receiving STAT signal express some level of SLBO protein (shown alone in bottom panels). DAPI staining indicating nuclear DNA is shown for all cells, and the cell-surface marker, Armadillo, highlights the border cells (circled) in top panels. Panel A and C show a wild type egg chamber at late stage 8. Panel B and D show an egg chamber at early stage 9, in which the border cells have already clustered at the anterior end, ready to migrate. These cells maintain a high level of STAT activity, as demonstrated by the high SLBO expression. The cell indicated by the arrow represents a cell that once turned on STAT, and remains physically close to the activating signal from the polar cells (bracket), but is no longer activated due to its inability to reach the threshold. attempts to address all the molecular interactions among the factors involved as well as the stimulatory and inhibitory interchanges between them.

We were interested in understanding potential cell-intrinsic mechanisms that could generate two major differences in cell fates in response to very small differences in STAT activity levels. Our current model of the JAK/STAT signaling pathway is based on the law

of mass action, which attempts to address not only the positive and negative relationships between regulators, but also the molecular interactions that exist among these players. By invoking the biological function of the players into the model, we can gain a more mechanistic insight of the system, and establish a more comprehensive understanding of the migratory transition of *Drosophila* border cells. Thus, we explored the possibility of achieving a bistable system with a minimal number of components using our mathematical model. We demonstrated that bistability can be achieved by the cross-repressional interactions between APT and SLBO.

MOTIVATION AND CONSTRUCTION OF A MATHEMATICAL MODEL

Previous work has been done heuristically modeling the system described in Fig 2A (Starz-Gaiano et al., 2009; Starz-Gaiano et al., 2008; Yoon et al., 2011). However, while the functional forms of those equations capture the positive or negative interactions between species, those forms are generic and not necessarily the most appropriate forms for the system. Our goal is to utilize the elementary reactions of this system leading to a more detailed, mechanistic mathematical model that captures the underlying architecture of the system.

We develop a set of reaction equations that captures the underlying mechanisms controlling the JAK/STAT pathway. Using this set of reactions, we create a system of differential equations that tracks the production and activation of the molecules in the pathway. We relate this new mechanistic mathematical model to the biological system to identify where the terms originate and how they impact the behavior of the model, where previously it was not possible to relate each term in the equations back to the biological model. The approach results in new insight into the mechanisms of cell motility and helps to inform new testable hypotheses.

The Law of Mass Action is a mathematical representation of chemical reactions that is useful in predicting and explaining the behavior of interacting molecules in a solution (Keener and Sneyd, 1998). Using the reaction scheme in Fig 2C we developed, using the Law of Mass Action, a system of differential equations that track each molecule through time. We systematically analyze each component of the system and write down its reaction equation.

We first consider the activation of the pathway by the ligand, UPD. UPD binds JAK to form activated JAK (JAK*) at a rate k^f_{UJ}. The reverse reaction occurs at a rate k^b_{UJ}. This activated JAK then binds two STAT monomers to form a complex, c_1. An activated STAT dimer (referred to as S^*_2) dissociates from this complex, leaving activated JAK, at a rate k_{c_1}. The dedimerization of activated STAT dimer back into STAT monomers occurs at a rate $k_{S_2{}^*}$. We have written the following set of reaction equations:

$$\text{UPD} + \text{JAK} \underset{k^b_{UJ}}{\overset{k^f_{UJ}}{\rightleftharpoons}} \text{JAK*}$$

$$\text{JAK*} + 2\text{STAT} \underset{k^b_{c_1}}{\overset{k^f_{c_1}}{\rightleftharpoons}} c_1 \xrightarrow{k_{c_1}} \text{JAK*} + S_2{}^*$$

