

## Lindsey Garver- Negative Regulator and Transcription factors Project Outline

My initial intent was to determine whether IgSF family members were regulated by the well-known immune gene transcription factors Rel1 and Rel2. However, after establishing a method to over-activate the factors in the absence of microbial challenge, I stumbled upon a striking phenotype involving Rel2's regulation of *Plasmodium falciparum* development. At the same time, I was publishing the IgSF data and so decided to leave the IgSF work in its current state and focus primarily on the pathway work which is summarized below.

### **Background**

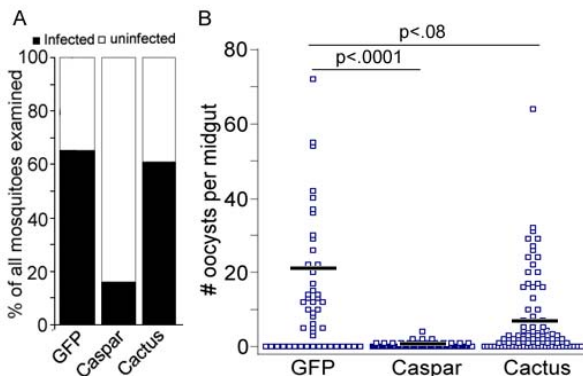
As a necessary part of the *Plasmodium* parasite lifecycle, the mosquito has been a favorable target for intervention. In fact, the only successful wide-spread malaria eradication programs have involved control of the mosquito vector rather than chemical or immunological methods in humans. Malaria control via the vector has recently undergone a renaissance as genome sequence, microarray chips and other advances have moved the insect into the post-genomic era. Molecular methods have given rise to more intricate study of *Plasmodium-Anopheles* interactions; some of the most promising and well-studied interactions being the mosquito immune response toward the parasite. Over millions of years, a balance has been struck between the mosquito's ability to survive infection by limiting parasite numbers and the parasite's ability to survive to continue transmission to the mammalian host. We and others aim to manipulate the mosquito's immune system such that this balance is skewed toward parasite limitation with the hope of generating sustainable populations of Plasmodium-refractory mosquitoes. It is the goal of our lab to understand how the mosquito's anti-*Plasmodium* defenses operate and to identify the best immune components to manipulate for generating refractory insects.

### **Rationale and Preliminary Data**

Much research has focused on immune effector genes (such as anti-microbial peptides) as having dual or unique anti-*Plasmodium* functions. While many show some degree of effect, so far none are capable of completely halting parasite development. Transcription of these effector genes is largely controlled by conserved intracellular signaling pathways so we hypothesized that manipulating pathway components, we may be able to induce transcription of multiple effector genes at once, creating a synergistic anti-*Plasmodium* response. To this end, I developed a research project focused on the negative regulators of the Toll and Imd pathways, two major insect immune signaling pathways. Both pathways have been implicated in directing the anti-*Plasmodium* response and a high degree of conservation between mosquito and fruit fly pathways allows us to hypothesize about the mosquito using what's already known in *Drosophila*. Our overall hypothesis is that by silencing *cactus* and *caspar*, the negative regulators of Toll and Imd, respectively, we will "turn on" immune signaling via transcription

factor nuclear translocation thereby inhibiting parasite development.

Our initial screen used an RNAi method to do just that: silence each negative regulator and follow by feeding mosquitoes on blood infected with *P. falciparum*. *caspar*-silenced



mosquitoes were almost completely unable to support parasite development to the oocyst stage; 85% of mosquitoes had no parasites at all while those that did had just 1-4 oocysts. *cactus*-silencing also affected oocyst development but to a lesser degree: the prevalence of infection was no different from controls but infection intensity was reduced by 66%. See figure at left for representation of this data. (Panel A represents the prevalence of infection in each silenced group while Panel B represents the intensity of infection. Blue dots indicate individual midgut oocyst counts while the black represent the average for the treatment group.)

