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Dissection of QTL on Host Chromosome 12 Uncovers Candidate Gene and Missense Polymorphism Associated with Porcine Circovirus 2 Susceptibility

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DISSECTION OF QTL ON HOST CHROMOSOME 12 UNCOVERS CANDIDATE GENE AND MISSENSE POLYMORPHISM ASSOCIATED WITH PORCINE CIRCOVIRUS 2 SUSCEPTIBILITY

By

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DISSECTION OF QTL ON HOST CHROMOSOME 12 UNCOVERS CANDIDATE GENE AND MISSENSE POLYMORPHISM ASSOCIATED WITH PORCINE CIRCOVIRUS 2 SUSCEPTIBILITY

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Porcine circovirus 2 (PCV2) is a small single stranded DNA virus responsible for a group of detrimental diseases referred to as Porcine Circovirus Associated Diseases (PCVAD). Observed variation in incidence and severity of PCVAD between pigs suggests a host genetic role in facilitating PCV2 pathogenesis. This study builds on prior research by Engle et al. (2014), who performed a large-scale genome-wide association study of 974 crossbred pigs experimentally infected with a PCV2b isolate and provided evidence of a host genetic role in PCV2 viremia, immune response, and growth. Two major Quantitative Trait Loci (QTL) were identified for viral load located on chromosome 7 (SSC7) near the swine leukocyte antigen complex class II (SLAII) locus and the proximal end of chromosome 12 (SSC12). The SNP with largest association, *ALGA0110477* (SSC12), explained 11.1% of the genetic variance and 7.4% of the phenotypic variance for viral load.

Dissection of the SSC12 QTL region using gene annotation and both genomic and RNA sequencing uncovered a novel missense polymorphism within *SYNGR2* (*p.Arg63Cys*) that exhibited the largest association with PCV2 viremia and immune response. *In vitro* gene silencing of *SYNGR2* was performed on porcine kidney 15 cell line (PK15) using siRNA designed against the *SYNGR2* mRNA sequence. A substantial

decrease in *SYNGR2* mRNA expression (82.2%) was achieved and corresponded with a significant reduction in PCV2 titer beginning at 48 hours post infection ($P<0.05$) compared to scramble siRNA and non-transfected control cells, indicating a role of *SYNGR2* in viral replication. The *SYNGR2 p.Arg63Cys* mutation is located within a protein domain conserved across mammals and results in an amino acid substitution (Arg→Cys) unique to swine. The impact of *SYNGR2* on PCV2 replication and location of a non-conservative substitution within a key domain provides strong evidence that the *SYNGR2 p.63Cys* variant underlies the observed genetic effect on viral load by potentially interfering with *SYNGR2* activity. These findings provide important insight into the role of host genetics in PCV2 pathogenesis.

Key Words: PCV2, susceptibility, viremia, immune response, replication, siRNA, synaptogyrin-2, SYNGR2

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CHAPTER 1

LITERATURE REVIEW

History of PCV2

Today, Porcine Circovirus 2 (PCV2) is recognized as a key threat to swine populations worldwide. However, its characterization as a distinct and disease causing pathogen did not occur until many years after its suggested emergence in 1962 [1] . The virus was first identified in 1974 as a contaminate of a porcine kidney cell line (ATCC-CCL31) that caused no observable cytopathic effects [2, 3]. This contaminant was initially believed to be a picornavirus-like virus, but upon further analysis was determined to be a single-stranded DNA virus with a covalently closed-circular genome, about 1.76kb in length [3, 4]. The composition and properties of this virus were different from any known animal pathogen and specific antibodies were found in sera collected exclusively from pigs, indicating they were the likely species of origin [4]. As a result, the unique virus was given the name porcine circovirus (PCV) and has since been assigned to the *Circoviridae* family [4, 5].

Initial experimental infection of pigs with the PK-15 PCV isolate induced no signs of clinical disease or evidence of viral replication, however, PCV specific antibodies were detected in blood samples collected from the infected pigs [6]. Another experimental infection using colostrum-deprived pigs validated the lack of pathogenesis, but was able to detect PCV antigen in tissues throughout the body indicating viral replication [7]. PCV specific antibodies were also present in asymptomatic conventionally raised Canadian pigs and wild boars from the Berlin region of Germany. Thus, PCV was deemed a non-pathogenic virus common of all swine [2, 6, 7].

In 1991, a new disease emerged in a previously healthy herd of Canadian pigs free of any major enteric and respiratory diseases [8, 9]. Clinical signs appeared in

affected piglets about 2-3 weeks post-weaning and included wasting, dyspnea, jaundice, and lymphoid depletion [10]. This disease soon became known as Post-Weaning Multisystemic Wasting Syndrome (PMWS), with the first description of PMWS given in 1996 [10]. PMWS cases have since been documented worldwide in piglets 5-12 weeks of age and are associated with increased rates of mortality [8]. Subsequent investigations into the culprit behind PMWS uncovered a potential link between disease onset and a novel type of PCV, overturning the precedent that PCV was strictly non-pathogenic.

In 1998, a virus similar in size and morphology to the PK-15 PCV isolate was found in tissue samples collected from Californian and French pigs with clinical signs of PMWS, but not in healthy controls [11]. The ability to detect PCV-like nucleic acid using *in situ* hybridization with a PCV genomic probe indicated that this virus was genetically similar to the PK-15 PCV isolate, however, a difference in reactivity to monoclonal antibodies suggested that it possessed distinct antigenic properties [11]. Genomic comparison revealed that the nucleotide sequence similarity was less than 80% between isolates from PMWS affected pigs and the PK-15 PCV isolate [12, 13]. Based on these findings, a proposal was made to break PCV into two groups with the new potentially pathogenic form classified as Porcine Circovirus type 2 (PCV2) and the non-pathogenic PK-15 isolate as Porcine Circovirus type 1 (PCV1) [12].

Although PCV2 was detected in archived tissue samples collected as early as 1962 from pigs of Northern Germany, PCV2-associated pathological changes and lesions did not appear in samples until 1985 [1]. In fact, the ability to detect viral DNA in samples between 1962 and 1984 was highly variable with an estimated prevalence of about 2.5%. However, this estimate sky-rocketed to over 30% in samples from 1985 and

onward, coinciding with the first incidents of associated lesions and reports of PMWS [1, 14]. Specifically, the prevalence of PCV2 within PMWS affected farms was found to be much higher than in non-affected farms as well as in PMWS affected pigs compared to non-affected pigs [15]. PCV2 has since been implicated in a variety of other diseases and symptoms aside from PMWS, such as Porcine Respiratory Disease Complex (PRDC), Porcine Dermatitis and Nephropathy Syndrome (PDNS), weight loss, reproductive failure, and lymphoid depletion [16-19]. In 2006, these diseases became collectively referred to as Porcine Circovirus Associated Diseases (PCVAD) [20].

Porcine Circovirus Associated Diseases

According to the American Association of Swine Veterinarians (AASV), PCVAD may be subclinical or consist of one or more clinical manifestations including: multisystemic disease with wasting, respiratory problems, PDNS, enteric conditions, reproductive disorders, and high mortality that are present solely or combined in a group of animals [20]. The Veterinary Diagnostic Laboratory at Iowa State University, classifies systemic infection and PCV2-associated pneumonia, enteritis, reproductive failure, and PDNS under PCVAD in the diagnostics database [20]. In an individual pig, PCVAD is diagnosed by microscopic lesions with an abundance of PCV2 antigen. Evaluation of intestine, lung, and lymphoid tissue for the presence of PCV2 antigen by immunohistochemistry must be performed in order to determine specific PCVAD manifestation [20].

a) *Subclinical PCV2 Infection*

A diagnosis of subclinical PCV2 infection is characterized by low levels of PCV2 antigen that are not responsible for any clinical disease. There may be no or minimal lesions, with infection often limited to one or two lymph nodes [20].

b) *Post-Weaning Multisystemic Wasting Syndrome (PMWS) and Systemic Infection*

Systemic PCV2 infection is characterized by lymphohistiocytic or granulomatous inflammatory lesions in lymphoid tissues, lung, intestines, liver, and/or heart [20]. The first symptom is often weight loss, jaundice, or failure to thrive [20]. In order for PCV2 infection to be diagnosed as systemic, PCV2 antigen must be found in more than one lymphoid tissue, one lymphoid tissue and one organ system, or two organ systems [17, 20]. If found in only one organ system than the diagnosis would be one of the other PCVAD manifestations and not systemic infection [20]. PMWS falls within the realm of systemic PCV2 infection. In order for a pig to be diagnosed with PMWS the following requirements must be met: 1) wasting, weight loss, and failure to thrive with or without labored breathing or jaundice, 2) histological lesions with lymphoid depletion or granulomatous inflammation present within any organs, and 3) detection of PCV2 infection within these lesions [21, 22].

c) *Porcine Respiratory Disease Complex (PRDC)*

Respiratory disease cases that involve multiple pathogens are often characterized as PRDC [16]. Characteristic symptoms of PRDC include decreased growth rate and feed efficiency, anorexia, cough, labored breathing, and fever [20]. In 2000, over 3,000 cases

of swine pneumonia were diagnosed at the Iowa State University Veterinary Diagnostic Laboratory, with 694 cases classified as PCV2-associated pneumonia [16]. Of the 694 cases, 56% presented with concurrent Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) infection, 19% with *Mycoplasma hyopneumoniae*, and 12% with Swine Influenza Virus (SIV) [16]. Three case studies, revealed that concurrent infection with PCV2 and PRRSV, *M. Hyopneumoniae*, or SIV resulted in PRDC [16]. High levels of PCV2 antigen found in lesions associated with granulomatous bronchointerstitial pneumonia with bronchiolitis and bronchiolar fibrosis lend support for role of PCV2 in prolonged respiratory disease [16, 20].

d) *Porcine Dermatitis and Nephropathy Syndrome (PDNS)*

Characteristic symptoms of PDNS include prominent raised purple skin lesions commonly distributed on the hind legs, lethargy, and fever [20, 23]. Renal lesions are also common and indicative of glomerulonephritis believed to be due accumulation of antigen-antibody aggregates and immune complexes within specific tissues [20, 23]. PDNS is often fatal, with animals succumbing after only a short period of clinical disease [23]. In a case control study, PCV2 was found in 100% of PDNS affected pigs predominantly in the lymph nodes and tonsils [24]. Extremely high levels of PCV2 antibodies were consistently found in the serum of affected pigs and were significantly higher compared to control pigs [24]. Accumulation of IgG and IgM antibodies as well as complement factors and CD8+ cells were also found in the kidneys of affected animals, indicating that excessive amounts of PCV2 antibodies caused by PCV2 infection may induce PDNS [24].

e) *PCV2-Associated Enteritis*

Clinically, PCV2-associated enteritis mimics ileitis due to *Lawsonia intracellularis* presenting with diarrhea, thickened intestinal mucosa, and enlarged mesenteric lymph nodes [20]. Six weaned pigs were diagnosed with PCV2-associated enteritis using histopathology, viral isolation, and *in situ* hybridization [25]. From this, the authors propose that in order to diagnose PCV2-associated enteritis the following criteria must be met: 1) presence of diarrhea, 2) typical lesions are found in Peyer's patches, but not other lymph nodes, and 3) PCV2 DNA or antigen must be detected within these lesions [25].

f) *PCV2-Associated Reproductive Failure*

Although rare amongst breeding populations, affected herds present with increased number of abortions, stillbirths, mummifications, and preweaning mortalities [20, 26]. Field cases indicate that myocarditis is the most common lesion to be found in stillborn or neonatal pigs along with elevated levels of PCV2 antigen [19]. Reproductive abnormalities have also been induced through experimental intrauterine infection of fetuses with PCV2, supporting the classification of PCV2 as a reproductive pathogen [27].

Taxonomy, Characteristics, and Genomic Organization

PCV1 and PCV2 belong to the *Circoviridae* family and are classified under the *Circovirus* genus. Members of the *Circoviridae* family are small, non-enveloped, single-stranded DNA viruses known to infect both pigs and birds [5, 28]. They have circular genomes approximately 1.8 to 2.3kb in length with a common stem loop structure

containing the origin of replication (Ori) [20, 29, 30]. The genomic organization of circoviruses and their mechanisms of replication closely mirror those of plant geminiviruses and nanoviruses [30]. In fact, further analysis of the Rep protein revealed a substantial degree of similarity between the n-terminal regions in circoviruses and nanoviruses [31]. However, the c-terminal region of the PCV Rep protein more closely resembles an RNA binding protein of a vertebrate RNA calicivirus [31]. Therefore, it has been proposed that PCV evolved after a recombination event between a nanovirus and calicivirus.

There are currently 11 species of circoviruses including both types of PCV and various avian circoviruses [30]. PCV1 and PCV2 are the smallest of the circovirus species as well as among the smallest known viruses able to infect mammals and carry out autonomous replication [30, 32, 33] . The PCV virion is composed of 60 capsid protein subunits arranged in 12 pentamer clustered-units to form an icosahedron with T=1 symmetry that is 17±1.3nm in diameter [14, 30, 32]. The circular single-stranded DNA genome packaged within these viral particles is approximately 1.7kb in length, encoding only what is required for replication and infection [30].

Recently a new genotype of porcine circovirus, PCV3, was identified in pigs from Missouri, Minnesota, and South Dakota with cardiac and multi-systemic inflammation [34]. The strain was identified by metagenomic analysis. The Rep and Cap proteins of PCV3 share the most homology, 55% and 35% respectively, with a circovirus found in bat feces and less homology with PCV1 and PCV2, indicating PCV3 to be a novel member of the *Circovirus* genus and the third found in pigs [34].

Both PCV1 and PCV2 have 11 potential Open Reading Frames (ORFs), two of which are necessary for viral replication and assembly [35]. ORF1 encodes the replicase proteins, Rep and Rep', and ORF2 encodes the capsid protein. The PCV genome is considered ambisense because ORF1 and ORF2 are aligned in opposite directions, such that ORF1 is oriented in the sense direction and ORF2 in the antisense direction relative to the Ori [14, 30]. The Rep protein is encoded by the full ORF1 transcript (35.6 kDA), while Rep' results from an alternatively spliced ORF1 transcript (19.2 kDA) [29, 36]. Both proteins are required for replication in PCV1 and PCV2 with the capacity to instigate replication at heterologous Ori [37]. The proteins differ slightly in size between PCV1 and PCV2, however, four conserved sequence motifs were identified and deemed crucial for Rep to initiate replication with any truncation or point mutation within a motif leading to protein inactivation [36]. Three of these motifs are typical for proteins utilized in rolling circle replication and the other contains the consensus sequence for a dNTP-binding box (GKS) [29, 36]. Additionally, an interferon-stimulated response element (ISRE) was discovered in the Ori of the PCV2 genome that was shown to affect *in vitro* interferon-mediated enhancement of PCV2 replication when present in an intact virus [30, 38]. The capsid protein is approximately 30 kDA in size and identified as the primary structural protein with the ability to self-assemble into capsid-like particles that encapsulate the PCV genome [39].

There are two other ORFs that encode unique non-structural viral proteins not involved in replication, but in other mechanisms of PCV2 infection. The ORF3 protein differs significantly in size between PCV1 (23.2 kDA) and PCV2 (11.9 kDA), with only about 62% sequence homology [35]. It is primarily found in the nucleus of infected cells,

peaking around 48 hours post infection and has been implicated in PCV2-induced apoptosis via activation of caspase-8 and caspase-3 pathways [40]. As a result, it is believed to facilitate local viral spread by catalyzing early viral release from infected cells as well as systemic dissemination by inducing macrophage phagocytosis of apoptotic cells [41]. ORF4 completely overlaps ORF3 and encodes a 13.3 kDA protein in PCV1 and a 6.5 kDA protein in PCV2, with 83% sequence homology [35]. It is believed to counter virus-induced apoptosis by regulating expression of the ORF3 protein as well as reduce viral replication via interactions with ORF1 mRNA during early stages of infection [42]. In fact, cells infected with ORF4-deficient PCV2 mutants showed increased ORF3 expression, caspase activity, and early viral growth [42].

Genetic Variation in PCV2 Strains

Although closely related, PCV2 shares only about 68-76% sequence similarity with PCV1 [13, 14, 30]. A greater proportion of sequence discrepancies appear in ORF2 than ORF1 with nucleotide homologies of approximately 67% and 83% respectively [13]. As a result, the amino acid sequences of the ORF1 protein, Rep, is more conserved with 85% sequence homology between PCV1 and PCV2 while the Cap protein encoded by ORF2 is more variable, sharing only about 65% similarity [13]. The overall nucleotide sequence similarity between PCV2 isolates is significantly higher with 95% homology or greater [12]. However, majority of differences once again occur within ORF2 with approximately 90% nucleotide and amino acid homology across isolates [14]. Therefore, it is believed that variation in the cap protein may contribute to differences in isolate pathogenicity [43].

PCV2 isolates cluster into distinct sub-groups or genotypes designated by lower case letters [44], which are subsequently split into clades [45]. However, classification of PCV2 isolates is not always clear cut due to genetic recombination between PCV2 strains during coinfection, which has been illustrated in multiple studies to generate chimera isolates resembling multiple sub-groups [46] and clades [47, 48]. Furthermore, novel PCV2 strains continue to emerge at a rapid pace due to a high mutation rate [49]. In fact, following the comparison of 160 PCV2 genomes, Firth et al. (2009) deduced that PCV2 has the highest nucleotide substitution rate of any ssDNA virus to date, reaching levels more commonly achieved by RNA viruses [50].