$$S_2{}^* \xrightarrow{k_{S_2{}^*}} 2S$$

This dimerized STAT enters the nucleus and activates transcription of *apt* upon binding its enhancer. Here, *apt's* sensitivity to activation by STAT is described by k_α, which is equal to the ratio of transition rates of activation and deactivation, $k^f{}_\alpha / k^b{}_\alpha$. Transcription of apt can also be activated by the protein Eyes absent (EYA). The gene is transcribed into mRNA at a rate k_{m_α}. The *apt* gene may also be activated by other, STAT independent factors, producing a basal level of apt mRNA at a low, constant rate, $m^o{}_\alpha$. The mRNA from all sources is then translated into protein at a rate k_A. Over time the mRNA and protein degrade at rates δ_α and δ_A, respectively. These interactions with APT are captured in the reactions:

$$\begin{aligned}
\text{EYA} + \text{apt} &\underset{k^b_\beta}{\overset{k^f_\beta}{\rightleftharpoons}} \text{apt*} \\
\text{apt*} &\xrightarrow{k_{m_\alpha}} m_{\text{apt}} + \text{apt*} \\
\text{apt} &\xrightarrow{m^o_\alpha} m_{\text{apt}} \\
m_{\text{apt}} &\xrightarrow{k_A} \text{APT} + m_{\text{apt}} \\
m_{\text{apt}} &\xrightarrow{\delta_\alpha} \varnothing \\
\text{APT} &\xrightarrow{\delta_A} \varnothing
\end{aligned}$$

Similarly, transcription of *slbo* is activated by STAT binding and the rate of activation also has a certain sensitivity to STAT,

k_β. The activated gene is transcribed into mRNA, m_{slbo} which is then translated into SLBO protein. The mRNA and gene product both degrade in the cell at rates δ_β and δ_B , respectively. These interactions with SLBO are captured in the reactions:

$$S_2^* + \text{slbo} \underset{k_\beta^b}{\overset{k_\beta^f}{\rightleftharpoons}} \text{slbo}^*$$

$$\text{slbo}^* \xrightarrow{k_{m_\beta}} m_{\text{slbo}} + \text{slbo}^*$$

$$\text{slbo} \xrightarrow{m_\beta^o} m_{\text{slbo}}$$

$$m_{\text{slbo}} \xrightarrow{k_B} \text{SLBO} + m_{\text{slbo}}$$

$$m_{\text{slbo}} \xrightarrow{\delta_\beta} \emptyset$$

$$\text{SLBO} \xrightarrow{\delta_B} \emptyset$$

STAT protein (monomer) is also produced auto catalytically. Activated, dimerized STAT binds its own enhancer and initiates transcription. The *stat* gene is also assumed to be activated at low levels by other transcription factors producing STAT mRNA (m_{stat}), at a rate m^o_σ, and which is assumed to degrade in the cell at a rate δ_σ. The following reactions describe these interactions:

$$S_2^* + \text{stat} \underset{k_\sigma^b}{\overset{k_\sigma^f}{\rightleftharpoons}} \text{stat}$$

$$\text{stat}^* \xrightarrow{k_{m_\sigma}} m_{\text{stat}} + \text{slbo}^*$$

$$\text{stat} \xrightarrow{m^o_\sigma} m_{\text{stat}}$$

$$m_{\text{stat}} \xrightarrow{k_S} \text{STAT} + m_{\text{stat}}$$

$$m_{\text{stat}} \xrightarrow{\delta_\sigma} \emptyset$$

$$\text{STAT} \xrightarrow{\delta_S} \emptyset$$

We also model the cross inhibition of SLBO and APT and the feedback inhibition of APT on STAT using the same method. SLBO represses APT post-transcriptionally while APT antagonizes SLBO by repressing its transcription. APT has also been shown to turn on transcription of a microRNA intermediate that disrupts the transcription of the *stat* gene. These interactions can be written as:

$$m_{\text{apt}} + \text{SLBO} \xrightarrow{\delta_{B\alpha}} \text{SLBO} + \emptyset$$

$$m_{\text{slbo}} + \text{APT} \xrightarrow{\delta_{A\beta}} \text{APT} + \emptyset$$

$$\text{APT} + \text{slbo} \underset{k_{\beta\alpha}^b}{\overset{k_{\beta\alpha}^f}{\rightleftharpoons}} \text{slbo}^R$$

$$m_{\text{stat}} + \text{APT} \xrightarrow{\delta_{A\sigma}} \text{APT} + \emptyset$$