Further, it seems that these infection phenotypes are conserved with respect to mosquito, but not parasite, species. *caspar* knock-down also inhibits *P. falciparum* development in 2 additional divergent Anopheline species: *A. stephensi*, an Asian vector, and *A. albimanus*, a South American vector (median oocyst count = 0 for both). However, it is Cactus, not Caspar, depletion that causes a drastic decrease in *P. berghei* oocyst loads in *A. gambiae*. Therefore, while Rel2 seems to be the dominant nuclear factor among Anophelines controlling immune gene expression effective against human parasites, it is Rel1 occupying that role for rodent parasites. This also has serious implications for the validity of *P. berghei* as a substitute for *P. falciparum* in immune assays.

So, it seems that both Rel1 and Rel2 are active against *P. falciparum* but in different ways. To begin to address this, I performed microarray-based gene expression analysis to determine the downstream gene expression caused by Cactus or Caspar silencing treatments. This gene expression is likely to be the root of the anti-*Plasmodium* phenotype and is therefore, extremely important for understanding the mechanism. We identified hundreds of genes regulated by either pathway, largely in immune functional groups, and some overlaps exist between the pathways. This is interesting because crosstalk between pathways has long been suspected but is only supported associatively. This is one more line of evidence that points to that conclusion. It is also interesting that genes from other functional groups are well represented. The Toll pathway in particular is known to play multiple roles but array data indicates maybe other processes such as cytoskeletal rearrangement or metabolism are involved in the physiological immune response to the malaria parasite. We coupled quantitative real time PCR assessment of some key immune genes and found the array data to be sound.

Interestingly, we found transcription of several known anti-*Plasmodium* effector genes to be significantly enhanced soon after *caspar* silencing. Thinking that perhaps these effectors are regulated by Rel2 and therefore are responsible for the infection phenotype resulting from *caspar*-silencing, I co-silenced these genes with *caspar* and found that, indeed, depletion of each of three downstream effectors significantly ablate the effect of *caspar* silencing. That is, resistance caused by Caspar depletion is nullified with depleting these effectors strongly suggesting that these factors are regulated by Rel2 and controlling, at least in part, the observed resistance phenotype. Likely, the resistance caused by *caspar* silencing is so complete because all of these effectors and possibly others are enriched at once.

We think that *caspar* or *rel2* would make excellent candidate genes for manipulation in a transgenic mosquito. Unlike other candidate gene products, either *caspar* or *rel2* is a single target that would activate multiple effectors thereby minimizing the genetic engineering complexity while avoiding pitfalls of single gene manipulation like development of resistance,

reversion or compromise of the anti-*Plasmodium* phenotype and incomplete refractoriness. Targeting Caspar or Rel2 as regulators of multiple immune effector genes may also allow us to halt development of different stages of the parasite within the mosquito and initiate anti-*Plasmodium* responses in multiple mosquito tissues. In this way, Caspar or Rel2 transgenics could avoid the limitations of stage and tissue specificity that have hindered efforts using other molecular targets in the vector. However, there exists one main problem: chronic immune induction is associated with reduced fitness which could compromise the ability of the mosquitoes to survive or procreate as well as native mosquitoes. To address this, I performed survival and fecundity experiments on *caspar*- and *cactus*-silenced mosquitoes alongside controls and found that *cactus* silencing had a noticeable effect on survival and fecundity while *caspar* silencing had no effect on these measures of fitness. This data suggests Caspar manipulation, at least under the kinetics of RNAi treatment and laboratory conditions, has no fitness cost. This is probably because RNAi-mediated silencing of *caspar* produces a short-lived but intense immune response that wanes as the dsRNA is degraded.

### ***Current Goals***

Further investigate Imd pathway components and their relationship to the *caspar* infection phenotype.

Further investigate the parasite species-specific effect of *caspar* silencing.

Consider the role of endogenous bacteria in this system.

Determine tissue, timing and parasite stage specificities related to *caspar* silencing and Rel2 activation.

Design and create transgenic *Anopheles* based on this strategy.