Currently, PCV2 is divided into three subgroups denoted PCV2a, PCV2b, and PCV2c, with PCV2a and PCV2b found worldwide. The existence of two more groups, PCV2d and PCV2e, has been proposed based on isolates from China [51], however, the methodology used in this study was determined insufficient to support classification as separate sub-groups [52]. Analysis of archived samples dating back to the early 1980s indicate that PCV2a precluded PCV2b in Northern Germany and the United Kingdom [1, 53] with similar reports corroborating this pattern in other countries, such as Denmark [54] and China [51]. Although PCV2a and PCV2b were present in European countries and China prior to 2003, phylogenetic analysis indicated a dramatic shift in predominance towards the PCV2b sub-group at this time [14, 30]. Shortly after, PCV2b also emerged in North America and Canada coinciding with increased incidences of PCVAD [55, 56]. PCV2c was isolated from archived tissue samples between 1980-1990 from Danish pigs prior to PCVAD outbreaks in 2003 and 2004. Phylogenetic analysis revealed a shift from PCV2c to PCV2a in the 1990s followed by another shift to PCV2b

in the early 2000s [54]. Therefore, PCV2c may represent a precursor to both PCV2a and PCV2b while PCV2b may represent the most adapted sub-group associated with increased pathogenicity [14, 54].

PCV2 Transmission

The transportation of asymptomatic animals is believed to have enabled the rapid dissemination of virulent PCV2 strains around the world as the geographic patterns of PCV2 appearance mirror those of the global pig trade [49]. Transmission of PCV2 often occurs via direct contact through oronasal, fecal, and urinary tracts [20]. In one study, direct contact with pigs infected with PCV2 42 days prior resulted in transmission to three out of three colostrum deprived control pigs [57]. Shedding patterns of PCV2 were analyzed using PCR for detection of viral DNA in samples collected from 16 PCV2 experimentally infected pigs and 313 field cases [58]. The presence of PCV2 was detected one day after inoculation in all oropharyngeal swabs, nasal swabs, and feces samples and 7 days after inoculation in whole blood and serum samples obtained from the experimentally infected pigs and remained positive for 70 days post infection for most of the samples [58]. In field cases, detection of PCV2 DNA using PCR occurred in 30.4% of whole blood samples, 19.2% of nasal swabs, and 20.4% of fecal samples with the frequency of positive samples increasing in post-weaning pigs approximately 3-4 months old [58]. Another study, including 146 pigs classified as either PMWS-affected, PCV2 subclinically infected, or PCV2 negative, found that in general trachea-bronchial swabs had the highest PCV2 load followed by serum, tonsillar, nasal, fecal, and urinary swabs [59]. Different levels of PCV2 were detected between the three groups, with

PMWS affected animals exhibiting significantly higher proportions compared to the subclinically infected or negative groups [59]. Therefore, the authors conclude that PCV2 is excreted through respiratory and oral secretions as well as in feces and urine of both PMWS affected and subclinically infected pigs [59].

Evidence of vertical PCV2 transmission has also been illustrated both experimentally and in the field [20]. Specifically, transplacental transmission was shown in cases of reproductive failure, late-term abortions, and stillbirths in which PCV2 antigen was detected in various tissues of dead piglets [19, 60]. Another study focused on the ability of PCV2 to infect and its effects on late term fetuses [27]. Of the 37 fetuses infected intramuscularly through the uterine wall between 86 and 93 days of gestation, 24 appeared phenotypically normal and 13 were unthrifty at farrowing, mummified, or stillborn. PCV2 infection was detected in all 37 piglets, providing experimental evidence that PCV2 can infect late term fetuses as well as induce reproductive abnormalities [27]. Additionally, intermittent shedding of PCV2 in semen of infected boars has been illustrated by several studies [61-63] with PCV2 predominantly found within the seminal fluid and non-sperm cell fraction [61]. Despite sporadic long term shedding in semen of infected boars, no morphological or detrimental changes to sperm cells were observed in the presence of PCV2 [63]. It is not known whether PCV2 in semen is infectious or capable of transmission via artificial insemination, however, the potential threat this poses to breeding programs worldwide is cause for concern.

PCV2 Cellular Pathogenesis

The molecular mechanisms underlying PCV2 pathogenesis remain somewhat elusive due to inconsistencies in PCV2 virulence that make it difficult to pinpoint exactly how PCV2 elicits disease symptoms. However, pathogenesis is believed to begin with PCV2 entry via oral and nasal cavities followed by viral replication in local lymphoid tissues and subsequent systemic dissemination of the virus to other tissues and organs [64]. Circulating PCV2 can be detected in sera by 7 days post infection (dpi), but peaks between 14 and 21 dpi [9, 26, 65]. Widespread detection of PCV2 in a variety of tissues and organs can be achieved beginning between 7 and 14 dpi, however, the highest viral titers are most often found in lymphoid tissues [9, 66]. Lymphoid depletion is a defining characteristic associated with PCV2 infection that causes reduced immune function and increased susceptibility to secondary pathogens [10]. Thus, efficient primary infection of lymphoid tissues may play an essential role in PCV2 induced immunosuppression and overall pathogenesis.

PCV2 predominantly infects host epithelial, endothelial, and monocytic cells by binding via capsid proteins to glycosaminoglycans (GAGs) on the cell surface [67]. Specifically, two GAGs, heparan sulphate and chondroitin sulphate B, found on the surface of all cell types serve as the primary receptors of PCV2 attachment [67, 68]. Following attachment, PCV2 enters monocytic cells via clathrin-mediated endocytosis [69] and epithelial/endothelial cells via an actin and GTPase-dependent pathway [70]. Once internalized, PCV2 virus-like particles (VLPs) were found to organize into double-membrane bound intracytoplasmic inclusion bodies likely formed through lysosomal engulfment of endocytic vesicles [71]. During this first cytoplasmic phase, VLPs were

also found to be associated with both the inner and outer membranes of mitochondria, suggesting a potential role of this organelle in facilitating PCV2 replication and pathogenesis [71]. In fact, mitochondria with diminished functionality containing PCV2 VLPs were observed in the lymph nodes of PMWS-affected animals [72].

PCV2 translocation from the periphery of infected cells to the nucleus is essential for replication. To do this, PCV2 commandeers a mechanism of intracellular transport involving the molecular motor dynein, which is used by the host to transport endosomal cargo along microtubules to the nucleus [73]. Exactly how PCV2 recruits the host machinery is unclear, however, the viral Cap protein has been shown to play a vital role in the translocation process. Specifically, the Cap protein induces acetylation of microtubular α-tubulin [74] and interacts directly with the Intermediate Chain 1 (IC1) subunit of dynein via residues 42-100 of the Cap protein following disassembly and release from early endosomes [73]. Inhibition of dynein activity, knockdown of the IC1 subunit, and disruption of the microtubular network were all individually found to significantly decrease PCV2 replication without affecting internalization and endosomal uptake of the virions [73].

Upon nuclear entry, VLPs aggregated into non-membrane bound intranuclear inclusion bodies often in close proximity to networks of protein-like structures [71]. It is believed that following replication, these protein networks are involved in the encapsidation of immature virions near the nuclear periphery prior to release back into the cytoplasm [71]. During this second cytoplasmic phase, double-membrane and triple-membrane bound intracytoplasmic inclusion bodies or viral factories containing VLPs appear with the assistance of organelle membranes such as the Golgi complex and rough

endoplasmic reticulum, indicating a potential role of these organelles in the final stages of viral assembly [71]. Three possible mechanisms of cellular release have been observed for mature PCV2 virions: 1) VLPs move as a unit within inclusion bodies utilizing organelle membranes to the plasma membrane for release via budding, 2) PCV2 particles are released from cytoplasmic inclusion bodies and exit the cell through exocytosis, or 3) complex cytoplasmic inclusion bodies are formed with various subcellular irregularities leading to cell lysis and release of PCV2 progeny [71].

The goal and conditional requirements of PCV2 depend on the type of cell infected. Inhibition of endosome acidification was shown to reduce PCV2 infection in monocytic cells [69], but enhance PCV2 infection and replication in epithelial cells [75]. This discrepancy in optimal pH may indicate that different proteases are involved in the disassembly of PCV2 in epithelial and monocytic cells [67]. Furthermore, despite prolific infection of monocytic cells, PCV2 replication was found to only occur within the nuclei of epithelial and endothelial cells [76-78]. The reason behind this is believed to be due to the virus's reliance on host cell machinery and mitosis for replication since the viral genome does not encode its own replicative tools and is unable to autonomously penetrate the nuclear envelope [79]. PCV2 can only achieve nuclear entry at the end of mitosis when it is incorporated into the nuclei of daughter cells, after which, it must wait until the cell enters S-phase to hijack host replicative machinery for replication [79]. As a result, PCV2 likely depends on cells with higher mitotic rates for adequate replication.

Although monocytic cells are not primary sites of viral replication, they are still main targets of PCV2. Monocytic cells are an essential component of innate immunity, but also play an important role in initiating adaptive immunity. Specifically, dendritic

cells (DCs) and macrophages engulf antigens at the site of infection, release cytokines that enhance the immune response, and then travel to lymph tissues where they present these processed antigens via surface proteins to activate general and pathogen-specific T lymphocytes. PCV2 is believed to have immunosuppressive capacity as indicated by the presence of lymphoid depletion, lesions, and susceptibility to secondary infection. Immunomodulation of monocytic cells may represent a potential mechanism of PCV2-induced immunosuppression by compromising a vital link between the innate and adaptive immune response to PCV2 and other pathogens.

The ability of PCV2 to accumulate to such high levels in the cytoplasm of monocytic cells without viral replication indicates inadequate degradation of the virus following internalization [80]. However, this increase in cytoplasmic PCV2 does not correspond with increased cell death. Vincent et al. (2003) found that PCV2 was able to persist within myeloid dendritic cells (DCs), following *in vitro* inoculation, without losing infectivity or inducing cell death. They also found no evidence of surface protein modulation in infected DCs or viral transmission to activated T-lymphocytes [78]. A similar result was observed in alveolar macrophages (AM); however, the apoptotic rate was lower in infected AMs compared to controls [81]. It is believed that the silent and persistent infection of myeloid DCs and capacity to escape degradation in various monocytic cells may represent a mechanism of PCV2 immune evasion essential for sustained infection [78, 80]. It has also been proposed that PCV2 utilizes monocytic cells for systemic dissemination by taking advantage of their normal circulation throughout the body [64, 78]. Therefore, survival of infected monocytic cells may be imperative for adequate viral spread and systemic infection.

Even though PCV2 caused no observable modulation to myeloid DCs, it does possess immunomodulatory capacity in other types of DCs. Vincent et al. (2005) assessed PCV2 interactions with a variety of DC subsets, including DC precursors, myeloid DCs, conventional blood DCs, and plasmacytoid DCs[82]. Once again, no modulation in myeloid DC antigen presentation or maturation was observed in the presence of PCV2. However, PCV2 infection of plasmacytoid DCs, also known as natural interferon-producing cells (NIPCs), was found to interrupt a normal costimulatory function required for myeloid DC maturation *in vivo* by inhibiting NIPC production of IFN-alpha and TNF-alpha following interaction with CpG-oligonucleotides (CpG-ODNs). The exact manner in which PCV2 disrupts production of these cytokines is unknown, but discovery of stimulatory and inhibitory CpG motifs within the PCV2 genome may provide some explanation [83]. One motif in particular was found to inhibit the production of IFN-alpha via TLR9 signaling in porcine PBMCs even in the presence of other stimulatory CpG motifs from the PCV2 genome [83, 84]. However, in a subsequent study the inhibitory effect of PCV2 was found to not only target CpG-ODN-induced cytokines, but responses induced by TLR7 and TLR9 agonists as well as other viral pathogens [85]. Also PCV2 DNA and CpG-ODNs did not co-localize within NIPCs during this study, suggesting they target distinct receptors. Therefore, PCV2 may modulate NIPCs via unique mechanism to disrupt both autocrine and paracrine maturation of myeloid DCs, impairing the ability of the host to recognize and respond to simultaneous or secondary infections [80, 85].

PCV2 has also demonstrated immunomodulatory capacity in alveolar macrophages (AMs). Chang et al. (2006) found that infected AMs exhibited reduced

phagocytosis and microbicidal capacity leaving the host vulnerable to secondary lung infections. The decreased microbicidal capacity is believed to be due in part to impaired production of reactive oxygen species (ROS) and may illustrate a potential mechanism utilized by PCV2 to escape degradation within these cells [81]. The infected AMs also exhibited increased production of cytokines and chemokines involved in enhancing the inflammatory response and recruiting polymorphonuclear leukocytes (PMNs) and monocytes to the infection site, such as TNF-alpha, AMCF-II, IL-8, MCP-1, and G-CSF [81]. Increased expression of these immune signals have also been observed in lymphoid tissues of PMWS-affected pigs [25, 86, 87], indicating modulation of monocytic cytokine and chemokine production may contribute to lesion formation by recruiting acute phase inflammatory cells and other monocytic cells to PCV2-infected tissues [81].

Furthermore, there is a correlation between increased levels of PCV2 in lymphoid organs of naturally PMWS-affected pigs and lymphocyte depletion and histiocytic lesions resulting from monocyte infiltration [88]. Darwich et al. (2002) found that the levels of CD8+ (cytotoxic) and CD4+CD8+ (double positive) T-cells were significantly decreased in PCV2-positive animals compared to PCV2-negative and healthy controls. Additionally, the magnitude of lymphocyte depletion and reduction in levels of CD8+ T cells and IgM+ B cells in peripheral blood were found to correspond with the level of PCV2 antigen in lymphoid tissues [89]. The mechanisms behind PCV2-induced lymphocyte depletion remain speculative. As reviewed by Darwich and Mateu (2012) and X.J. Meng (2013), some studies suggest that apoptosis may be a prominent mechanism leading to lymphocyte depletion while others provide evidence implicating reduced proliferation rather than apoptosis as the root source.

Whether these depletion mechanisms are induced directly by the virus or cytokines is also highly debated. Studies have shown damage due to PCV2 replication can directly deplete B and T lymphocytes [90, 91]. However, cytokine imbalances and alterations have been observed in many lymphoid organs of PMWS affected animals as well [87]. Specifically, levels of IL-10 have been associated with disease severity and viral load [92, 93] and over expression of IL-10 mRNA in the thymus was found to correlate with the degree of lymphocyte depletion and thymic atrophy [87]. PCV2 has been shown to induce similar alterations *in vitro* by increasing production of IL-10 and other pro-inflammatory cytokines and suppressing production of IL-2 and IL-4 by PBMCs collected from both PMWS-affected and healthy pigs as well as repressing IL-2, IL-4, and IFN-y production by PBMCs from PMWS-affected pigs compared to healthy pigs in response to mitogen or superantigen stimulation [86, 94]. Therefore, it is unclear whether these cytokine imbalances *in vivo* are caused by PCV2 modulation of infiltrating monocytes that actually induce depletion or if they are simply a reactive response resulting from depletion via direct viral-induced mechanisms.

Factors Affecting PCV2 Virulence, Pathogenesis, and Disease Progression

There is significant variation in the virulence of PCV2 isolates, such that simply being infected with PCV2 does not mean subsequent clinical manifestation of PCVAD. Assessing virulence is further complicated by the fact that experimental infection with a PCV2 isolate alone, regardless of its virulence in naturally infected herds, often results in subclinical infection and rarely in disease [15, 30]. As a result, the virulence of PCV2 isolates is generally quantified based on the level of PCV2 replication and histological

changes rather than symptomatic status [14]. There are four major factors believed to influence virulence and related pathogenesis: virus-dependent, host-dependent, secondary infection, and modulation of innate and adaptive immunity [20]. Since multiple external factors are presumed to be involved, obtaining consistent findings on isolate virulence and identifying regions of the PCV2 genome associated with increased virulence has proven difficult.

a) *Virus-Dependent: Variation in Strain Virulence*

Grau-Roma et al. (2008) isolated PCV2 from sera of pigs from PMWS affected farms and non-PMWS affected farms in Spain. They compared the ORF2 sequences of the 87 isolates and found they could be divided into two different genotypic groups corresponding to PCV2b (genotype 1) and PCV2a (genotype 2). PCV2b strains were detected exclusively in PMWS affected farms while PCV2a strains could be found in both affected and non-affected farms with several pigs from affected farms infected with both PCV2b and PCV2a strains [15]. Similar reports were made in North America, with the PCV2 strains isolated from diseased pigs exhibiting high sequence similarity with PCV2b isolates from Europe and Asia [56, 95]. However, there were also reports that contradicted these findings. Phylogenetic analysis of 70 PCV2 strains, including 34 strains isolated from pigs with a variety of clinical conditions, found that strains associated with PMWS were dispersed throughout the phylogenetic tree and often grouped with strains associated with other diseases and healthy pigs [43]. Experimental infection of 113 pigs with one of four different PCV2 isolates, two PCV2a and two PCV2b isolates, showed no difference in virulence between genotypic groups, however,

differences within genotypic groups were observed [96]. Dual inoculation of 20 germ-free pigs 7 days apart with PCV2a and PCV2b (2a|2b or 2b|2a), PCV2b (2b|2b), or PCV2a (2a|2a) showed that heterologous inoculation resulted in increased severity and incidence of disease compared to inoculation with PCV2a or PCV2b alone [97]. This indicates that co-infection with PCV2a and PCV2b leads to increased virulence compared to singular infection and may explain the elevated pathogenicity observed with the emergence of PCV2b since majority of herds were already infected with PCV2a [8, 14, 97].