This complete reaction scheme was rewritten as a system of differential equations, which describes the production, activation, inactivation, and degradation of each chemical in time based on the reaction equations. Fig 4 shows the full system of differential equations (1) - (15). In each equation, the production, activation, or inactivation is modeled with A and B denoting APT and SLBO, with m denoting the mRNA of the gene defined in the subscript (e.g., mRNA of *apt* is written as m_α, where the subscript α implies *apt*). Furthermore, α, β and σ represent the fraction of their respective gene that is transcriptionally inactive. We assume conservation so that the proportion of each gene that is inactive, repressed, and active totals one.

$$\frac{dJ^*}{dt} = k^f_{UJ} U J - k^b_{UJ} J^* - k^f_{c_1} J^* S^2 + k^b_{c_1} c_1 + k_{c_1} c_1 \tag{1}$$

$$\frac{dJ}{dt} = -k^f_{UJ} U J + k^b_{UJ} J^* \tag{2}$$

$$\frac{dS}{dt} = -2k^f_{c_1} J^* S^2 + 2k^b_{c_1} c_1 + 2k_{S_2^*} S_2^* + k_S m_\sigma - \delta_S S \tag{3}$$

$$\frac{dc_1}{dt} = k^f_{c_1} J^* S^2 - k^b_{c_1} c_1 - k_{c_1} c_1 \tag{4}$$

$$\frac{dc_3}{dt} = k^f_{S_2^* A} S_2^* A - k^b_{S_2^* A} c_2 \tag{5}$$

$$\frac{dS_2^*}{dt} = k_{c_1} c_1 - k_{S_2^*} S_2^* - k^f_{S_2^* A} S_2^* A + k^b_{S_2^* A} c_2 \tag{6}$$

$$\frac{dA}{dt} = k_A m_\alpha - \delta_A A - k^f_{S_2^* A} S_2^* A + k^b_{S_2^* A} c_2 \tag{7}$$

$$\frac{dB}{dt} = k_B m_\beta - \delta_B B \tag{8}$$

$$\frac{dm_\alpha}{dt} = k_{m_\alpha}(1-\alpha) - \delta_{m_\alpha} m_\alpha + m^o_\alpha - \delta_{B\alpha} B^2 m_\alpha \tag{9}$$

$$\frac{dm_\beta}{dt} = k_{m_\beta}(1-\beta-\beta^R) - \delta_{m_\beta} m_\beta + m^o_\beta - \delta_{A_\beta} A m_\beta \tag{10}$$

$$\frac{dm_\sigma}{dt} = k_{m_\sigma}(1-\sigma) - \delta_{m_\sigma} m_\sigma + m^o_\sigma - \delta_{A_\sigma} A m_\sigma \tag{11}$$

$$\frac{d\alpha}{dt} = -k^f_\alpha S_2^* \alpha + k^b_\alpha (1-\alpha) - k^f_{\alpha Y} E \alpha + k^b_{\alpha Y}(1-\alpha) \tag{12}$$

$$\frac{d\beta}{dt} = -k^f_\beta S_2^* \beta + k^b_\beta (1-\beta-\beta^R) + k^b_{\beta A} \beta^R - k^f_{\beta A} A \beta \tag{13}$$

$$\frac{d\beta^R}{dt} = k^f_{\beta A} A \beta - k^b_{\beta A} \beta^R \tag{14}$$

$$\frac{d\sigma}{dt} = -k^f_\sigma S_2^* \sigma + k^b_\sigma (1-\sigma) \tag{15}$$

FIG. 4 Full system of differential equations used to model the JAK/STAT pathway. Each equation tracks the production, activation, inactivation and degradation of one component of the pathway shown in Fig 2C. Throughout the system a superscript of an asterisk implies activation and a superscript of an 'R' denotes repression.

