**Application of Cell Based Assays to Evaluate Gene Function in the North American Gray Wolf (*Canis lupus*)**

**Introduction:** With the advent of next generation sequencing technologies, the identification of genes under selection has become a central focus of evolutionary studies. Yet, such studies are often speculative and without independent support. I intend to develop a cell culture-based method, utilizing CRISPR-Cas9 technology to causally connect candidate genes with their function under controlled conditions *in vitro*. This research builds on resources developed by Ph.D. candidate Rachel Johnston while in Dr. Robert Wayne's lab. This resource consists of 26 cell lines and two CRISPR-Cas9 edited cell lines established from gray wolves (*Canis lupus*), which I will use to develop a system to test hypotheses of gene function. I hypothesize that canine β-defensin 3 (*CBD103* or the K locus) is involved in conferring immune response to viruses. To test this, I propose to challenge three wolf cell lines of differing *CBD103* genotype with an ecologically relevant pathogen encountered by the wild population, canine distemper virus (CDV).

*CBD103* is a particularly interesting and suitable gene to consider given: **1)** a mutation in *CBD103* has undergone a selective sweep in gray wolves, leading to an easily observable phenotype (coat color) in wild populations; **2)** the mutation has been well-described and can be manipulated in a laboratory setting; and **3)** the functional role that *CBD103* plays in innate immune response can be tested using *in vitro* methods. A 3 base-pair (bp) in-frame deletion in an exon of *CBD103* [1] is known to cause a change in coat color, in which the wild type allele ($k^Y$) codes for a gray coat in wild type homozygous wolves and yellow coat in dogs, whereas a dominant mutant allele ($K^B$) causes a black coat color in both species [1, 2]. This mutation was introduced to wolves through ancient hybridization with dogs and underwent a selective sweep uniquely in North America [2]. Although this deletion confers a change in coat color, the actual phenotype under selection may not be related to coat color, as heterozygote black wolves have higher survivorship and lifetime reproductive success in a variety of environments [3]. Moreover, black homozygotes have very low fitness and are rare in populations [3]. Apparently, having a single copy of $K^B$ paired with the wild type confers high fitness, whereas having two copies of $K^B$ greatly impairs fitness [3], offering discrete states for testing in a cell-based assay.

I predict that *CBD103* is involved in the wolf immune system, as it is a β-defensin and these genes generally function as part of the innate immune system [4]. Human β-defensin 3 (*DEFB103*), the human homolog to *CBD103*, has antibacterial and antiviral properties, and incites a pro-inflammatory response to microbial antigens [4, 5]. The role of *CBD103* in immunity is yet unknown, but population modeling suggests it may be important. CDV has led to short-term decreases in wolf populations surveyed in Yellowstone National Park (YNP) due to the high mortality rate of infected pups and thus may elicit a selective pressure [6]. Interestingly, black wolves ($K^B/ k^Y$ and $K^B/ K^B$) in YNP have higher survivorship rates than gray wolves in years when CDV rates have been high (Wayne, pers. comm.). Thus, I predict that $K^B/ k^Y$ genotype confers higher immunocompetence to CDV. I propose to evaluate this possibility using *in vitro* methods that have been established in model species [e.g. 7].

**Resources:** To investigate the effect of this mutation in *CBD103*, epithelial cells from a homozygous wild type individual ($k^Y/k^Y$) were immortalized and manipulated with CRISPR-Cas9 to produce two cell lines carrying the 3 bp deletion: a heterozygous ($K^B/ k^Y$) and a homozygous mutant ($K^B/ K^B$). Because these three cell lines are derived from one individual and thus have the same genomic background, they serve as a unique control for mitigating the effect of genetic factors other than the changes in the *CBD103* gene.

**Methods:** I will utilize the CDV 5804 strain, which has been altered to express green florescent protein (GFP) to allow for assessing infection rates [8]. Total RNA will be collected from infected (experimental) and non-infected (control) cells in 12 hour increments ranging from 0 to 48 hours, and will be processed for RNA-seq. Infection rates and virus propagation will be assessed by: 1) measuring GFP expression in cells 3-5 days following infection and 2) harvesting virus from the supernatant. Analysis following the collection of RNA-seq data will include establishing gene expression networks and testing for differences in gene expression response to CDV between cell lines. If the $K^B$ allele confers a functional response to CDV, I expect the gene expression network observed in the wild type cells to display differences between control and experimental cells, particularly in genes associated with viral response pathways. Furthermore, if a heterozygote advantage exists, I expect heterozygotes will **1)** have faster and larger changes in expression in genes identified in wild type cells and **2)** have lower levels of viral infection reflected in a smaller percentage of cells expressing GFP and lower viral titers in the supernatant. If differences in functional response to CDV is not observed between cell lines, it is possible *CBD103* genotype does not provide a functional advantage to CDV, but may provide protection against other antigens and/or may serve a functional role beyond coat color in other tissue types.

**Intellectual Merit:** This research will explore the impact of *CBD103* genotype in gray wolf populations when challenged with a naturally occurring pathogen CDV using a cell-based assay. My study will allow for further experiments such as testing the impact of other pathogens (e.g. canine parvovirus), developing cell lines from other tissues, and investigating the unexplored role of DNA methylation in mediating gene expression responses to environmental challenges in these cells. This cell-based assay will allow for further exploration into the ecological challenges facing this keystone species, the ability of grey wolf populations to tolerate them, and potentially contribute to population management decisions.

**Broader Impact:** This project has the potential to establish and validate an assay for a much-needed protocol to investigate the functional genomics of non-model multicellular systems that can be applied across taxa and fields. Specifically, my research will show that a challenge experiment can test genotype specific response in CRISPR-Cas9 edited cell lines. Other candidate genes under selection in a wide variety of systems beyond wolves can be tested with challenges such as pathogens, varied temperatures, or altered cell conditions to test functional hypotheses to more definitively build a case for genotype-phenotype relationships. I intend to use my Ph.D. training and this research to engage with the public on the importance of research in the molecular sciences and environmental conservation of our nation's wildlife. Completion of this project will assist me in my goal of becoming a professor and training future scientists, especially under represented minorities.

[1] Candille SI, Kaelin CB, Cattanach BM, et al. (2007) A ß-defensin mutation causes black coat color in domestic dogs. *Science* 318, 1418. [2] Anderson TM, vonHoldt BM, Candille SI, et al. (2009) Molecular and evolutionary history of melanism of North American gray wolves. *Science* 323, 1339. [3] Coulson T, Macnulty D, Stahler DR, et al. (2012) Modeling effects of environmental change on wolf population dynamics, trait evolution, and life history. *Science* 334, 1275. [4] Wilson SS, Wiens ME, & Smith JG. (2013). Antiviral mechanisms of human defensins. *J Mol Biol* 425, 4965. [5] Harvey LE, Kohlgraf, KG, Mehalick, LA, et al. (2013). Defensin DEFB103 bidirectionally regulates chemokine and cytokine responses to a pro-inflammatory stimulus. *Sci Rep* 3, 1232. [6] Almberg ES, Mech LD, Smith DW, Sheldon JW, & Crabtree RL. (2009). A serological survey of infectious disease in Yellowstone National Park's canid community. *PLoS One* 4, e7042. [7] Pickrell JK, Marioni JC, Pai AA, et al. (2010). Understanding mechanisms underlying human gene expression variation with RNA sequencing. *Nature* 464, 768. [8] von Messling V, Milosevic D, & Cattaneo R. (2004). Tropism illuminated: lymphocyte-based pathways blazed by lethal morbillivirus through the host immune system. *Proc Natl Acad Sci USA* 101, 14216.