Additionally, PCV2a strains have also been associated with clinical symptoms and exhibit variation in virulence. Opriessnig et al. (2006b) experimentally infected 42 specific-pathogen-free (SPF) pigs with a PCV2a strain isolated from a pig with no PCV2-associated lesions (PCV2-4838) and a PCV2a strain isolated from a pig with PCV2-associated lesions and disease symptoms (PCV2-40895). Sequence comparison revealed a total of 20 nucleotide differences between the two strains: 5 in ORF1, 14 in ORF2, and one in ORF3 resulting in 99.4%, 96.1%, and 99.7% amino acid sequence homologies, respectively. They found significant evidence based on severity of microscopic lesions and level of PCV2 and antibodies in serum that the PCV2-40895 isolate was more virulent than the PCV2-4838 isolate, indicating that PCV2a can be pathogenic and variation in virulence between even genetically similar isolates exists [9].

The occurrence of novel strains with increased virulence pose a constant threat due to the high mutation rate of PCV2 [49]. Majority of genetic differences between strains present today are located within ORF2, occasionally altering the amino acid sequence of the capsid protein. Additionally, the ORF2 sequences of PCV2a strains were

found to be more variable than PCV2b strains, indicating PCV2b may represent the evolution of PCV2a towards increased virulence [15, 96, 98]. In fact, comparison between archival PCV2 strains from 1970-1971 and contemporary PCV2 strains uncovered a consistent 9bp sequence difference in an immunogenic epitope of ORF2, altering configuration of the nucleocapsid from hydrophilic in archival strains to hydrophobic in contemporary strains [99]. Experimental infection of gnotobiotic pigs with PCV2 clones illustrated that the archival PCV2 strains were avirulent compared to contemporary PCV2 strains [99]. Additional support for the role of Cap sequence variation in isolate pathogenicity is given by several studies that illustrate as few as one or two amino acid substitutions in the Cap protein is sufficient to cause alterations in PCV2 virulence [100-102]. Thus, comparison of ORF2 sequences between current PCV2 isolates has been a major area of focus in attempt to identify specific regions associated with virulence.

Larochelle et al. (2002) identified three different regions of the Cap protein with increased amino acid heterogeneity, positions 59-80, 121-136, and 180-191 [43]. Two of these regions overlap with dominant immunoreactive areas at positions 65-87 and 113-147 previously identified using Pepscan [103]. In fact, majority of differences between isolates from healthy and diseased pigs are located within immunogenic epitopes of the Cap protein [30, 45]. However, after further analysis no consistent amino acid motifs within these regions were found that could be associated with PCV2 isolates from pigs with PMWS or other disease symptoms [43]. In another study, Grau-Roma et al. (2008) identified three similar regions of high heterogeneity between ORF2 sequences at amino acid positions 57-91, 121-136, and 185-191. Alignment revealed amino acid sequence

patterns in these regions that were specific to either PCV2a or PCV2b. As previously stated, the PCV2a sequences were more variable within these regions while the PCV2b sequences were more conserved between isolates. Once again they were unable to detect any association between the PCV2b sequence found in individual pigs and disease status [15]. Similar outcomes were also reported following phylogenetic analyses of Dutch [53] and French isolates [98]. This recurrent inability to identify genetic signatures of PCV2 virulence suggests that external factors have significant influence over virulence and the outcome of infection.

b) *Innate and Adaptive Immunity in PCV2 Infection*

PCV2 infection has been shown to induce a humoral immune response, a facet of adaptive immunity illustrated by antibody production, which in subclinically infected pigs results in decreased viral titers [65, 88, 104]. Generally, specific IgM antibodies are the first to appear in serum and represent recent or on-going infection, while specific IgG antibodies are detected later on and represent the animals immune status for a particular pathogen, such as prior exposure or vaccination. Progranichnyy et al. (2000) intranasally and intramuscularly inoculated five 8-week old colostrum deprived pigs free of any major swine pathogens with a strain of PCV2 isolated from a pig diagnosed with PMWS. PCV2 viremia could be detected at 7, 14, and 21 dpi, but not at 28 dpi. PCV2-specific antibodies were first detected at 14 dpi, however, the presence of PCV2-neutralizing antibodies was not observed until 28 dpi [104]. A similar result was obtained by McKnite et al. (2014), with PCV2-specific IgM antibodies present at 14 dpi, peaking at 21 dpi and dropping again at 28 dpi and PCV2-specific IgG or neutralizing antibodies present at 21

dpi followed by an increase in levels at 28 dpi. Although viremia was still detected at 28 dpi in this study, it had significantly decreased in level compared to 14 and 21 dpi [65]. In fact, a positive phenotypic correlation was found between overall viral load and IgM ($r=0.26$ to 0.34, 14-28 dpi, $P<0.0001$) and IgG ($r=0.17$ to 0.20, 21-28 dpi, $P<0.01$) during the challenge [65]. However, variation in the level and type of adaptive immunity between animals has been observed and may play a significant role in the efficacy of PCV2 replication and virulence.

Meerts et al. (2005) infected 12 gnotobiotic pigs with PCV2 and treated 4 of these pigs with cyclosporine A (CysA) in order to assess the influence of adaptive immunity on PCV2 replication. All CysA treated pigs had higher viral titers compared to the non-CysA treated pigs, suggesting adaptive immunity plays a central role in limiting viral replication [88]. They also observed several different replication and antibody profiles among the non-CysA treated pigs. No PCV2 could be detected in the two pigs with the highest neutralizing antibody titers present at 15 dpi along with a significant increase in IFN-y mRNA at 15 dpi. Five pigs with lower neutralizing antibody titers and no detectable increase in IFN-y, had low to moderate PCV2 titers at 15 dpi that either plateaued or decreased as time progressed. A single pig exhibited no neutralizing antibodies or rise in IFN-y and had a low PCV2 titer at 15 dpi that drastically increased at 21 dpi. Since a higher level of viral replication is associated with increased potential of disease progression, this study suggests that the outcome of infection may be partially contingent upon the adaptive immune response of the host [88].

Meerts et al. (2006) went on to further investigate the correlation between viral replication and lack of neutralizing antibodies in a subsequent study comparing

experimentally infected SPF pigs, naturally PMWS-affected pigs, and healthy controls. No PCV2-neutralizing antibodies were found in the naturally infected PMWS-affected pigs and only low neutralizing antibody titers could be observed between 7 and 14 dpi in the experimentally infected PMWS-affected pigs. This differed from the healthy and subclinically infected pigs, which exhibited neutralizing antibody titers corresponding with that of total antibodies [105]. They also found that the IgM profiles differed between healthy and affected pigs, with IgM increasing throughout the study in healthy/subclinically infected pigs and decreasing or remaining at low levels in affected pigs [105]. A similar result was found by Fort et al. (2007), such that PMWS-affected pigs had negative or significantly lower neutralizing antibody titers than healthy animals and a suggestive inverse correlation between neutralizing antibody titers and viral load. In addition, PCV2-specific IgG titers were positively correlated with neutralizing antibody titers ($r=0.76$, $P<0.0001$), while no correlation was observed between IgM and neutralizing antibody titers [106]. Together, these studies illustrate that there is a difference in the humoral response between subclinically infected pigs and clinically affected pigs with the level of viral replication and incidence of disease increasing in the absence of PCV2-neutralizing antibodies [88, 105, 106]. Whether these differences in adaptive immunity are simply due to variation in host immune responses or variation in efficacy of PCV2 immunomodulation between hosts is still unclear.

Natural mechanisms of active and passive immunity have also been shown to decrease host susceptibility to PCV2, including prior PCV2 infection [96] and PCV2-specific maternal antibodies [107, 108]. In the second part of the study conducted by Opriessnig et al. (2008), they initially inoculated pigs with either a PCV2a or PCV2b

isolate then re-inoculated the animals 5 weeks later with the other isolate and compared the responses to pigs without prior exposure. Viral replication of the secondary isolate was almost completely blocked in the re-inoculated pigs, indicating prior infection not only provided protection against subsequent infection by way of active immunity, but that cross-protection between PCV2a and PCV2b isolates also exists [96]. Several studies have also noted a potential litter effect for PMWS susceptibility and mortality [107, 109]. It is known that newborn piglets are protected by passively acquired maternal antibodies from a variety of viral pathogens [108]. Therefore, sow infection or vaccination status and as a result maternal antibody titer have also been associated with piglet disease susceptibility.

McKeown et al. (2005) experimentally infected 24 piglets split into 4 groups based on maternal antibody titer at 12 days of age: group A were negative, group B had low levels, and groups C and D had high levels of maternal antibodies. Groups A, B, and C were inoculated at day 0 with PCV2 and then re-infected at day 42. Group D was only infected at day 42. Viremia prior to day 42 was detected in nearly all group A and B piglets, but in only 2 of 8 group C piglets. Following re-infection or initial inoculation (group D) at day 42, viremia was observed between 7 and 21 dpi in nearly all group A, B, and D piglets, but only 3 of 8 group C piglets [108]. All groups except for group C tested negative for PCV2 maternal antibodies at day 42 [108]. Sow antibody titer was also associated with piglet mortality in naturally PMWS-affected farms, such that the piglets from sows with medium to high antibody titers had significantly lower death percentages than those from sows with low antibody titers [107]. These results indicate that higher titers of PCV2 maternal antibodies provides greater protection against early PCV2

infection and that a combination of high PCV2 maternal antibody titer and prior exposure to PCV2 decrease susceptibility to secondary PCV2 infection by extending the length of protection supplied by the maternal antibodies [108].

c) *Immunostimulation and Secondary Infections in PCVAD*

Coinfection with secondary pathogens or extraneous immunostimulation with adjuvants or vaccines have been shown to fuel the development and severity of PCVAD [30]. These factors are often required for PCV2 infected animals to display clinical symptoms, which has led many to question if PCV2 is actually the etiological agent behind these diseases. The widespread infection of PCV2 in even asymptomatic herds and variation in PCVAD symptoms further complicated its discovery and association with disease [110]. Studies have revealed that although PCV2 alone often results in subclinical infection, it is the common and crucial component of PCVAD development [20, 111].

In 2004, Opriessnig et al. (2004b) assessed the effect of dual infection with *Mycoplasma hyopneumoniae* and PCV2 on development of PMWS. They randomly assigned 67 pigs to 4 groups consisting of a control group (n=17), a *M. hyopneumoniae* group (n=17), a PCV2 group (n=16), and a dual *M. hyopneumoniae*/PCV2 group (n=17). The pigs were inoculated with *M. hyopneumoniae* at 4 weeks of age and/or PCV2 at 6 weeks of age. The dual-infected pigs presented with reduced weight gain, labored breathing, and lethargy. Compared to the other groups, the dual-infected pigs had significantly more severe macroscopic lung lesions, microscopic PCV2-associated lung and lymphoid lesions, and increased amounts of PCV2-antigens associated with the

lesions. Four of the 17 dual-infected pigs also had symptoms characteristic with PMWS such as severe lymphoid depletion, reduced growth rate, and granulomatous lymphadenitis associated with high levels of PCV2-antigen. These findings, indicate that *M. hyopneumoniae* exacerbates PCV2-associated lung and lymphoid lesion severity and promotes increased and sustained levels of PCV2 antigen leading to increased incidence of PMWS [17].

In 2002, pigs with systemic PCVAD were assessed for pathogens and it was found that *M. hyopneumoniae* was detected in 36% of the 484 cases, whereas singular PCV2 infection was detected in only 2% of cases [112]. Other common pathogens that were associated with PCV2 coinfection in this study included Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) found in 52% of cases, bacterial septicemia or pneumonia in 22% of cases, and Swine Influenza Virus (SIV) in 5.4% of cases [112]. Based on these findings, it is common opinion that there is no secondary pathogen that is solely responsible for inducing incidence and severity of PCV2-associated diseases [20]. Rather, it is believed several pathogens that vary from location to location are capable of eliciting PCVAD development [20].

Experimental infection of gnotobiotic pigs with PCV2 alone generates only subclinical to mild symptoms with little dissemination of viral antigen, however, when gnotobiotic pigs are co-infected with PCV2 and porcine parvovirus (PPV) they present with moderate to severe symptoms and widespread dissemination of PCV2 antigen in lymphoid tissues and histiocytic lesions [113]. Since experimental infection of conventional pigs with PCV2 has been occasionally successful at inducing PMWS symptoms, but a secondary pathogen is required to elicit disease symptoms in gnotobiotic

pigs, Krakowka et al. (2001) proposed that increased PCV2 replication and dissemination *in vivo* requires immune stimulation mediated by monocytic cell activation in lymphoid tissues. To test this hypothesis, they performed a short-term experiment to assess the effect of local immunostimulation on viral replication and dissemination and a long-term experiment to assess the effect of systemic immunostimulation on incidence of PMWS [110].

For the short-term experiment, Krakowka et al. (2001) inoculated 7 piglets with PCV2 at 1 day of age. At 3 and 7 days of age, piglets were injected in the left hip and shoulder with either the immunostimulant keyhole limpet hemocyanin (KLH), an irrelevant antigen, emulsified in incomplete Freund's adjuvant (ICFA) or KLH in saline. Viral load was then assessed at 13-14 dpi in local lymph nodes draining injection sites and contralateral lymph nodes, so that each pig served as its own control. The amount of recovered infectious virus was highest in draining lymph nodes of KLH/ICFA injection sites compared to contralateral lymph nodes and compared to pigs injected with KLH in saline. Furthermore, PCV2 was found in distal lymph nodes of animals injected with KLH/ICFA, but only sparsely distributed in distal lymph nodes of animals injected with KHL in saline. For the long-term experiment, they inoculated 10 piglets at 1 day of age with PCV2. At 3 and 7 days of age, piglets were injected in both hips and shoulders to achieve systemic immunostimulation with either KHL/ICFA alone or KHL/ICFA followed by intraperitoneal (IP) injections of thioglycollate broth (glycan) at 10 and 20 days of age to stimulate peritoneal macrophages. All immunized pigs (both KLH/ICFA and KLH/ICFA-IP glycan) infected with PCV2 developed PMWS by 35 days of age while none of the infected pigs that were not immunized developed PMWS. Through

these experiments, Krakowka et al. (2001) illustrated that immunostimulation enhanced PCV2 replication in local lymph nodes as well dissemination to distal lymphoid tissues and that systemic immunostimulation, with or without macrophage activation, triggered the development of PMWS in PCV2-infected gnotobiotic pigs, indicating that immune activation is essential for PCV2 pathogenesis [110].

Immunostimulation by way of vaccination or adjuvant administration increases lymphocyte proliferation [114]. This mechanism could explain the correlation between increased viral antigen and immunostimulation since PCV2 replication may increase in dividing cells. However, another study assessing the effects of immunostimulation on PMWS development in SPF piglets did not find any difference in incidence or severity of disease between piglets that had been subjected to the immunizing agent, KLH/ICFA, and those that had not, suggesting immunostimulation is not an important factor in PMWS development [115]. In 2005, a reverse transcription PCR (RT-PCR) assay able to detect the presence of PCV2 capsid mRNA was developed and allowed the researchers to be able to discern between replicating PCV2 and PCV2 virions within PK-15 cells [116]. This knowledge was used in subsequent studies to quantify the amount of Cap mRNA within PBMCs using a quantitative real time PCR (qPCR) assay in order to better assess the impact of immunostimulation [114, 117].

In vitro inoculation with PCV2 showed that viral replication was significantly increased in PBMCs that had been stimulated with concanavalin A (ConA) compared to resting PBMCs [117]. However, the magnitude of increase in viral replication within stimulated PBMCs was later found to depend on the mitogen used and independent of cell proliferation since similar levels of Cap mRNA were found in proliferating and non-

proliferating cells [114]. Interestingly, they also found that PCV2 infected PBMCs stimulated with pokeweed mitogen (PWM) exhibited increased apoptosis compared to uninfected or unstimulated infected PBMCs. In fact, PBMCs infected with PCV2 alone without stimulation exhibited reduced levels of apoptosis compared to non-infected PBMCs [114]. These findings indicate that immunostimulation increases PCV2 replication, supporting the *in vivo* study conducted by Krakowka et al. (2001). However, the mechanism that induces this replication appears to be independent of lymphocyte proliferation [114]. In addition, certain stimulation increased apoptosis compared to infection alone [114]. Although this has not yet been tested *in vivo*, it may indicate that immunostimulation plays an even greater role in PCV2 pathogenesis than originally thought.

The question of whether PCV2 immunosuppression enables secondary infections that exacerbate disease symptoms or secondary infections stimulate PCV2 replication and pathogenesis is analogous to the age old question of what came first: the chicken or the egg? The answer seems to change depending on the angle from which you view it. The illustrated importance of immunostimulation and wide variety of secondary pathogens capable of eliciting PCVAD symptoms suggest that secondary infections may simply provide the required stimulation of the immune system to enhance PCV2 pathogenesis. In this case, pathogenesis would not be contingent upon a specific secondary pathogen, but on the type of stimulation provided by that secondary infection. Immunosuppression was also shown to promote PCV2 replication and spread, however, when a stronger immunosuppressant was used, characteristic PCVAD inflammatory reactions were not observed [118, 119]. Therefore, the degree of immunosuppression, immunostimulation,

and time of secondary infection may all contribute to the pathogenic outcome of PCV2 infection.

d) *Host-Dependent: Role of Host Genetics in PCV2 Susceptibility*

Although PCV2 infection is widespread amongst all pigs, the progression from subclinical infection to PCVAD has been shown to vary between breeds and individuals. This indicates that genetic variation between breeds, populations, and individuals may play a significant role in host susceptibility to PCV2 infection and subsequent incidence and severity of disease.