ESTABLISHMENT AND ANALYSIS OF BISTABILITY IN THE MATHEMATICAL MODEL

The previous mathematical model (Starz-Gaiano et al., 2008) was constructed to exhibit certain observed features of the biological system, such as bistability. Because our model was built from the elementary interactions, we began our mathematical analysis of our model by determining if it exhibited bistability for any family of parameters. In addition to determining if the system was bistable, we also wanted to isolate which components of the system were sufficient for bistability. We suggest that the simple cross repression system of APT and SLBO (depicted in Fig 5) is the key factor in causing bistability. To determine if this minimal system was responsible for bistability we simplified our equations to only include those components involved in the cross repression.

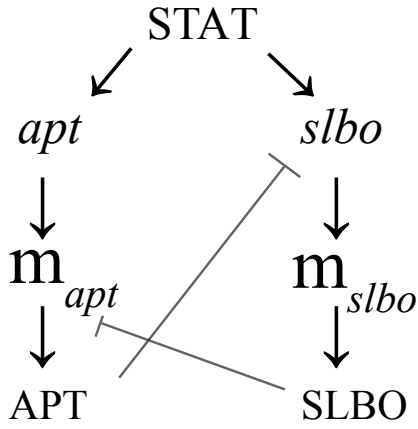

FIG. 5 Genetic circuit explaining the minimal cross repression system used to investigate bistability. SLBO represses *apt* at the gene level and APT represses SLBO at the mRNA level. Arrows indicate activation and lines indicate repression.

We isolate the equations that show STAT activating *apt* and *slbo*, their transcription into mRNA, and translation into protein. We also incorporate APT's inhibition of slbo at the gene/mRNA level and SLBO inhibition of APT post-transcriptionally (Starz-Gaiano et al, 2008). This gives the following system of differential equations to describe the simplified system in Fig 6, (16) - (22).

$$(16)\quad \frac{dA}{dt} = k_A m_\alpha - \delta_A A$$

$$(17)\quad \frac{dB}{dt} = k_B m_\beta - \delta_B B$$

$$(18)\quad \frac{dm_\alpha}{dt} = k_{m\alpha}(1-\alpha) - \delta_{m_\alpha} m_\alpha + m^o{}_\alpha - \delta_{B\alpha} B^2 m_\alpha$$

$$(19)\quad \frac{dm_\beta}{dt} = k_{m\beta}(1-\beta-\beta^R) - \delta_{m_\beta} m_\beta + m^o_\beta - \delta_{A\beta} AmB$$

$$(20)\quad \frac{d\alpha}{dt} = k^f_\alpha S^*_2 \alpha + k^b{}_\alpha (1-\alpha)$$

$$(21)\quad \frac{d\beta}{dt} = k^f_\beta S^*_2 \beta + k^b_\beta (1-\beta-\beta^R)$$

$$(22)\quad \frac{d\beta^R}{dt} = k^f_{\beta A} A\beta - k^b_{\beta A} \beta^R$$

FIG. 6 System of differential equations that captures the simple cross repression system. Note that cooperativity of SLBO to repress APT was found to be essential for bistability and is included in the last term of equation (4).

In order to determine the parameters that produce bistability, we placed this system of differential equations into a steady state (i.e., all of the derivatives are set to zero). We then solved the system of equations for A and B. This gives us the following set of equations.

$$(23)\quad A = \frac{k_A}{\delta_A}\frac{k_{m_\alpha}}{\delta_{m_\alpha}}\left[\frac{\frac{S^*_2}{S^*_2 + k_\alpha} + \frac{m^o{}_\alpha}{k_{m_\alpha}}}{1 + \frac{\delta_{\beta\alpha}}{\delta_{m\alpha}} B^2}\right]$$

$$(24)\quad B = \frac{k_B}{\delta_B}\frac{k_{m\beta}}{\delta_{m\beta}}\left[\frac{\frac{S^*_2 + A/k_{\beta R}\,(1/k^f_\beta - 1)}{S^*_2 + k_\beta + A/k_{\beta R}\,(1/k^f_\beta - 1)} + \frac{m_{o_\beta}}{k_{m_\beta}}}{1 + \frac{\delta_{A\beta}}{\delta_{m\beta}} A}\right]$$

FIG. 7 Steady-state equations

Then we substituted the first equation above into the second equation to generate one equation for B in terms of B. When the left side of this equation is equivalent to the right side the system is in a state of equilibrium. Our goal was to find biologically reasonable param-

eters that achieve three mathematical equilibria, two stable equilibria separated by one unstable equilibrium, indicating bistability. We also sought a system in which decreasing STAT would put the system in only one low steady state, while increasing STAT would lead to one elevated steady state.

Our initial equations (not shown) were unable to produce these results. However, a review of our model and further testing revealed that if we included cooperativity of SLBO to repress APT, then the system exhibits the ability to be bistable where without such a change, the system could never be bistable for any set of parameters. The parameters that we used for the basal bistable system are provided in Table 1.

Initial Parameters		
Parameter	Symbol	Value
Rate of APT Translation	k_A	0.5
Rate of transcription of apt	k_{m_α}	0.9
Rate of STAT independent production of APT mRNA	$m^o{}_\alpha$	0.52
Rate of degradation of APT	δ_A	1
Rate of degradation if mRNA of APT	δ_{m_α}	0.05
Degradation rate of mRNA of APT due to miRNAs	δ_{A_σ}	0.1
Binding rate of STAT to apt	k^f_α	1
and dissociation rate	k^b_α	9
Rate of SLBO translation	k_B	2
Rate of transcription of slbo	k_{m_β}	0.9
Rate of STAT independent production of SLBO mRNA	m^o_β	0.03
Rate of desgradation of SLBO	δ_β	1
Rate of degradation of mRNA of SLBO	δ_{m_β}	0.3
Degradation rate of mRNA to SLBO due to miRNAs	δ_{B_σ}	0.5
Binding rate of STAT to slbo	k^f_β	1
and dissociation rate	k^b_β	6
Rate that slbo transitions into repressed state	$k^f_{\beta R}$	2
and rate it transitions out	$k^b_{\beta R}$	0.522

TABLE 1 Initial parameters that were used to establish bistability in the simplified model. These parameters were perturbed in order to gauge the sensitivity of the bistability of the system. Here, *A* and *B* represent APT and SLBO, respectively. Furthermore, the fraction of transcriptionally inactive apt and slbo genes have been written as α and β .

Fig 8 shows that multiple steady states emerge as STAT increases. Specifically for intermediate STAT activation we have three intersections. Through stability analysis (data not shown), we find that

we have two stable steady states, separated by one unstable steady state. Here, the left side of the steady state equation is plotted using a dashed gray line and the right side is plotted in intermediate level of STAT (black, solid), a lower level of STAT (gray, solid), and with an increased level of STAT (gray, dotted). At a low level of STAT activation, there is only one intersection corresponding to low SLBO production and lack of cell motility. At high STAT levels the system can only be at the activated level of SLBO production and the cell will move. Importantly, for intermediate STAT levels, three intersections emerge, where the system could be either activated or inactivated depending on the initial state of the system. This result is consistent with a model in which there is a threshold, as we predicted.