Opriessnig et al. (2006a) compared the susceptibility of three different breeds of pigs to PCV2 infection. In this study, they selected 75 colostrum fed, purebred female pigs raised under identical conditions from a single farm consisting of Duroc (n=26), Landrace (n=25), and Large White (n=24). Twelve of these pigs (3 Duroc, 6 Landrace, and 3 Large White) were mock inoculated and the remaining 63 pigs (23 Duroc, 19 Landrace, and 21 Large White) were experimentally infected intramuscularly and intranasally with PCV2 at 5-7 weeks of age. The control pigs were kept together in a room separate from the infected pigs. Clinical evaluation was conducted daily while weights and serum samples were collected 7, 14, 21, 28, and 35 dpi. Half of the infected and control pigs from each breed were necropsied at 21 dpi and the other half at 35 dpi. Similar macroscopic and microscopic lesions including enlarged lymph nodes, granulomatous inflammation, and lymphoid depletion were observed in all 3 breeds. However, lymphoid depletion was significantly more severe in Landrace compared to Duroc and Large White. Additionally, 3 of the 19 Landrace pigs developed severe

lymphoid lesions containing high levels of PCV2 antigen, which are characteristic of PMWS. None of the Duroc or Large White individuals exhibited such lesions. Therefore, the 3 breeds were determined to be equally susceptible to subclinical PCV2 infection, but Landrace were more susceptible to developing clinical PMWS [120].

In a subsequent study, Opriessnig et al. (2009) went on to compare susceptibility of Landrace and Pietrain pigs to PCV2. Together they had 39 Landrace and 39 Pietrain pigs that were divided into 4 groups: Landrace non-inoculated negative control (n=13), Pietrain non-inoculated negative control (n=13), Landrace-PCV2 (n=26), and Pietrain-PCV2 (n=26). The Landrace-PCV2 and Pietrain-PCV2 groups were inoculated with PCV2 at 21 weeks of age and blood samples were collected weekly for 3 weeks post infection. Serum samples were tested for PCV2-specific IgG and IgM antibodies, neutralizing antibodies, and PCV2 viremia. Plasma samples were tested for certain cytokines. All groups of pigs were necropsied at 21 dpi. Clinical disease was not observed in any of the animals and the presence of macroscopic lesions did not differ between breeds. No differences in PCV2-specific IgG and IgM levels, neutralizing antibodies, viremia, or cytokine concentrations were observed between breeds. Both PCV2 groups had significantly more severe lesion scores than the control groups, but the Pietrain-PCV2 group had significantly less severe microscopic lesions than the Landrace-PCV2 group. Only about 34.6% of the Pietrain-PCV2 group had mild follicular depletion in lymph nodes along with a low level of PCV2 antigen while the remaining 65.4% exhibited normal lymphoid tissues. On the other hand, 30.7% of the Landrace-PCV2 group exhibited moderate to severe lymphoid depletion and histiocytic replacement along with moderate lymphohistiocytic infiltration of the heart and liver tissues. These results

indicate that there is a difference in susceptibility to PCV2-associated lesions between Landrace and Pietrain pigs [121], supporting their previous findings suggesting a genetic component to PCVAD susceptibility [120].

In 2005, Rose et al. conducted a study in France to assess the presumed protective effect of the Pietrain breed against PMWS. They selected 4 PMWS-affected farms in France and randomly inseminated sows with either pure Pietrain semen or non-Pietrain semen normally used on the farm (semen X). One of the farms had a third genetic type of semen (Y) that was used in addition to the Pietrain and X semen. This experiment was carried out in 2 batches per farm for a total of 8 batches. A total of 30 pigs per paternal genetic group stratified by litter (n=2) were selected from each batch. This resource population of 540 piglets were then housed in identical conditions, observed for clinical symptoms, and weighed from birth to slaughter. Blood samples were taken at age of weaning, 7, 13, 16, and 21 weeks of age and then again at slaughter and tested for PCV2-specific antibodies. A portion of the resource population that exhibited the best growth performance and assumed to be unaffected by significant disease were selected as a reference group used to account for inherent differences in growth performance between genetic groups. The average incidence rate of PMWS was 18% with no significant differences in age of onset or morbidity between the paternal genetic groups [21]. However, the time it took for PMWS to develop was found to be influenced by PCV2 seroconversion and associated with maternally acquired passive immunity [21], corroborating the impact of maternal effects on PMWS incidence and survival [107, 108]. Therefore, the inability to detect a significant paternal genetic effect in this study may have been due in part to confounding maternal effects. Taking this into account, the

observed difference in morbidity between Pietrain-derived and X-derived offspring, 16.3% and 17.3% respectively, may indicate a genetic source of reduced PMWS susceptibility in the Pietrain breed [21].

To further investigate the effect of host genetics on PCVAD progression, McKnite et al. (2014) performed the first genome wide association study (GWAS) to identify genomic regions associated with PCV2 susceptibility. The resource population was comprised of 4 batches of crossbred pigs derived from various commercial lines (Nebraska Index Line (NIL), Landrace, Large White, and Duroc). A total of 386 pigs across the batches were experimentally infected intranasally and intramuscularly at 5 weeks of age with PCV2b and were weighed and monitored for clinical symptoms. Blood samples were taken weekly over the course of the 28-day challenge to assess for PCV2 specific IgM and IgG antibodies and PCV2 viremia. All pigs were genotyped for approximately 61,177 SNPs using the Illumina Porcine SNP60 BeadArray. After filtering to remove low quality SNPs and DNA samples, a total of 370 samples and 56,433 SNPs were used for GWAS. Using a Bayes B model, SNP effects were measured in 1Mb windows and then combined to get the proportion of genetic variance for each indicator trait of PCVAD susceptibility: weekly viremia, PCV2-specific IgG and IgM antibody levels, and Average Daily Gain (ADG) as well as overall viral load and ADG. Host genetics was found to explain significant proportions of phenotypic variation for both viremia (35-59%) and PCV2-specific antibody levels (10-59%), however, the proportion varied across weekly measurements. In addition, multiple loci (1Mb windows) were found to contribute to the phenotypic variation for each of the target traits, with some unique to specific traits and common between traits. Specifically, windows on SSC1,

SSC7, and SSC12 affected viral load and/or specific weekly viremia measurements while windows on SSC12, SSC14, and SSCX affected weekly IgM and IgG [65].

McKnite et al. (2014) hypothesized that loci influencing viral replication and accumulation would indirectly affect the immune response and weight gain of individuals over the course of the PCV2 challenge. They assessed genomic correlations between PCVAD indicator traits and found that viral load had a significant negative genomic correlation with ADG ($r=-0.21$ to -0.24, 0-14 dpi, $P<0.0001$) and positive genomic correlation with IgM ($r=0.22$ to 0.54, 14-28 dpi, $P<0.0001$) and IgG ($r=0.29$ to 0.43, 21-28 dpi, $P<0.0001$). Single marker association was performed using 31 SNPs with significant effects on viral load to estimate pleiotropic effects across the indicator traits [65]. Of these SNPs, 41.9% had effects on two or more measurements of weekly viremia, 35.5% had effects on overall ADG or at least one measurement of weekly ADG, 16.1% had effects on one or more measurements of IgM, and 19.4% had effects on one or more measurements of IgG [65]. In addition, the combined effect of 3 SNPs located on SSC9 and SSC12 that captured the negative relationship between viral load and ADG indicated that individuals with more favorable alleles at these loci would have lower weekly viremia and viral load and higher weekly and overall ADG [65].

This study was expanded with the incorporation of additional maternal lines from North-American swine breeding programs, which doubled the amount of samples ($n=974$) used in the GWAS [122]. Following the same protocols as described in McKnite et al. (2014), this study found that the SNP genotypes explained a larger proportion of phenotypic variation for overall viral load (64%) than detected by the previous study [122]. Additionally, they uncovered two major SNPs on SSC12 and SSC7 that explained

11.5% and 2.8% respectively of the genetic variation for viral load [122]. Although, the proportion of phenotypic variation in ADG explained by the SNP genotypes was still limited, there was once again a negative correlation between viral load and overall ADG ($r=-0.36$, $P<0.0001$), partially explained by the major SNPs. Specifically, animals with the CC genotype for the major SSC12 SNP, exhibited lower viral load ($P<0.0001$) and higher overall ADG ($P<0.05$) compared to the heterozygous (CT) or alternate homozygous (TT) genotypes [122].

Building on the findings of McKnite et al. (2014) and Engle et al. (2014), a subsequent study went on to assess the role of host genetics in the level of the pro-inflammatory cytokine, TNF-alpha, known to be modulated in PCV2 infected animals [123]. Initially, the protein serum TNF-alpha levels were measured weekly in 6 pigs with high viral load and low ADG and 6 pigs with low viral load and high ADG. A significant difference in TNF-alpha level at 21 dpi was observed between the two groups. TNF-alpha levels were profiled from another 297 pigs within the resource population and found to be phenotypically positively correlated with viral load ($r=0.25$, $P<0.001$), viremia ($r=0.15$ to 0.28, 14-28 dpi, $P<0.05$), IgM ($r=0.20$ to 0.34, 14-28 dpi, $P<0.001$), and IgG ($r=0.11$ to 0.19, 21-28 dpi, $P<0.05$) antibody levels and negatively correlated with overall ADG ($r=-0.16$, $P<0.05$). The GWAS for TNF-alpha at 21 dpi revealed a substantial proportion of the phenotypic variation (73.9%) could be explained by the 56,433 SNPs. Major effect windows were located on SSC8, SSC10, and SSC14, however, no single window accounted for majority of the effect [123]. The lack of association surrounding the TNF-alpha locus on SSC7 suggests that the host genetic factors controlling this variation in

TNF-alpha at 21 dpi in response to PCV2 infection act through mechanisms of trans-modulation.

The ability to identify QTL associated with various indicator traits of PCV2 susceptibility support the notion that host genetics plays a critical role in disease progression. Specifically, QTL on chromosomes 7 and 12 were identified for weekly viremia, antibody levels, and overall viral load following experimental PCV2 infection [122]. Further investigation of QTL such as these may lead to the identification of genes and polymorphisms responsible for reduced PCV2 susceptibility and better our understanding of PCV2 pathogenesis. Together this knowledge has the potential to enable the development of more efficient molecular solutions to better combat and prevent PCV2 in the future.

Current PCV2 Management and Vaccines

PCV2 was found to be resilient to multiple common disinfectants, such as ethanol [124]. This resilience combined with persistent subclinical infection makes control and prevention of PCV2 outbreaks difficult. Even with effective disinfectants, such as bleach, it is impossible to completely disinfect facilities of PCV2 and prevent shedding by subclinically infected animals through fecal, urinary, and oronasal routes [58, 59, 124]. Therefore, although facility management is an important factor in preventing PCV2 outbreaks, there is only so much that can be done to prevent this elusive pathogen. As a result, majority of management practices at the present time revolve around vaccination regimes for the entire herd for both PCV2 and secondary pathogens.

There are a variety of commercial PCV2 vaccines currently on the market including: Ingelvac® CircoFLEX™, Porcilis® PCV, Suvaxyn PCV2® One Dose, Circumvent PCV™, and Circovac®. Both Ingelvac® CircoFLEX™ (Boehringer Ingelheim) and Circumvent PCV™ (Intervet) are subunit vaccines utilizing killed baculovirus vectors injected into the host to prevent against PCV2 replication and shedding. The antigen of these vaccines is generated by incorporation of the ORF2 gene that encodes the PCV2 capsid protein into the baculovirus vector. Ingelvac® CircoFLEX™ is a single dose (1 mL) vaccine administered intramuscularly to piglets 3 weeks of age or older (Boehringer Ingelheim). Circumvent PCV™ can be administered intramuscularly either as a single dose (2 mL) at 3 weeks of age or as a double dose vaccine (2 mL) as early as 3 days of age followed by a second injection (1 mL) 3 weeks later (Intervet). Circumvent PCV™ was tested in a randomized controlled clinical trial in a herd of pigs with history of PCVAD [125]. They found that vaccinated pigs had significantly lower mortality rates (50% reduction) and higher ADG (9.3% increase) during the finishing phase compared to control pigs. Vaccinated pigs were also found to weigh on average 8.8kg more at time of marketing [125]. Porcilis® PCV (Intervet International) is another subunit vaccine that contains the capsid protein of a PCV2b strain. This vaccine was found to prevent viremia against 4 different PCV2 genotypes originating from different geographic locations as well as reduce both nasal and fecal PCV2 shedding [126]. However, Porcilis® PCV is a double dose vaccine with the first injection (2 mL) administered at 3 weeks of age and the second injection (2 mL) administered 2-3 weeks later (Intervet), which may increase stress for the individual animal. Suvaxyn PCV2® One Dose is a killed-recombinant vector vaccine that contains a

porcine circovirus type 1-2 chimera as the antigen and is administered intramuscularly in a single dose (2 mL) at 3 weeks of age or older (Fort Dodge® Animal Health). Circovac® is a 'whole-virus' vaccine that contains inactivated porcine circovirus type 2 and a mineral oil adjuvant administered in two doses 3 to 4 weeks apart to gilts and sows prior to breeding (Merial).

Various studies have been conducted to compare the effectiveness of different commercially available vaccines. In 2009, Takahagi et al. compared the effects of Ingelvac® CircoFLEX™ and Porcilis® PCV on different PCV2 genotypes in Japanese commercial pig farms. Mean mortality rates 8 months after implementation of vaccination programs decreased in farms infected with PCV2a-2 (from 20.8% to 12.1%) and PCV2b (from 26.5% to 13.7%), but did not significantly change in farms infected with PCV2a-1 (from 14.7% to 14.1%). This indicates that vaccines can effectively reduce mortality rates associated with PCV2; however, the effectiveness may be dependent upon the PCV2 genotype in question [127].

In 2011, a study was conducted by Lyoo et al. that compared the effectiveness of Suvaxyn PCV2® One Dose, Circumvent PCV™, and Ingelvac® CircoFLEX™. A total of 80 pigs were divided into four groups, with three groups receiving one of the vaccines at 3-weeks of age following the manufacturers protocol and the other acting as a control group. All three vaccinated groups had significantly lower PCV2 viremia compared to the control group at 22 weeks of age [128]. In addition, groups that received Circumvent PCV™ or Ingelvac® CircoFLEX™ exhibited increased ADG (0.81kg or 0.80kg, respectively) compared to the control group (0.74kg, $P<0.05$). Another study compared the same three vaccines, however, also assessed the effects of single and double dose for

the Suvaxyn PCV2® vaccine [129]. A total of 60 3.5-week old piglets were divided into groups and received a different vaccine or vaccine dosage. Overall, they found that viremia was reduced by 78.5% in pigs receiving single dose vaccines (Ingelvac® CircoFLEX™ and Suvaxyn PCV2® One Dose) and 97.1% in pigs that received double dose vaccines (Circumvent PCV™ and Suvaxyn PCV2®). They also observed a reduction in microscopic lymphoid lesions by 78.7% for single dose and 81.8% for double dose vaccines [129]. Together, these results indicate that both single and double dose vaccines significantly reduce PCV2 viremia and improve the growth and overall wellness of the animals.

However, vaccines are not a cure all in terms of PCV2. As illustrated above, there are no commercially available vaccines capable of complete eradication of PCV2 viremia [130]. This poses a potential control problem since PCV2 can be transmitted or cause complications at even low levels. Specifically, one study identified a new PCV2 disease syndrome associated with herds vaccinated for PCV2, called Acute Pulmonary Edema (APE) [131]. This syndrome was first discovered in 8 farms across Kansas and Nebraska in piglets as young as 7-weeks of age. High levels of PCV2 DNA, but no other pathogens were detected in the sera and tissues of all APE affected pigs. Unlike other PCVAD, APE has preacute onset and results from transmission of PCV2 to young piglets prior to vaccination age that do not have adequate maternally derived-antibodies for protection [131].

In regards to female reproductive failure, re-vaccinating gilts 3-weeks prior to integration into the breeding herd did not influence the proportion of mummified or still born fetuses in affected herds with increased rates of reproductive failure attributed to

PCV2 [130]. Rather, findings based on an unaffected herd from this same gilt source suggested that flow strategies maybe a better solution. These gilts were integrated into the breeding herd 9 weeks younger than gilts from the affected herds and showed no increase in fetal mortality or mummification, indicating that when gilts are introduced into the breeding herd prior to breeding may impact PCV2-associated reproductive failure [130]. The reason being that younger gilts have time to recover from PCV2 exposure prior to breeding, which protects the fetuses from vertical PCV2 transmission [130]. Although current vaccines are fairly effective, they are not full proof, which is why further research to better understand PCV2 pathogenesis is crucial for future prevention of this small, but mighty pathogen.

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CHAPTER 2

ANNOTATION OF QTL REGION LOCATED ON CHROMOSOME 12 AND POLYMORPHISM DISCOVERY UNCOVERS CANDIDATE GENES AND NOVEL POLYMORPHISMS INFLUENCING *IN VIVO* PCV2 REPLICATION*

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2.1 Introduction

Anecdotal field data and initial experimental evidence [1, 2] described differences between breeds in both incidence and severity of PCVAD [3], supporting the role for host genetic variation in the etiology of the disease. In the first genome-wide association study (GWAS), host genetics was found to influence PCV2 titer and accounted for an important proportion of the phenotypic variation (~45%) for viral load [4]. In addition, substantial variation in the timing and magnitude of immune response was also observed [4, 5]. A subsequent study [5] extended this work by examining the influence of host genetics on the process of infection, based on a combination of the two previous study populations (n=974 F1 crossbred pigs originating from 14 genetic lines) challenged by experimental infection with the same PCV2b strain and genotyped with a high-density array (Porcine SNP60 BeadArray, 56,433 SNPs). The population structure provides substantial variation in linkage disequilibrium (LD) decay in order to potentially identify genomic regions that influence PCV2 susceptibility using a GWAS approach. In this study, we build on the findings of Engle et al. (2014) using both genomic and RNA sequencing, gene annotation, and polymorphism discovery to dissect the genomic regions associated with PCV2 viral load on chromosome 12 to further elucidate the role of host genetics in disease progression.

2.2 Materials and Methods

Experimental Design: Animals, Diets, and Housing

Experimental PCV2b challenge was conducted in nine batches that varied in size from 81 to 141 pigs. The genetic makeup of this resource population consisted of 974 F1 crossbred pigs produced by 14 genetic lines generated by seven genetic programs. Batches 1-4 (n=386) were produced by the UNL Swine Research Unit involving Large White, Landrace, and Duroc commercial pigs as well as pigs from the Nebraska Index Line (NIL), a line selected primarily for litter size [4]. Batches 5-9 (n=588) were produced with genetics from PigGen Canada Consortium, which used Large White dams and Landrace sires [5].

The dams of the experimental pigs had been vaccinated for PCVAD at 3 weeks of age with a single dose of Ingelvac CircoFLEX vaccine (Boehringer Ingelheim). The suppliers of the pigs also had vaccination programs for Porcine parvovirus, *Erysipelothrix rhusiopathiae*, *Clostridium perfringens*, Leptospirosis and Colibacillosis and tested negative for Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). Prior to experimental infection, the pigs tested negative for presence of PCV2 in peripheral blood by real time quantitative PCR (qPCR) and had a sample/positive ratio (S/P) lower than 0.4 for IgM and 0.3 for passive IgG, the PCV2-specific antibodies [4]. Following infection, experimental pigs were examined daily for clinical signs of disease; weights and blood samples were collected at 0, 7, 14, 21 and 28 days post infection (dpi). Details of the experimental procedures, phenotypic and sample collection are described in Engle et al. (2014) and McKnite et al. (2014).

A validation dataset consisting of a group of 71 maternal crossbred pigs representing all three *ALGA0110477* genotypes infected with the same PCV2b strain at 5 weeks of age was generated using the same experimental conditions. A group of 40 pigs (CT and TT genotypes) vaccinated for PCV2 at 3 weeks of age were used as controls. The vaccinated pigs were housed in the same room with the experimentally infected pigs, but in different pens.

PCV2b Isolate and Experimental Infection

The PCV2b strain (UNL2014001) used for the experimental infection was obtained from a pig with symptoms characteristic to PMWS, the most common PCVAD syndrome. The strain was sequenced (accession KP016747.1) using dye terminators and compared to PCV2 strains available in GenBank [5]. The strain was cultured in swine testicular cell lines as described [4]. At an average of 36 days all the pigs were inoculated with the UNL2014001 PCV2b strain with a titer of $10^{4.0}$ 50% tissue culture infection dose ($TCID_{50}$) intranasally and intramuscularly.

Serologic Profile: Quantification of Viral DNA and PCV2-specific Antibodies

PCV2 specific antibodies, IgM and IgG, were profiled weekly from serum using ELISA (Ingenasa) as described in McKnite et al. (2014). Estimates of the number of PCV2b copies, or viremia, was performed using viral genomic DNA isolated by QIAamp DNA Minikit (Qiagen) and quantified by qPCR using TaqMan Master Mix and ABI 7900 Real Time PCR System (Thermo Scientific). The viral load for each pig during the entire challenge was represented as area under the curve (AUC) based on an algorithm

that takes into account viral levels observed at each time point following infection (0, 7, 14, 21, and 28 dpi) fitting a smooth curve over the 28 days and summing the areas in 0.01 time increments [6].

Host DNA Isolation, Sequencing, Polymorphism Discovery and Genotyping

The DNA was isolated from ear and tail tissue clips using DNeasy or Puregene blood and tissue kits (Qiagen). The experimental animals were genotyped using either the first or second generation of the Porcine SNP60 BeadArray (Illumina) that contain 62,183 and 61,565 SNPs, respectively. Only the common SNPs present in both BeadArray versions (91.6%, 61,177) were mapped on *Sscrofa* 10.2 porcine reference genome assembly. The broad position of the unmapped SNPs on the original *Sscrofa* 10.2, such as *ALGA0110477*, was predicted by linkage disequilibrium estimates (r^2) with the mapped SNPs using "Linkage disequilibrium" option from GenSel software package [7]. An additional 7.2% of the previously unmapped SNPs, including *ALGA0110477*, were mapped on *Sscrofa* 11.1. DNA samples and SNP assays with a genotyping call rate below 80% were excluded from the analyses and a GenCall quality score of 0.40 was used as a minimum threshold for genotype quality [8], resulting in 56,433 SNPs (*Sscrofa* 10.2) used in the GWAS.

Targeted DNA sequencing of candidate genes in the SSC12 QTL region and their 2 – 4 kb region upstream of the transcription start sites (TSS) was performed using dye terminators and ABI PRISM 3100 Genetic Analyzer (Thermo Scientific) on high and low viremic samples. Discovery and validation of the polymorphisms detected by RNA-seq was based on alignment of DNA sequences using Sequencher software (Gene Codes).

Potential impact of the polymorphisms located in the proximal promoter on important regulatory motifs was evaluated using FIMO (version 4.11.3)[9] and the JASPAR transcription profile database (version 2016). Genotyping of polymorphisms located in transcribed regions and proximal promoters of candidate and surrounding genes was performed by multiplex assays using Sequenom MassARRAY platform and Sequenom iPLEX chemistry based on the manufacturer protocols (Sequenom, San Diego, CA).

Genome-Wide Association Studies

The proportion of phenotypic variance explained by host genetics for PCV2-viremia, PCV2-specific antibodies (IgM and IgG) and average daily gain (ADGi) during experimental infection was estimated based on Porcine SNP60 BeadArray genotypes using a BayesB model [10] and GenSel software [7]. The statistical model included litter, pen and batch as class variables and passive IgG and age at infection as covariates. Bayesian regression models fit multiple SNPs in genome-wide associations, assuming that the marker effects result from a mixture of a point mass distribution whereby SNP have null effects and a distribution of non-zero effects (e.g., normal, heavy tailed). Bayesian analyses were based on π equal to 0.99 that assumed a prior probability of 1% of the SNPs having a non-zero effect. Prior assumptions are made relative to the genetic and environmental variances and the proportion of markers that have a null effect on a specific trait of interest. These models are implemented via a Markov chain Monte Carlo (MCMC) sampling algorithm. The posterior means are averaged over the number of samples from the MCMC [11]. The Markov chain included 40,000 samples with the first 1,000 being removed as burn-in. Markov chain was set to use every 40^{th} sample to

estimate posterior distribution for the genetic variance explained by each 1 Mb window of the reference genome. This distribution was used to estimate the probabilities of each 1Mb window having a variance greater than 0 or greater than the average variance explained by each 1Mb window as described in McKnite et al. (2014).

Bayes Interval Mapping (BayesIM) was implemented to derive haplotype effects across the genome on PCVAD-related traits as described in Kachman (2015) [12]. Briefly, a hidden Markov model was used to generate 8 haplotype states based on SNP genotypes [13]. Phenotypic variation of the targeted traits was analyzed with a hierarchal Bayesian model. QTL were placed every 50 kb across the genome while average haplotype size was set to 500 kb. Genetic variances, haplotype effects, and model frequencies were estimated at each locus. There were 42,000 MCMC samples collected with the first 2,000 used for burn-in. The model included batch, litter and pen as random effects and IgG and age at infection as covariates. If a locus had an effect, haplotype effects for each cluster were modeled as independent normal random variables.

Associations between the single marker genotypes and phenotypic variation were tested using a linear mixed model fitted by JMP 10.0 (SAS Inst. Inc.) that included marker genotype and batch as fixed effects, litter and pen as random effects while age at infection and IgG were used as covariates. Additive and dominance effects were estimated for each of the targeted DNA polymorphisms. A similar model was used to estimate the interaction between SNPs.

Novel Assembly of the Proximal End of SSC12

Inverse PCR (iPCR), using four (AciI, AluI, HaeIII, HpaII, RsaII) and six cutter (EcoRI, HaeII, HincII, HindIII, KpnI, MfeI, MspA1l) restriction enzymes (New England Biolabs), T4 DNA ligase (New England Biolabs) and nested PCR using AmpliTaq Gold 360 DNA polymerase (Thermo Scientific), was employed to expand the genomic DNA sequence surrounding the short *ALGA0110477* sequence. A genomic scaffold (19 Mb) of the proximal end of SSC12 was constructed based on Pacific Biosciences sequencing reads [14]. The position of the extended *ALGA0110477* sequence and all SSC12 mapped and unmapped SNPs were determined on the genomic scaffold using BLAT. Annotation of the QTL region on the SSC12 scaffold was based on RNA-seq alignments and BLAST, but also *Ab initio* approaches such as GenScan [15, 16] in combination with pBLAST.

RNA-seq and Gene Expression Profiling

In order to profile transcriptome changes and sequence variation related to PCV2 infection, peripheral blood samples collected from the validation group of pigs that exhibited high (N_{TT}=6) and low (N_{CC}=5) viremic genotypes for *ALGA0110477* at 0, 7, and 14 dpi were subjected to RNA sequencing. RNA was extracted from peripheral blood collected in Tempus tubes using the Tempus Spin RNA Isolation Reagent Kit (Thermo Scientific). RNA samples were sequenced using Ion Proton technology as described in the manufacturer protocol (Thermo Fisher Scientific Inc.). The adaptor-free sequencing reads were trimmed and filtered using Trim galore (version 0.4) [17] with low-quality bases in the 5’ end being removed and nucleotides with quality call less than 22 being

trimmed from the 3' end. The filtered reads were initially aligned to the SSC12 scaffold (19 Mb) using the two-step alignment approach used for Ion Proton transcriptome data that includes both Tophat and local-Bowtie [18]. The reads were later also aligned to the new pig assembly *Sscrofa* 11.1. The number of reads mapped to each gene in the annotated QTL region was obtained using HTSeq (version 0.6.1p1)[19].

Expression of the candidate genes across time points following PCV2 infection was quantified using TaqMan Master Mix and CFX384 Real Time PCR. The qPCR assays were designed using IDT Realtime PCR Tool software (www.idt.com) and sequences generated based on RNA-seq alignments. RNA was extracted from peripheral blood samples collected in Tempus tubes from a subset of pigs representing all genotypes from the validation data set that displayed extreme viral load (n=40) from 0 to 21 dpi using the Tempus Spin RNA Isolation Reagent Kit (Thermo Scientific). Complementary DNA (cDNA) was obtained using a mix of random hexamers and poly dT primers using First Strand cDNA Synthesis Kit (GE Healthcare Bio-Sciences). Expression of ribosomal protein L32 (*Rpl32*) gene was used for normalization. Mean normalized expression (MNE) values were calculated based on cycle crossing thresholds (CT) obtained for the technical triplicates taking qPCR efficiencies into account [20]. MNE values for *SYNGR2 p.Arg63Cys* and *BIRC5 g.-343delA* genotypes and time points following infection were log10 transformed and compared by t-test.

2.3 Results and Discussion

Two Genomic Loci Influence Host Genetic Effect on PCV2 Infection

As specified in Engle et al. (2014), this study looked at multiple key phenotypes including weekly viremia and PCV2-specific antibodies, overall viral load, and growth rate as measures of PCV2 infection. During early infection, the proportion of phenotypic variation explained by SNP genotypes was low, however, increased following the observed surge in viremia and associated immune response. For example, proportion of phenotypic variation accounted for by the SNP genotypes went from 19% at 7 days post infection (dpi) to 52% at 14 dpi for viremia and from 14% and 3% at 7 dpi to 60% and 44% at 21 dpi for PCV2-specific IgM and IgG respectively (Table 1). However, the largest proportion of phenotypic variation explained by SNP genotypes was 64% for PCV2 viral load calculated across time points. Contrarily, the contribution of SNP genotypes to variation in Average Daily Gain (ADG, monitoring growth rate through body weight) remained low across time points throughout the challenge, 13% at 7dpi to 7% at 21 dpi, as well as for overall ADG, 16% (Table 1).

The initial GWAS association was performed using a BayesB model where individual SNPs and successive 1 Mb windows of the genome were evaluated for association with phenotypic variation [4]. Genome-wide average posterior distribution for the genetic variance was used to estimate the probability of each 1 Mb window having greater than the average genetic variance explained across PCV2-related traits. The GWAS revealed two windows with effects greater than the average effect associated with both viremia and antibody phenotypes ($Pr>0.90$) and therefore assumed to represent "true" Quantitative Trait Loci (QTL) (Figure 1). One QTL was located on SSC7 near the

Swine Leukocyte Antigen Complex Class II (SLAII) at 28-29 Mb and the other was putatively located near the proximal end of SSC12, at approximately 3-4 Mb. The top SNPs (*ALGA0039682 and ALGA0110477*) associated with the largest genetic variance for each window both explained over 94% of the genetic variance accounted for by the window.

The SNP, *ALGA0110477*, was associated with largest effect on PCV2 viral load, explaining 11.1% of the genetic variance and 7.4% of the phenotypic variance for viral load (Figure 2). In the 10.2 reference assembly, this SNP was located on an unplaced scaffold so linkage disequilibrium estimates between *ALGA0110477* and all other SNPs on the Porcine SNP60 BeadArray were used to determine its potential location. SNPs *ALGA0122316*, *ASGA0089708*, and *ASGA0090188* had the highest LD estimates (r^2<0.28) suggesting *ALGA0110477* is located on the proximal end of SSC12. However, despite LD these SNPs or any other within the region did not show any association with viral load in the initial analysis. Using a different Bayesian approach, BayesIM, [12] that fits haplotypes across the region rather than individual SNPs, we were able to detect an effect of this region even when excluding *ALGA0110477* from the analysis, supporting the initial identification of this region as a potential QTL for viral load (Figure 3).

Since the unplaced contig containing *ALGA0110477* was not annotated and the available sequence surrounding the SNP only extended 84 bp, inverse PCR (iPCR) was used to extend the proximal sequence to 1,252 bp. The elongated sequence was then compared with contigs from an early version of a long read-based genome assembly of a pig (accession NPJO00000000) [14], which uncovered a 19 Mb scaffold that provided precise location of *ALGA0110477* and context used for identification of candidate genes.

The recent release of a long read-based improved reference assembly, *Sscrofa* 11.1 (GenBank accession GCA_000003025), supported more accurate ordering and placement of markers, including *ALGA0110477* (SSC12, 3,673,576 bp), and facilitated finer analysis into genomic context with surrounding gene annotation. As a result, an enhanced profiling of the loci associated with PCV2-related phenotypes across time points was possible in order to distinguish the role of host genetics in innate and adaptive immunity.

This analysis utilizing *Sscrofa* 11.1 and a BayesIM approach, identified the same two locations on SSC7 and SSC12 as having the largest effects on viral load (Figure 4). In addition, haplotype effects estimated across the proximal end of SSC12 provided evidence of haplotypes with divergent effects in viral load, with a peak detected at 3.7 Mb (Figure 5). These QTL were also observed for other targeted traits (Figures 6-8). For viremia at 14 dpi, both QTL had similar effects, however, at 21 and 28 dpi the SSC7 QTL had a larger effect (Figure 6). An additional QTL on SSC8 that was not observed for overall viral load, had the largest effect for viremia at 14 dpi (Figure 6). For PCV2-specific antibodies, both QTL were associated with IgM variation (Figure 7), indicative of active infection, starting at 14 dpi and with IgG variation (Figure 8), representing previous PCV2 exposure or vaccination, starting at 21 dpi. Together these results provide evidence of two QTL located on SSC7 and SSC12 that represent the effect of host variation on PCV2 infection and associated immune response.

Fine Mapping and Annotation of the SSC12 QTL Region

The 19 Mb SSC12 scaffold was used to identify genes surrounding the top marker *ALGA0110477*. Annotation of this QTL region was achieved by combining *Ab initio* gene prediction, pBLAST, and RNA-seq analysis. Thirteen potential genes with an e value > $7e^{-64}$ and a pBLAST score > 200 were identified. Five of these genes were found to be expressed in RNA-seq data of peripheral blood from pigs subjected to PCV2. These five genes had functions in cytokine signaling (*SOCS3*), inhibition of apoptosis and regulation of cell proliferation (*BIRC5*), membrane trafficking and transport (*SYNGR2*), and transmembrane ion channels (*TMC6* and *TMC8*).

RNA-seq analysis of alternate *ALGA0110477* homozygotes with extreme high and low viral loads (n=22) were used for initial polymorphism discovery and uncovered 4 missense, 11 synonymous, and 10 UTR SNPs as well as 1 UTR indel across the five candidate genes. These polymorphisms were confirmed by targeted dideoxy sequencing of the genes. In addition, sequencing 1-2kb upstream of the Transcription Start Site (TSS) of *BIRC5*, *SOCS3*, and *SYNGR2* identified 32 SNPs and 4 short indels. These novel polymorphisms and 580 SNPs on the Porcine SNP60 BeadArray were mapped to the scaffold using BLAT. *ALGA0110477* had the highest LD with another marker from the BeadArray (*ASGA0086395*, r2=0.55) located about 24.5kb away followed by three SNPs within the *SYNGR2* gene (r2= 0.42-0.48) located 123.7kb away.