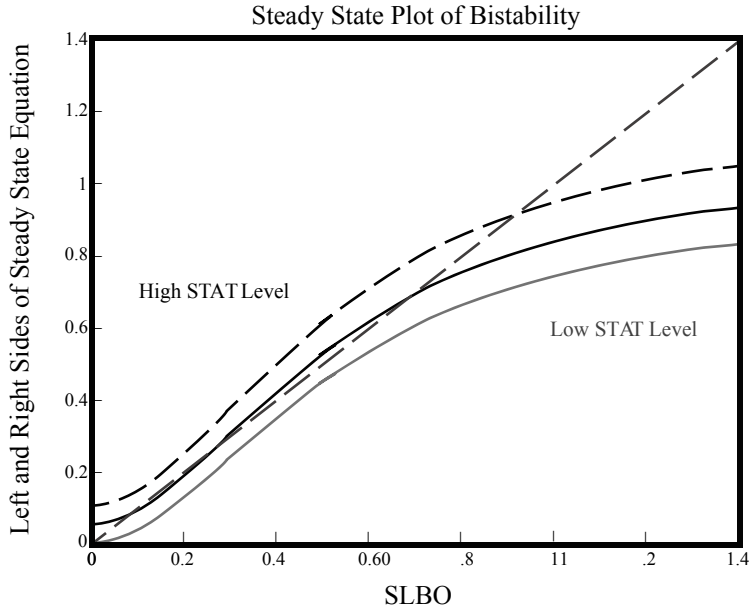

FIG. 8 A steady-state plot reveals bistability at an intermediate level of STAT activity. The left and right hand sides of the steady state equation plotted against SLBO for three levels of STAT activity. A high level of activated STAT causes only activation of the pathway, while a low level of activated STAT leads to only inactivation. However, if there is an intermediate level of STAT, the system could be either activated or inactivated.

This critical result shows that our reduced mathematical system of cross repression is sufficient to produce bistability. Since this model is based on the mechanisms that control the pathway, it helps to validate the observed biological result of bistability in the system. It reveals that this framework of simple cross repression is capable

of producing a bistable system, which is significant for our system and possibly for many others across the biological sciences as well, since many other systems are controlled by biologically similar mechanisms.

We continued our mathematical investigation by analyzing how sensitive the bistability of our model is to variation of the parameters. We wanted to see how the system responds to small changes to the parameters and how bistability was maintained. We did this, in part, because many of these parameters were not well established in previously published literature. We used, as a guideline, parameters from Harris et al. and Starz-Gaiano et al. to estimate several of our parameters. In Fig 8 STAT (which initially is equal to 1) is varied by 15 percent. This leads to a loss of bistability. By changing each parameter and noting how the endpoints of the bistable range move, we can get a sense of how flexible the system is.

We performed a local sensitivity analysis to quantify this change using two measures, 'Bistable Start' and 'Bistable Range' following the methodology of Harris et al (2011). 'Bistable Start' is the lowest STAT value that produces multiple equilibria and is a measure of how the left endpoint of the bistable domain moves in response to the perturbation. 'Bistable Range' is the measure of the change in the bistable range (distance from the left endpoint to the right endpoint) in response to the parameter perturbation. We computed these values for each parameter in the simplified system. Fig 9 shows these values. For example, k_A, the production rate of APT translation, shows a sizeable shift in the initial point of bistability to higher STAT and a significant increase in the range of bistability, while the degradation of APT, δ_A, shows a sizeable shift in the initial point of bistability to lower STAT and a significant decrease in the range of bistability. The reverse is true for the translation and degradation of SLBO. In general it appears that parameters related to enhancing APT increase the range of bistability and begin bistability at higher levels of STAT, while parameters related to enhancing SLBO tend to decrease the range of bistability and begin bistability at lower STAT levels. Furthermore, it is less critical to have exact parameter, given the range of values that can exhibit bistability.

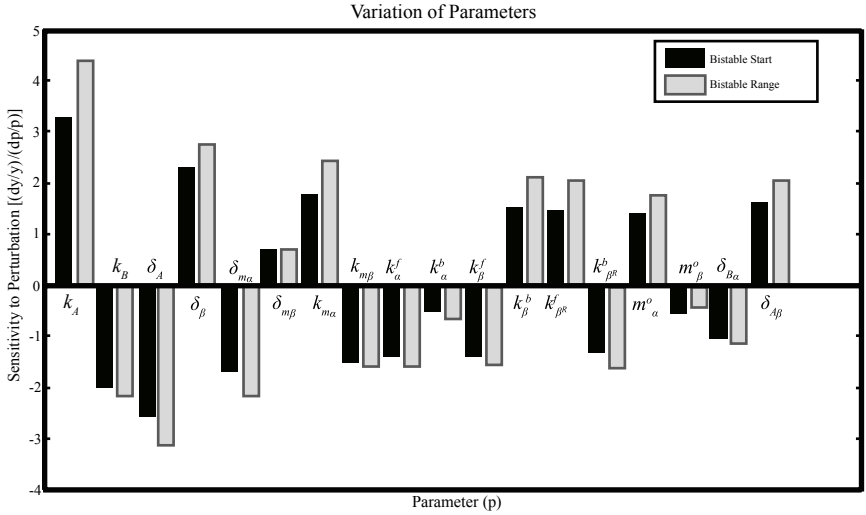

FIG. 9 A quantification of the sensitivity of bistability to parameter perturbation. Bistable Start is [((R$_1$-R$_o$)/Ro)/(**Δ**p/p)], where R$_o$ is the starting right limit point, R$_1$ is the right limit point after perturbation and p and **Δ**p are the parameter value and change in parameter value (10%) respectively. Bistable Range is [(((R$_1$-L$_1$)-(R$_o$-L$_o$))/(R$_o$-L$_o$))/(**Δ**p/p)], where L$_o$ and L$_1$ are the starting and perturbed left limit point respectively. They inform where the range of bistability begins and how it responds to perturbation.

Ultimately, we have shown that the downstream effectors of the JAK/STAT pathway are capable of producing a bistable switch in cell fate. This result, coupled with the results from the bistability assay, confirms that the current model contains all of the important components. We also showed that with the simplified framework must have cooperativity of SLBO to repress APT in order to obtain bistability in the mathematical model. This observation leads us to believe that the full model likely needs to satisfy the same condition in order to achieve bistability, hence the B^2 in equation (9) of the full system.

Several testable hypotheses resulted from the mathematical analysis. One hypothesis is that cooperativity is likely in the repression system. One way cooperativity could occur is by the dimerization of two SLBO monomers or by the auto-regulation of *slbo* transcription, which could be tested in biological assays. Another finding is that the rate of translation is an important component in bistability. The parameter sensitivity analysis showed that the production of APT protein is particularly sensitive, which leads us to consider which biological components could affect this production. We hypothesize that one or more microRNAs, which can control translational rate,

are important. Using biological techniques we are working to determine if there are microRNAs that mediate the negative interactions between APT and SLBO.

MICRO-RNAS

While the initial biological circuit shown in Figure 2A demonstrates the basic relationships among the factors, in reality, the molecular interactions are much more complicated. In fact, we suspect that the inhibitory pathways are regulated by intermediates as well. We believe that there is a possibility that SLBO may be inhibiting APT by upregulating the expression of a microRNA (miRNA) that targets the apt message. miRNAs are short RNA sequences of about 22 base pairs that are normally encoded within the genome. They bind to the 3' untranslated regions (UTRs), which are the regulatory regions, of mRNAs, and block protein translation by either inhibiting the translational process itself or by degrading the mRNA. Whether a given mRNA is degraded or translationally inhibited depends on the number of complementary base pairs between the microRNA and the mRNA itself (Enright et al., 2003). We believe that this type of molecule may play an important role in mediating the cross repression of APT and SLBO because both APT and SLBO are transcription factors. In fact, APT is known to inhibit STAT via upregulation of a miRNA (Yoon et al., 2011).