A subset of samples with extreme high and low viral loads (n=307) were genotyped for all novel polymorphisms. These genotypes and the Porcine SNP60 BeadArray SNPs that were mapped to the scaffold (n=632) were included in an additive linear mixed model analysis, which found a missense SNP (*p.Arg63Cys*) in *SYNGR2* and

a 1 bp insertion (*g.-343delA*) located 343 bp upstream of the *BIRC5* TSS have the largest effects on PCV2 viral load (Figure 9, F-ratio >47, P<0.0001). In addition, the phenotypic variance explained by each of the novel mutations was substantially larger (21-23% +/- 6.1-6.4%) compared to *ALGA0110477* (12.6 +/- 4.8%). These polymorphisms were also associated with large effects on all weekly viremia measures (P<0.0001) and PCV2-specific antibodies, starting from 14 dpi for IgM and 21 dpi for IgG (P<0.0001). The effects on growth during the challenge were most evident after 14 dpi as well as during the entire challenge period (0 – 28 dpi, P<0.0005).

SYNGR2 and *BIRC5* are Potential Candidate Genes Influencing PCV2 Replication

The *SYNGR2 p.Arg63Cys* SNP is located in the first loop of the *SYNGR2* protein in a region conserved across mammals [21] known to be crucial for formation of microvesicle membrane fraction [22]. The *Arg* residue is prevalent in other species (e.g., human, rat, cow, horse) sometimes being replaced by *His* (Rhesus macaque, dog), *Lys* (prairie vole, Chinese hamster) or *Gln* (mouse, golden hamster) while the *Cys* residue appears to be specific to swine (Figure 10). The substitution of *Arg* to *Cys* determines a change in charge and hydrophobicity of the loop (Figure 11). The *SYNGR2 p.63Cys* allele is favorable with the viral load of the homozygous genotype (Least Square Mean = 54.3 units) being lower compared to the heterozygote (67.03 units, $P = 0.005$) and alternate homozygote (*p.63Arg,* 79.54 units, $P < 0.0001$). The favorable homozygous genotype was also associated with lower weekly viremia (P < 0.0001, Figure 12), IgM (> 14 dpi, P < 0.0001, Figure 13), IgG (> 21 dpi, P < 0.0001, Figure 14) and higher growth (overall 0 – 28 ADG and > 14 dpi, P < 0.001) compared to the alternate homozygote. Expression of

SYNGR2 did not differ across *SYNGR2 p.Arg63Cys* genotypes or time points following *in vivo* PCV2 challenge.

The 1bp deletion located 343 bp upstream of the TSS of *BIRC5* (*BIRC5 g.-343delA*) was found to be in high LD ($r^2 = 0.83$) with *SYNGR2 p.63Cys* allele and as expected was associated with low viral load ($P < 0.0001$). The deletion was predicted to affect a potential motif for *NR5A2*, a DNA-binding zinc finger transcription factor. However, no significant difference in expression was observed between *BIRC5* genotypes across time points following *in vivo* PCV2 challenge ($P > 0.17$). At 14 dpi the homozygotes for the insertion exhibited an elevated nominal expression compared to the other genotypes, but the difference was not significant ($P = 0.061$).

2.4 Conclusion

In this study, host genotypes explained a substantial proportion of the phenotypic variation for PCV2 viremia, viral load, and immune response, with two major QTL identified on SSC7 and SSC12. The inability to uncover a QTL located on SSC12 in the previous report [4] was based on 1) a genetic structure with a very limited number of homozygotes for the *SYNGR2 p.63Cys* allele (Q = 1.2%) in the initial study compared to the Engle et al. (2014) dataset (Q = 18.3%), which is less favorable for detecting associations in additive statistical models, and 2) lower ability of *ALGA0110477* to capture the low viremic effects of *SYNGR2 p.63Cys*. While in the Engle et al. (2014) dataset the presence of the *ALGA0110477 C* variant had a 65% probability of being located on the same haplotype with the *SYNGR2 p.63Cys* allele, in McKnite et al. (2014) this variant is found in similar proportions in haplotypes that carry different *SYNGR2* alleles (e.g. *SYNGR2 p.63Cys*; P = 55.9%).

Dissection of the SSC12 QTL based on gene annotation, genomic and RNA sequencing uncovered a non-conservative substitution in a key domain of the *SYNGR2* gene and a single base pair insertion upstream of the *BIRC5* gene associated with PCV2 viremia and immune response. Located 41.9 kb apart, the high LD observed between *SYNGR2 p.Arg63Cys* and *BIRC5 g.-343delA* ($r^2 = 0.83$) hampered the ability to distinguish their individual effects in the *in vivo* challenge dataset. In contrast, the LD between *SYNGR2 p.Arg63Cys* and other *SYNGR2* SNPs was limited ($r^2 < 0.26$), as well as that with *BIRC5 g.-343delA* and other *BIRC5* polymorphisms ($r^2 < 0.16$). Since there was no significant change in *BIRC5* expression throughout the challenge period, we hypothesize that the missense *SYNGR2 p.Arg63Cys* mutation is the functional mutation

associated with PCV2 replication and not *BIRC5 g.-343delA.* We believe that the observed effect of *BIRC5 g.-343delA* on PCV2 viral load is due to the tight linkage of this polymorphism with *SYNGR2 p.Arg63Cys.* Additionally, we propose that the effects on growth and PCV2-specific antibodies are a result of the variation in viremia modulated by *SYNGR2.*

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CHAPTER 3

SYNAPTOGYRIN-2 INFLUENCES *IN VITRO* PCV2 REPLICATION IN PORCINE KIDNEY 15 CELL LINE*

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3.1 Introduction

Synaptogyrin-2 (*SYNGR2*) is a non-neural member of the synaptogyrin family, a group of genes primarily expressed in the membrane of synaptic vesicles of neuronal cells with roles in vesicle biogenesis, exocytosis and recycling via endocytosis [1, 2]. There is limited information about the functional role of this member of the gene family. Recently, *SYNGR2* was implicated as an active player in promoting viral RNA replication and immune evasion of thrombocytopenia syndrome virus (SFTSV), a novel tick-borne bunyavirus in humans [3]. *SYNGR2* interacted with non-structural viral proteins to promote the formation of lipid–based inclusion bodies, which become virus factories within the cytoplasm of infected cells. *SYNGR2* mRNA had been upregulated more than 200-fold at 36 hours post infection (hpi) with SFTSV. *In vitro* silencing of *SYNGR2* resulted in a decrease in viral replication and a reduction in the number and size of the inclusion bodies, further substantiating the role of *SYNGR2* in facilitating SFTSV infection [3].

Following PCV2 uptake, virus-like particles (VLP) were found to be integrated into intracytoplasmic inclusion bodies in infected cells, in both *in vivo* and *in vitro* experiments [4-6]. The VLP are localized in endosomes at the initial phase of infection. After release from early endosomes, the viral capsid of PCV2 has been shown to induce acetylation of microtubular α-tubulin interacting directly with dynein, a molecular motor used to transport VLP cargo along microtubules to the nucleus for replication [7]. PCV2 is a DNA virus and replication of the viral DNA takes place in the nucleus [8]. As a result, the involvement of *SYNGR2* in endocytosis indicates its potential role in modulating PCV2 replication and disease progression. *SYNGR2 p.Arg63Cys*, the only

missense polymorphism identified in *SYNGR2* (Chapter 2) and characterized by a predicted change in charge and hydrophobicity of the first loop that connects two essential transmembrane domains, is located in a region conserved across mammals (Chapter 2). In rats, the first intraluminal loop and C-terminus of *SYNGR2* were found to be crucial for successful incorporation of the protein into vesicular membranes and vesicle formation [1]. Replacement of residues 67–73 in the first loop led to protein degradation, with residues 70–73 having the largest impact [1]. In pigs, this segment of four residues is analogous to amino acids 60–63.

Considering that the location of the *SYNGR2 p.Arg63Cys* substitution is in a conserved domain involved in vesicle formation [1] and recent literature support of *SYNGR2* affecting replication of a tick-borne human RNA virus [3], we hypothesize that *SYNGR2* may play a role in the internalization and early release of PCV2 from endosomes influencing efficient trafficking of VLP to the nucleus for replication. In order to validate a role of *SYNGR2* in facilitating PCV2 replication, we transfected Porcine kidney 15 cell line (PK15) with small interfering RNAs (siRNA) targeting the mRNA of *SYNGR2* and subsequently inoculated the silenced cells with the same PCV2b isolate used *in vivo* (UNL2014001).

The PK15 cell line has an epithelial origin and is a well-established model system for PCV2 innate immunity and cellular pathogenesis [9]. The purpose of transfecting the cells with *SYNGR2*-specific siRNA was to silence or suppress the expression of *SYNGR2* by utilizing a natural process known as RNA interference [10]. The siRNA are 21 to 23 nucleotides long with sequence specificity for certain mRNA. Once integrated into the cell, these siRNA assemble with proteins forming an endonuclease complex and act as

guide RNAs targeting complementary mRNA for degradation [10]. Thus, siRNA modulate gene expression by degrading mRNA prior to translation, preventing generation of proteins. However, exogenous delivery without endogenous expression of the desired siRNA results in transient suppression lasting about a week *in vitro* [10]. By silencing *SYNGR2*, we could provide evidence of its role in PCV2 infection and impact on viral replication.

3.2 Materials and Methods

PK15 Cell Culture, Sample Collection, and Genotyping

The porcine kidney cell line (PK15), was grown in DMEM high glucose media supplemented with 10% FBS and 1% Penicillin-Streptomycin (5,000 U/mL). Cell samples were collected from culture plates in PBS and centrifuged for ~1 min at 16,000xg to pellet the cells. PBS was removed and pelleted cells were stored at -80°C. DNA and RNA were extracted using TRIzol reagent following the manufacturers protocol (Thermo Fisher). The cDNA was generated from extracted RNA using First strand cDNA Synthesis Kit (GE Healthcare Bio-Sciences). Dideoxy sequencing of the cDNA and genomic DNA was used to profile the gene sequences and genotype the *BIRC5* and *SYNGR2* variants in the PK15 cell line.

In vitro PCV2 Infection of PK15 Cells and Expression Profiling

Cells were cultured in 12-well plates (4 cm^2) with $5.0x10^5$ cells per well and infected with UNL2014001 PCV2b strain ($TCID_{50}=10^4$) when 80-100% confluent at MOI=0.00025. One hour following infection, cells were washed and fresh media was added (DMEM high glucose and 2% FBS). The cells were incubated at 37 ˚C with 5% CO_2 for up to 5 days. Control cells were maintained the same way and mock inoculated with plain DMEM high glucose media. Supernatant and cells were collected at specific time points (0, 12, 24, 48, 72, and 96 hpi) and frozen at −80 ˚C. Viral DNA was extracted from supernatant using QIAamp DNA Mini kit (Qiagen). RNA, viral and host DNA was extracted from PK15 cells using AllPrep DNA/RNA Mini kit (Qiagen). TaqMan Master Mix and CFX384 Real Time PCR Detection System (BioRad) were used for

quantification of PCV2 and expression profiling of *BIRC5* and *SYNGR2* from PK15 cells. Expression of ribosomal protein L32 (*Rpl32*) gene was used for normalization of gene expression. MNE values of *SYNGR2* and *BIRC5* were log10 transformed and compared by t-test between control and infected cells.

In vitro Silencing of *SYNGR2* in PK15 Cells

PK15 cells were transfected 24 hours after plating in 12-well plates (4 cm^2) with $2.5x10^5$ cells per well when ~80% confluent with two siRNA oligos targeting *SYNGR2* mRNA (siRNA-01: sense 5'-CUACAAGGCCGGAGUGGAUUU-3', and antisense 5'-AUCCACUCCGGCCUUGUAGUU-3'; siRNA-03: sense 5'-CCACAAGUCCGGAGAGCAGUU-3', and antisense, 5'-CUGCUCUCCGGACUUGUGGUU-3', Dharmacon Research) and the AllStars Negative Control siRNA (scramble, Qiagen) at 10nM and 20nM concentrations. Transfection was performed using Lipofectamine RNAiMAX transfection reagent (Invitrogen) following the manufacturer's protocol. Cell samples were collected 24, 48, 72, and 96 hours post transfection and stored at -80°C. RNA was extracted using RNAeasy Mini kit (Qiagen). Real Time PCR was used to profile *SYNGR2* expression. Following initial testing, the siRNA-01 and AllStars Negative Control siRNA both at 20nM concentrations were used for subsequent transfections. Cells were inoculated 24 hours after transfection following the same infection protocol described above. Statistical differences in viral titer between cell lines across time points were tested using a linear model fitted by JMP 10.0 (SAS Inst. Inc.) that included batch and cell line as fixed effects. Pairwise comparisons between least-squares means of the viral titers were based on the Tukey test.

3.3 Results and Discussion

BIRC5 and *SYNGR2* Gene Structure and Variants in PK15 Cells

Targeted cDNA and genomic DNA sequencing of the PK15 cell line for both genes revealed no differences in transcript structure compared to the RNAseq data for either *SYNGR2* or *BIRC5*. Interestingly, only one *BIRC5* isoform was observed in the PK15 cell line compared to four isoforms present in the RNAseq data from pig sera collected during the experimental challenge. The isoform includes 4 complete exons and was the predominate isoform observed *in vivo*. The PK15 cell line was also determined to be homozygous for the unfavorable alleles associated with increased viral load for both *SYNGR2 p.Arg63Cys* (*p.63Arg*) and *BIRC5 g.-343delA* (*g.-343insA*).

In vitro PCV2 Infection had No Effect on Expression of *BIRC5* or *SYNGR2*

Successful infection of PK15 cell culture with PCV2b inoculate was observed, with consistent infection profiles across three independent replications in both supernatant and cell samples (Figures 15 and 16). In the supernatant we saw an increase in viral copies from 0 to 12 and 24 to 72 hpi, with plateaus observed from 12 to 24 hpi and at 96 hpi (Figure 16). In the cell samples we observed a decrease in viral copies per well between 0 and 12 hpi, which is expected because at 0 hours the cells are saturated with PCV2 virions, however, only a portion of the virions will successfully enter the cell and able to be observed at 12 hpi (Figure 15). The rest of the virions will be released and present in the supernatant. From 12 hours onward we see a consistent increase in PCV2 copies, indicating successful PCV2 replication and proliferation, with PCV2 titer peaking at 72 hpi and decreasing slightly at 96 hpi likely due to cell loss (Figure 15).

Expression profiling of *BIRC5* and *SYNGR2* across time points post infection showed no significant difference in expression for either gene between infected and control cells, corroborating *in vivo* findings. Although expression of *SYNGR2* increased and *BIRC5* decreased over the 4-day period following infection, these changes were observed in both infected and control cells (Figures 17 and 18). The lack of evidence to suggest that PCV2 infection triggers changes in gene expression strengthened our hypothesis that *SYNGR2 p.Arg63Cys* is the functional source responsible for variation in PCV2 replication.

Viral titer was Decreased in *SYNGR2*-Silenced Cells Infected with PCV2

We evaluated two siRNA (siRNA-01 and siRNA-03) at two different concentrations (10 nM and 20 nM) and found that siRNA-01 was the most efficient to knockdown mRNA level of *SYNGR2* compared to the cells subjected to a scramble siRNA control, with an average reduction of 82.2% in *SYNGR2* mRNA level observed starting 24 hours after transfection (≈0 hpi, Figure 19). PK15 cells with the expression of *SYNGR2* silenced were then infected with PCV2 24 hours after transfection. A reduction in viral titer was observed in the *SYNGR2* silenced cells subjected to PCV2 starting at 48 hpi when compared to scramble siRNA and non-transfected control cells, indicating a role of *SYNGR2* in viral replication ($P < 0.05$, Figure 20). The viral titer across time points was not statistically different between the scramble siRNA and non-transfected control cells ($P > 0.54$, Figure 20).

3.4 Conclusion

In our study, we observed similar results to those reported by Sun et al. (2016), in that silencing of *SYNGR2* expression in PK15 cells was associated with a significant reduction in PCV2 titer, indicating a role of *SYNGR2* in promoting viral replication. Although, we did not observe an increase in *SYNGR2* mRNA levels following *in vitro* (like in Sun et al. study) or *in vivo* infection with PCV2, this may simply reflect important distinctions between SFTSV and PCV2. For instance, SFTSV is an RNA virus with the capacity to replicate within intracytoplasmic inclusion bodies, or viral factories. As Sun et al. (2016) illustrated*, SYNGR2* is a component of these vesicles and necessary for their formation. As SFTSV replicates, more viral factories will be required for viral proliferation, resulting in increased levels of *SYNGR2*. PCV2, on the other hand, is a DNA virus and can only replicate in the nucleus of host cells. Therefore, the potential role of *SYNGR2* in PCV2 infection likely takes place prior to viral replication and may not require such an increase in *SYNGR2* expression, but rather specific *SYNGR2*-PCV2 interactions. Since the position of this substitution is clearly located in a loop domain and not part of a transmembrane region as indicated by Janz and Sudhof (1998) and predicted by PSIPRED [11], an interaction between *SYNGR2* and a ligand is favored compared to the potential impact of *SYNGR2 p.Arg63Cys* on overall protein folding or loop conformation (Figures 21 and 22). Therefore, as long as there is an adequate level of *SYNGR2* protein, efficient PCV2 infection is possible.