We performed a database search to determine candidate microRNAs (Betel et al 2010.; Betel et al., 2008; Enright et al., 2003). We took advantage of the fact that the entire genome of Drosophila melanogaster is sequenced and annotated in detail, as well as the fact that the DNA binding site consensus sequence is known for both SLBO and APT. Thus, we were looking for any miRNAs that were complementary to either the *slbo* or *apt* message, and in particular those that also had upstream sequences that matched the consensus binding site for the reciprocal protein. Since the mechanism by which SLBO represses APT is unknown, we are particularly interested in candidates upregulated by SLBO that bind to the 3' UTR of the *apt* mRNA. Strikingly, we found 14 candidates that matched our criteria. This strongly supports the idea that cross-repression between SLBO and APT is very important and therefore highly regulated. The summary of this data is shown in Table 2. Based on available reagents, microRNAs 87and 284 will be the focus for further studies.

microRNA	Binds to	Number of SLBO binding sites	Number of APT binding sites
miR-962	Slbo 3' UTR	1	-
miR-9c	Slbo 3' UTR	1	(1*(9bp))
miR-9b	Slbo 3' UTR	-	(1*(9bp))
miR-927	Slbo 3' UTR	2	(1*(8bp))
miR-315	Slbo 3' UTR	3	2
miR-274	Slbo 3' UTR	-	-
miR-964	Slbo 3' UTR	2	-
miR-1002	Slbo 3' UTR	4	-
miR-252	Slbo 3' UTR and apt 3' UTR	2	2
miR-970	Apt 3' UTR	1	-
miR-87	Apt 3' UTR	1	1
miR-8	Apt 3' UTR	4	-
miR-284	Apt 3' UTR	1	1
miR-1	Apt 3' UTR	-	-
Let-7	Apt 3' UTR	3	-

TABLE 2. Putative microRNA that mediate the cross repressional relationship between SLBO and APT. The top half of the table shows the microRNAs that bind to the 3' untranslated region of the *slbo* mRNA, and the bottom half of the table shows the microRNAs that bind to the 3' untranslated region of the *apt* mRNA. The microRNAs 87 and 284 are the intermediates currently under investigation.

The current mathematical model can accommodate that microRNAs may play a role in the negative relationship between APT and SLBO. In fact, the mathematical formalisms can be virtually the same. We showed that for the reduced system to have bistability there likely is cooperativity of SLBO to repress APT at the gene level. If microRNAs do play a role in this system then it is straightforward to incorporate into the mathematics, but the result from the minimum bistability model will still hold. Based on the cooperativity of SLBO in suppressing APT supported by our mathematical model, we might even predict that SLBO would dimerize to initiate their transcription.

Essential biological processes are highly regulated, and often with redundant mechanisms that ensure robustness. We have used a combination of mathematical and biological strategies to dissect a molecular switch that sets a threshold between two different cellular behaviors. Our work has revealed many levels of regulation that contribute to this system, and we have established a set of

minimal required components to establish bistability. This minimal cross-repression system requires one of the components to act with cooperativity, which we can examine further in vivo.

In addition to the minimal system, the full system can also display bistability, and it is likely that both systems contribute to the outcomes in vivo, accentuating output of the entire network. We also showed that the physical removal of an activating signal via migration is not required for bistability, however it may provide a backup mechanism to help regulate the overall system. Finally, since the minimal bistability model indicated the importance of cross-repression, we searched for molecules that could mediate this regulation. We identified many new miRNA candidates that likely have a role the specification of motile cells, and these will be a focus of further investigation.

Mathematical modeling of bistable systems has been of interest in many aspect of biology. The JAK/STAT signaling pathway, likewise, is important in many contexts, and is well known for a role in stem cell maintenance. Many of the components we have studied in the ovary are also essential in stem cells in other fly tissues, and we hope to extend our modeling analysis to provide insight to these other situations.

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