While further research is required to define the mechanism in which *SYNGR2* and its alleles alter PCV2 multiplication, we propose the following theories based on current findings and the nature of PCV2 infection. We hypothesize that the interaction between a

ligand and the protein encoded by the *SYNGR2 p.63Cys* allele may 1) reduce the ability of the virus to enter the cell through the endocytic pathway or be internalized into endosomes following cellular entry, 2) alter capsid release from early endosomes, thus preventing migration of the virus to the nucleus for DNA replication or 3) decrease the efficiency of virus uncoating, an essential step required for successful replication. Additional studies utilizing gene editing technology will also be necessary to provide direct experimental evidence of the impact of allelic substitution at the *SYNGR2 p.Arg63Cys* locus on PCV2 replication, however, based on our data we believe the *SYNGR2 p.Cys63* variant decreases the ability of PCV2 to replicate following infection. The significant decrease in viral titer observed following an 80% reduction in *SYNGR2* expression *in vitro*, despite presence of the unfavorable *BIRC5* variant, provides strong evidence of the involvement of *SYNGR2* in PCV2 infection. Together with the lack of expression differences for *BIRC5 in vivo* and *in vitro* in response to PCV2 infection, strongly suggests the only missense mutation, *SYNGR2 p.Arg63Cys*, to be a plausible quantitative trait nucleotide (QTN) for PCV2 susceptibility. These findings not only contribute to a better understanding of the critical players involved in pathogenesis of PCV2 infected cells, but also the role of host genetics in viral disease susceptibility.

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TABLES

Table 1. Proportion of phenotypic variance explained by SNPs from Porcine SNP60 BeadArray using Bayes B of PCV2-related traits days post infection (dpi) with PCV2.

Trait/dpi	**7**	**14**	**21**	**28**	**0-28**
Viremia	0.19	0.52	0.45	0.39	0.64
IgM	0.14	0.60	0.44	0.52	
IgG	0.03	0.08	0.44	0.38	
Trait/dpi	**0-7**	**7-14**	**14-21**	**21-28**	**0-28**
ADG	0.13	0.11	0.07	0.07	0.16

FIGURES

Figure 1. Probability of a 1 Mb window to have an effect above the average effect of the genome-wide 1 Mb windows estimated using BayesB on overall Average Daily Gain (ADG) and PCV2 viral load (AUC). The genetic variance for PCV2 viral load explained by the top two 1 Mb windows that included *ALGA0039682* (SSC7) and *ALGA0110477* (SSC12) have effects above the 1 Mb window average effect (P > 0.90).

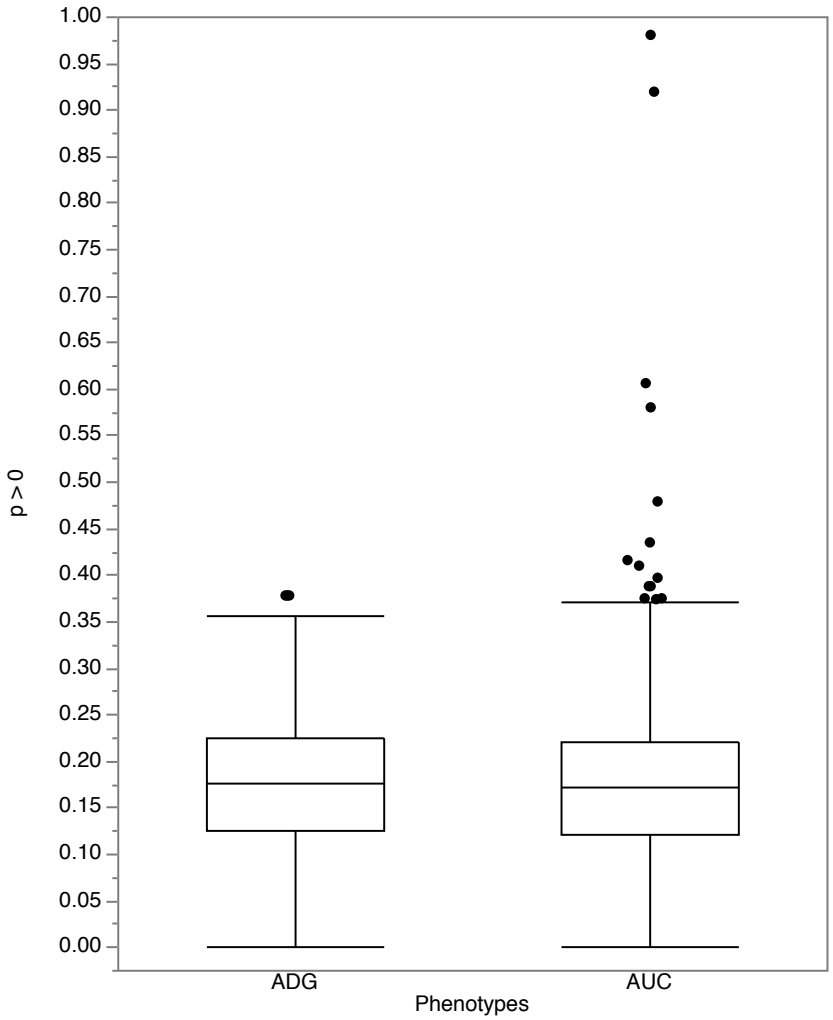


Figure 2. Genome-wide association between 56,433 SNPs and PCV2b viral load using BayesB. Each dot represents the proportion of genetic variance explained by an individual SNP. The x-axis represents the position of each SNP in the swine genome using *Sscrofa* 10.2 assembly. The y-axis represents the contribution of each SNP to the genetic variance. Alternate colors represent autosomes, from SSCs 1 to 18, chromosome X and Y followed by a set of SNPs without a genomic location. *ALGA0110477* is located in the last group of SNPs with the genomic location unassigned, but characterized by a model frequency of 0.96.

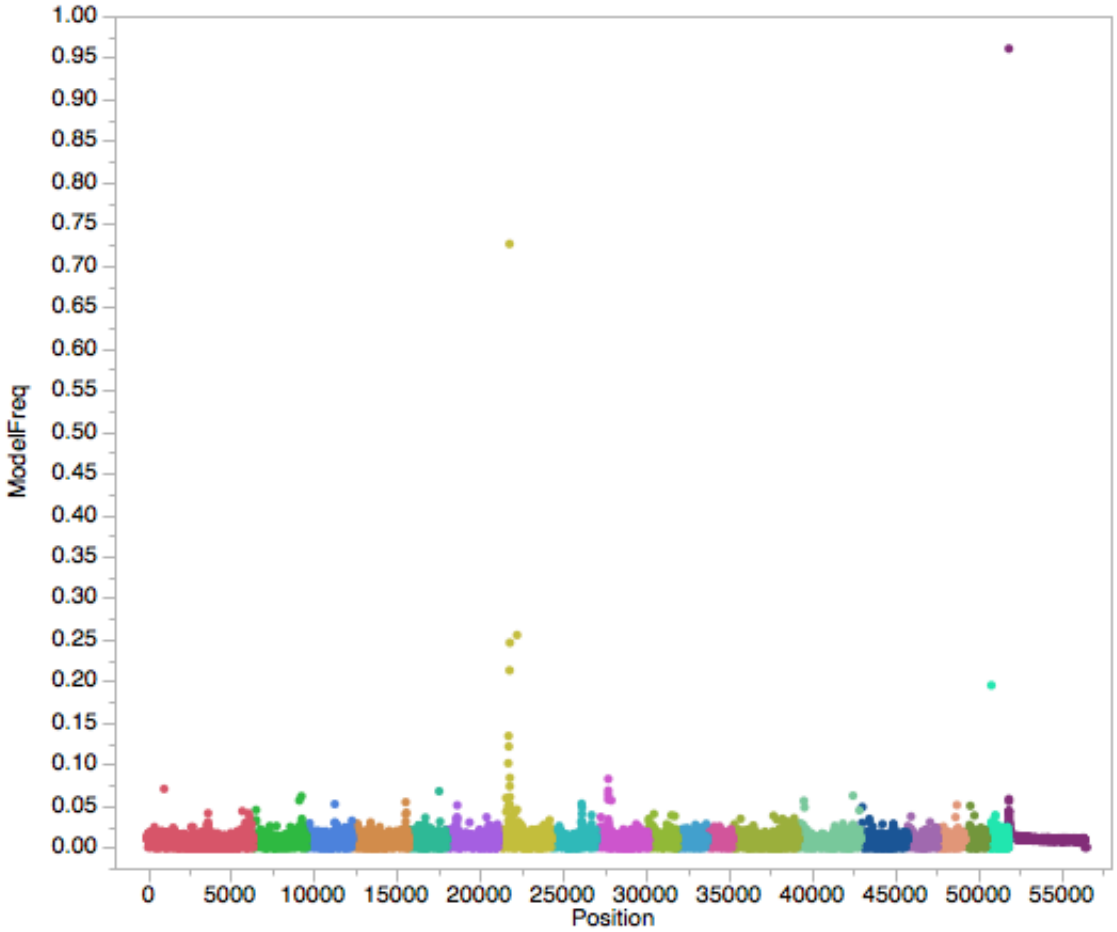


Figure 3. Genome-wide association between 54,946 SNPs and PCV2b viral load using BayesIM. Marker *ALGA0110477* and all other unmapped SNPs were excluded from the analysis. Each dot represents the model frequency associated with each 50kb QTL. The X-axis represents the position of the QTL across the swine genome using *Sscrofa* 10.2 assembly. The Y-axis represents the model frequency of the association between a QTL and PCV2 viral load. Alternate colors represent autosomes, from SSC1 to 18.

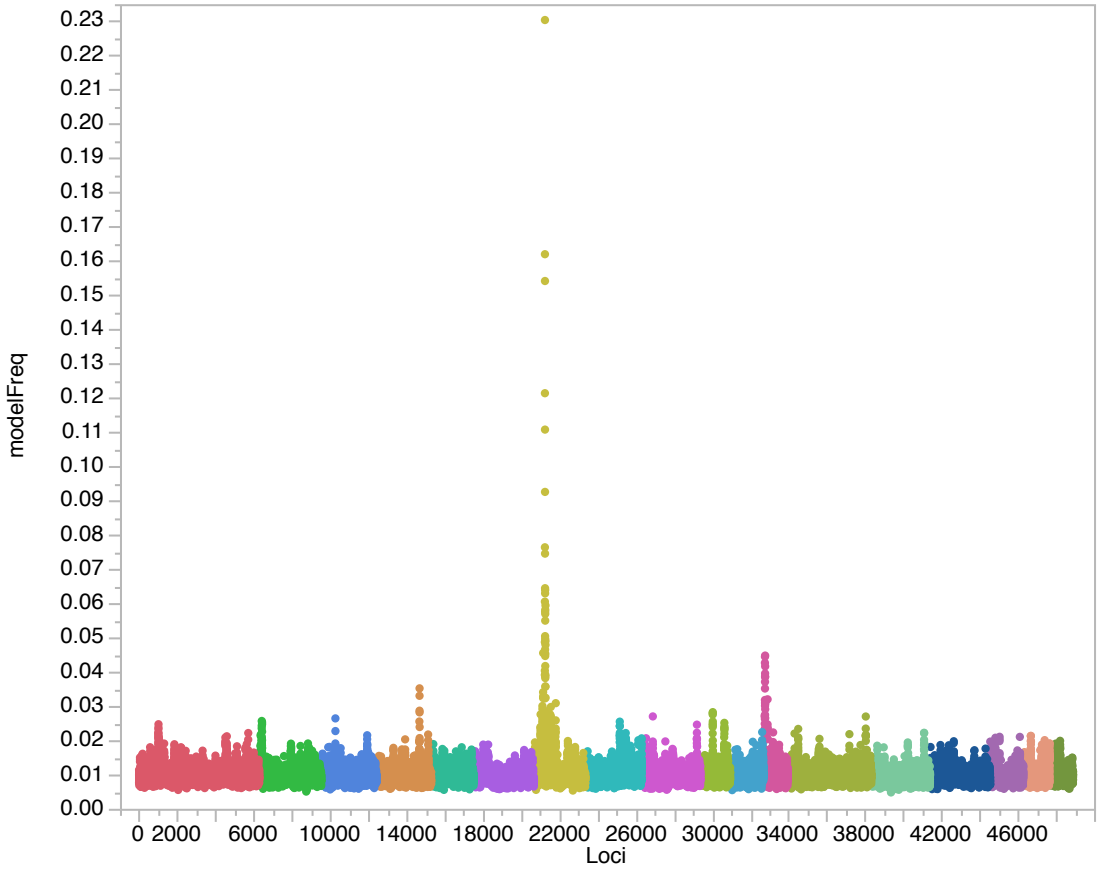


Figure 4. Genome-wide association between 51,592 SNPs and PCV2b viral load using BayesIM. Each dot represents the model frequency associated with each 50kb QTL. The X-axis represents the position of the QTL across the swine genome using *Sscrofa* 11.1 assembly. The Y-axis represents the model frequency of the association between a QTL and PCV2 viral load. Alternate colors represent autosomes, from SSC1 to 18.

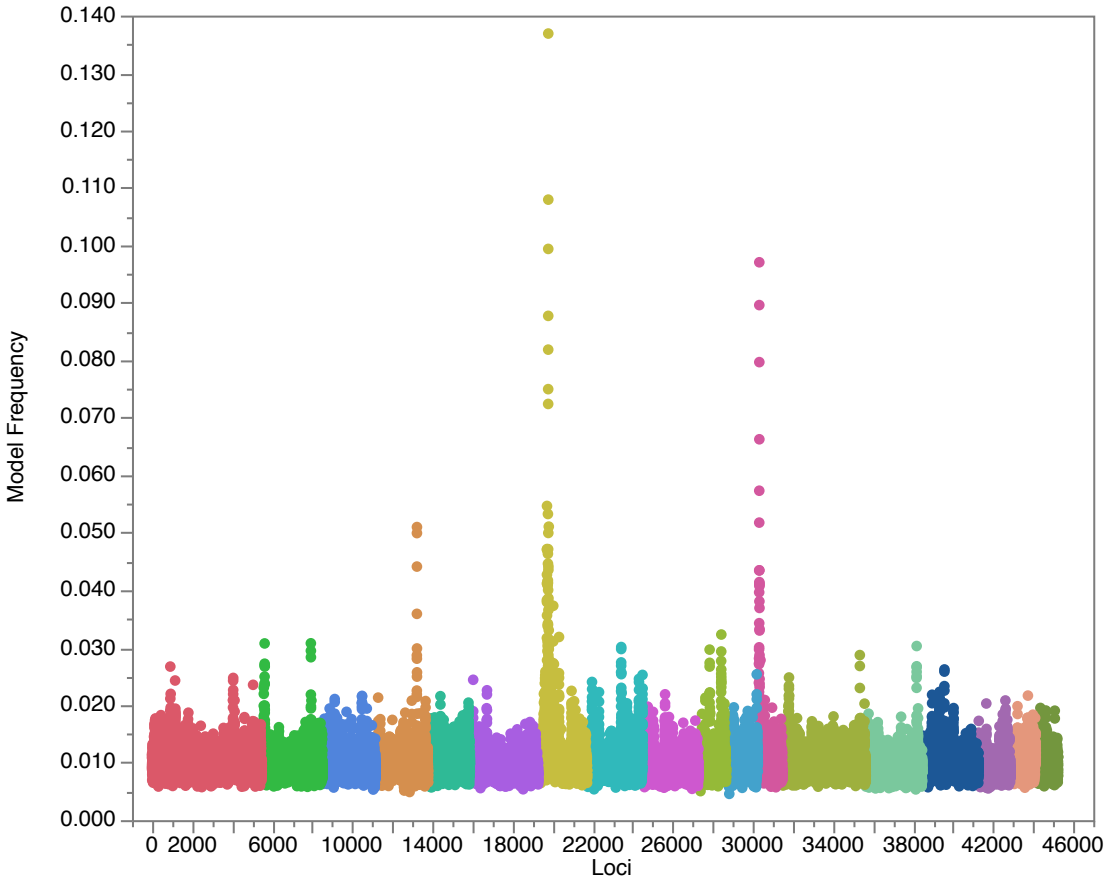


Figure 5. Haplotype effects for PCV2 viral load across the proximal end of SSC12 estimated using BayesIM model.

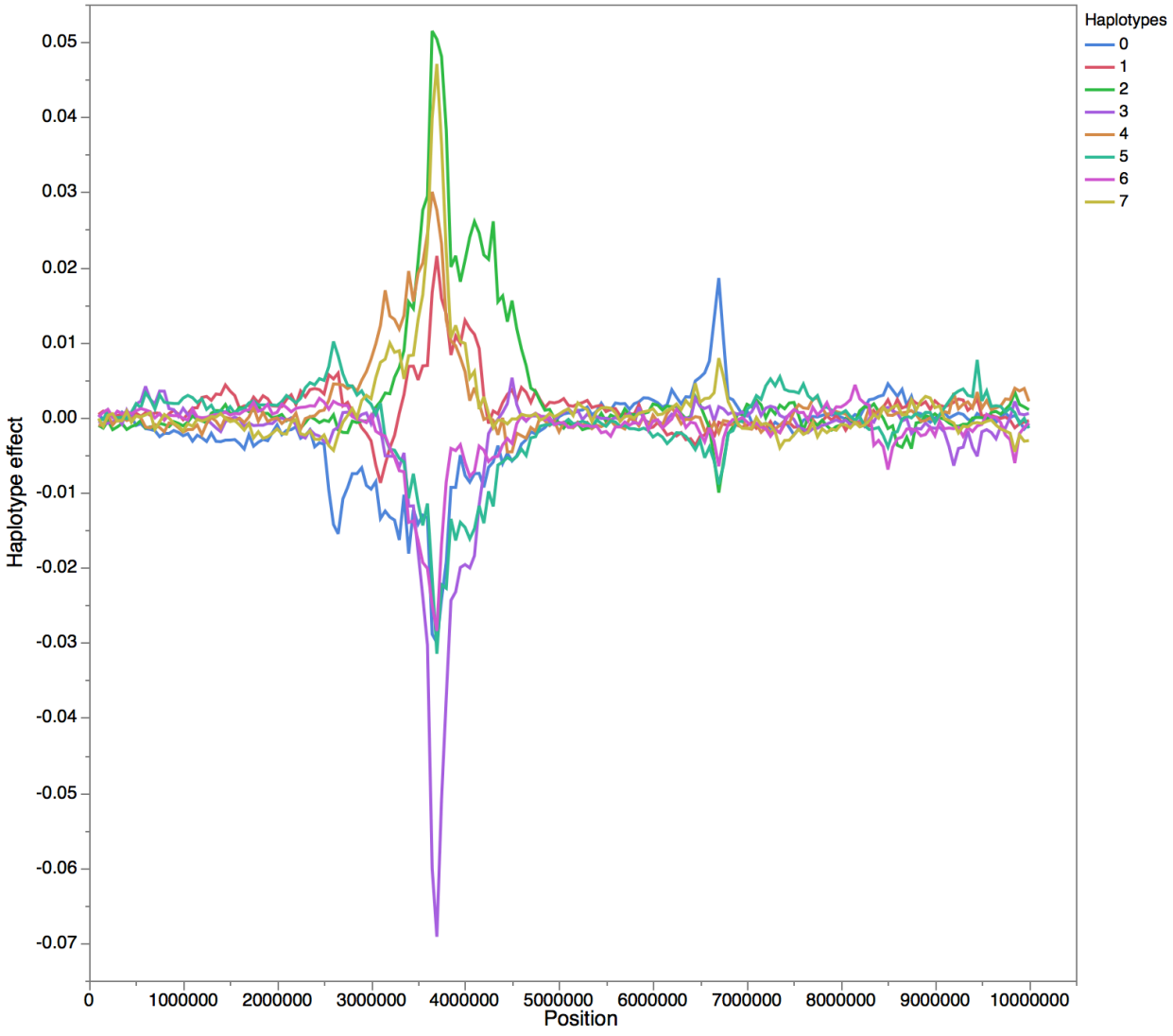


Figure 6. Genome-wide association between 51,592 SNPs and PCV2b viremia using BayesIM. Each dot represents the model frequency associated with each 50kb QTL. The X-axis represents the position of the QTL across the swine genome using *Sscrofa* 11.1 assembly. The Y-axis represents the model frequency of the association between a QTL and PCV2 viremia. Alternate colors represent autosomes, from SSC1 to 18.

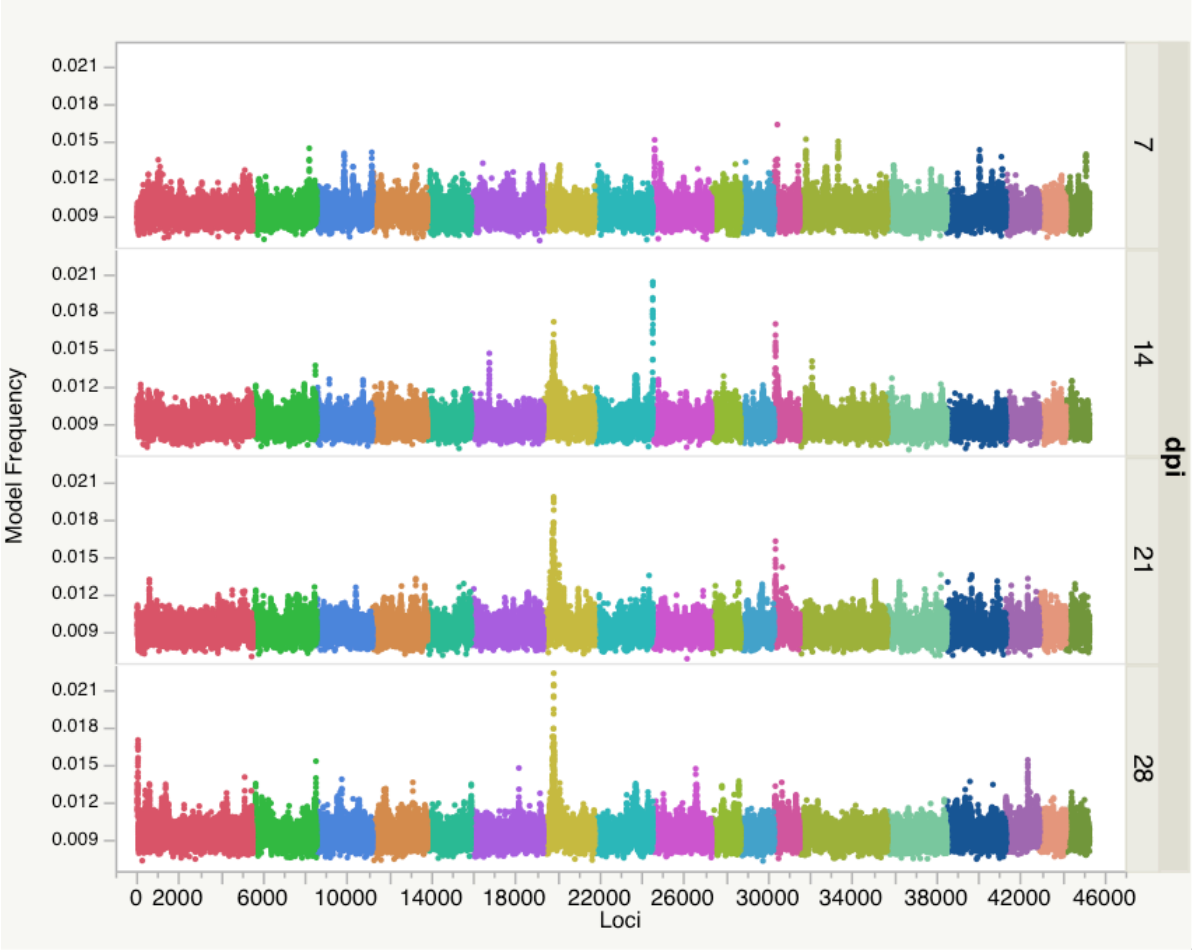


Figure 7. Genome-wide association between 51,592 SNPs and PCV2-specific IgM using BayesIM. Each dot represents the model frequency associated with each 50kb QTL. The X-axis represents the position of the QTL across the swine genome using *Sscrofa* 11.1 assembly. The Y-axis represents the model frequency of the association between a QTL and IgM following PCV2 infection. Alternate colors represent autosomes, from SSC1 to 18.

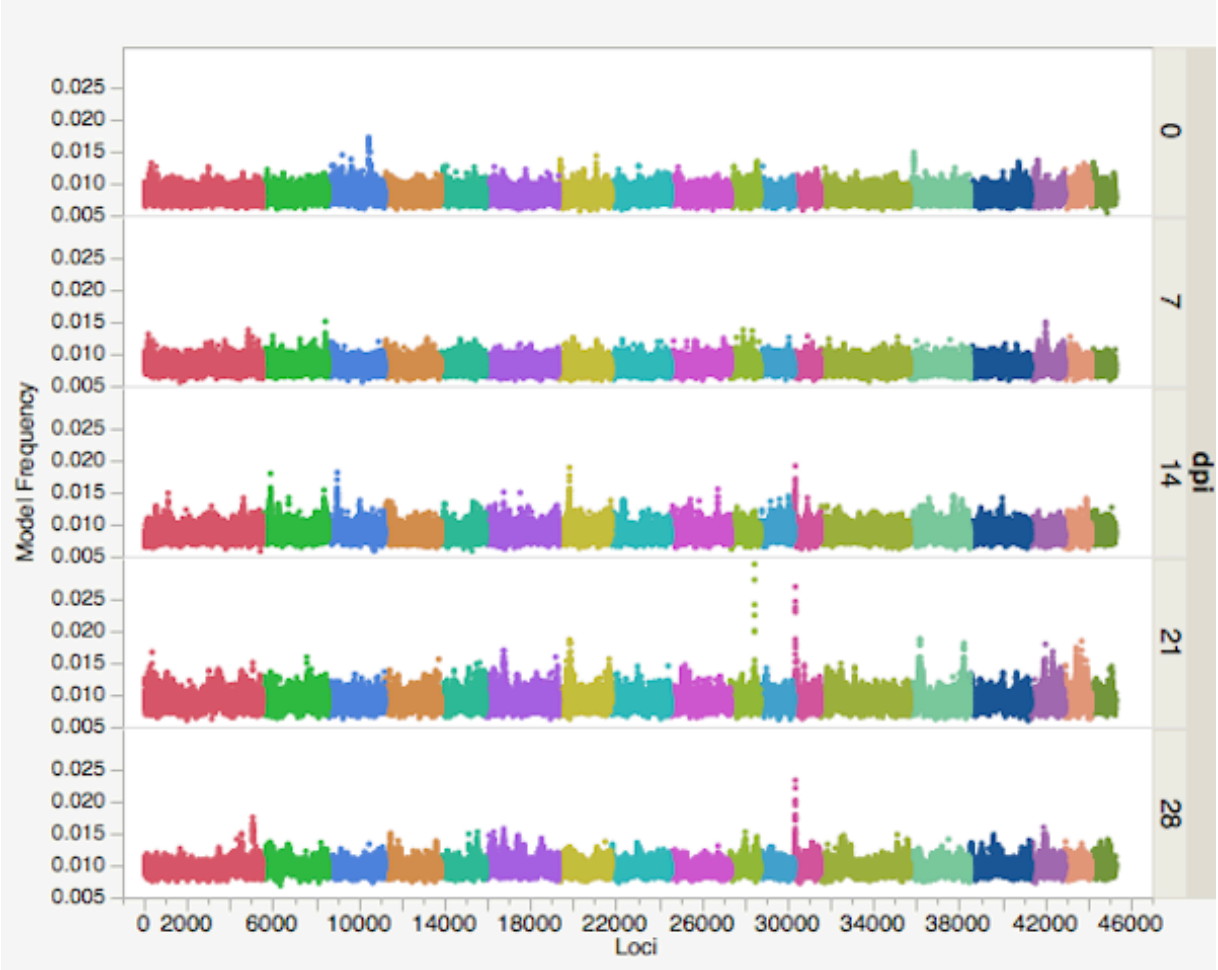


Figure 8. Genome-wide association between 51,592 SNPs and PCV2-specific IgG using BayesIM. Each dot represents the model frequency associated with each 50kb QTL. The X-axis represents the position of the QTL across the swine genome using *Sscrofa* 11.1 assembly. The Y-axis represents the model frequency of the association between a QTL and IgG following PCV2 infection. Alternate colors represent autosomes, from SSC1 to 18.

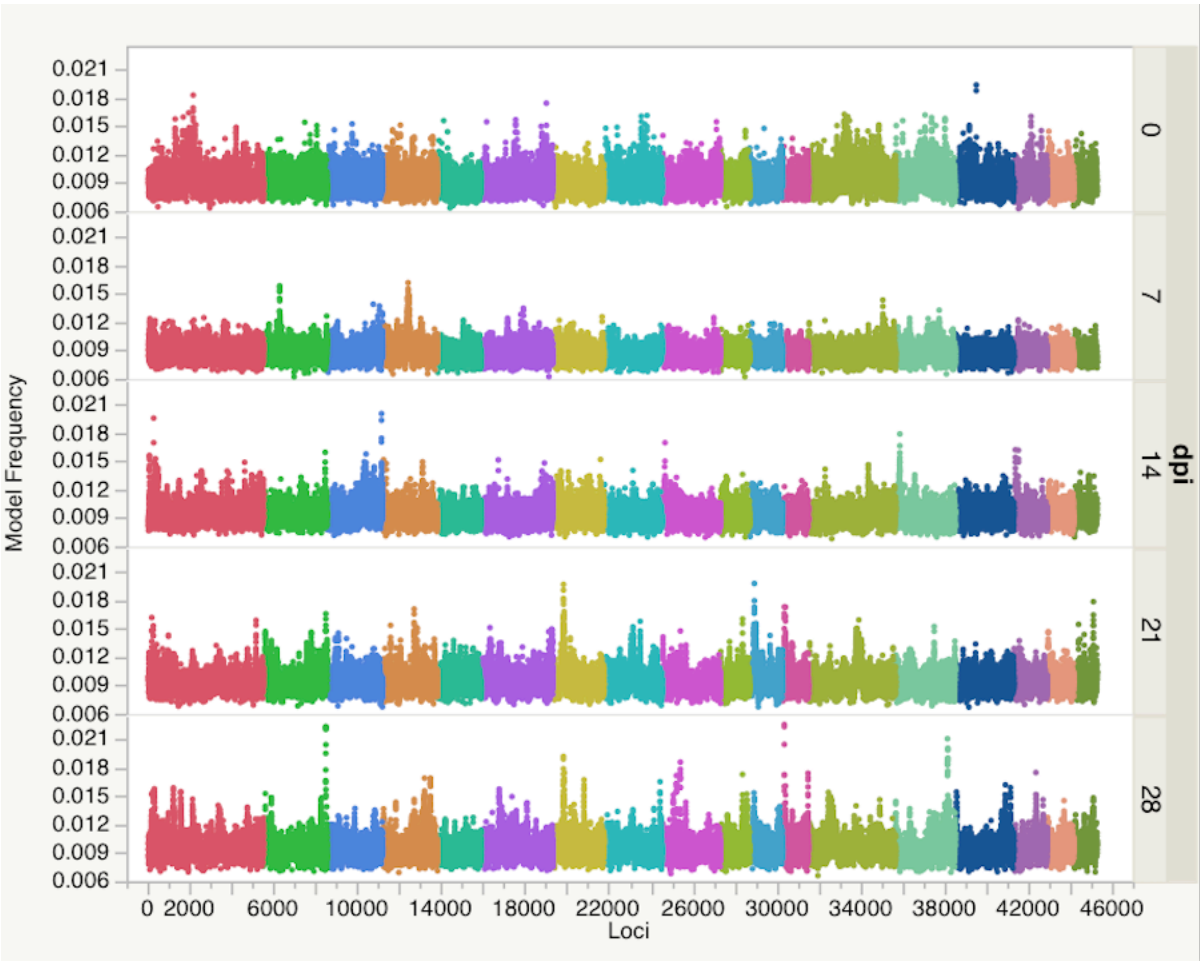


Figure 9. Association results between the genotypes of the DNA polymorphisms mapped to the 19 Mb scaffold of the proximal end of SSC12 and PCV2 viral load using a linear mixed additive model. The line represents a smoother with a default λ of 0.05. *SYNGR2 p.Arg63Cys* and *BIRC5 g.-343delA* were associated with the largest effects on PCV2 viral load (F-ratio > 47, $P < 0.0001$).

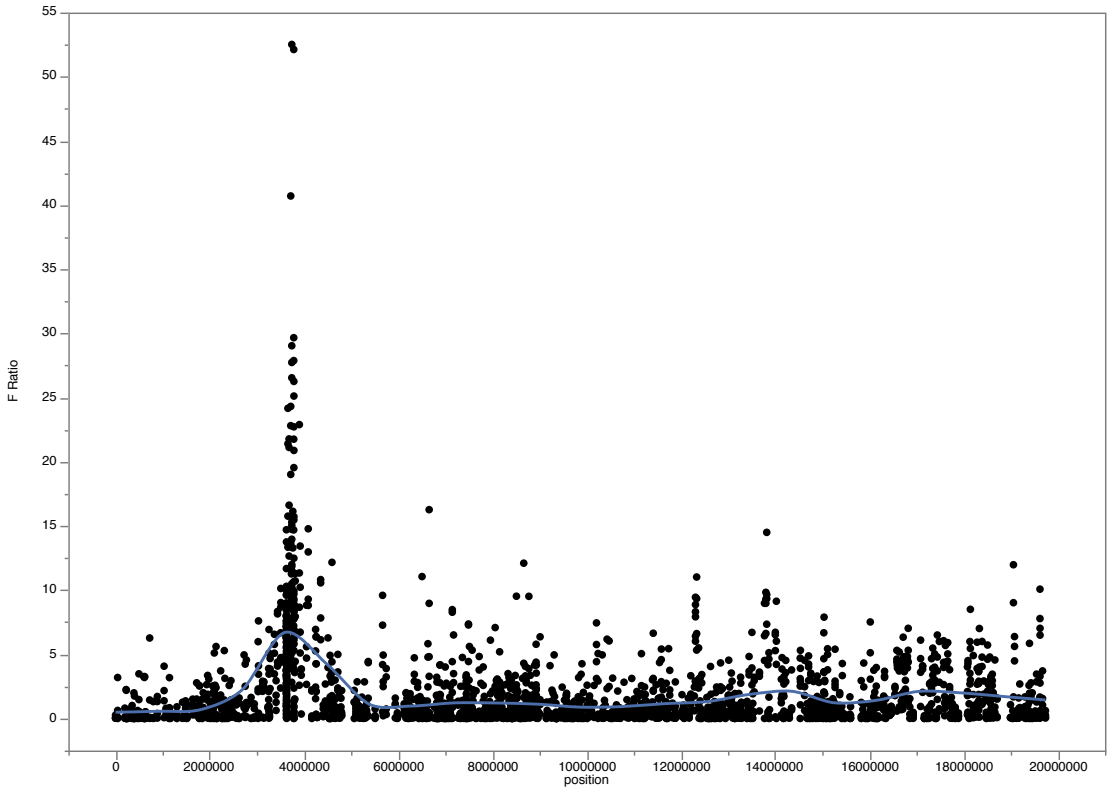


Figure 10. Sequence alignment of the first loop of *SYNGR2* across mammalian species. The red block indicates a conserved region demonstrated to be important in successful incorporation of the protein into vesicular membranes and vesicle formation.

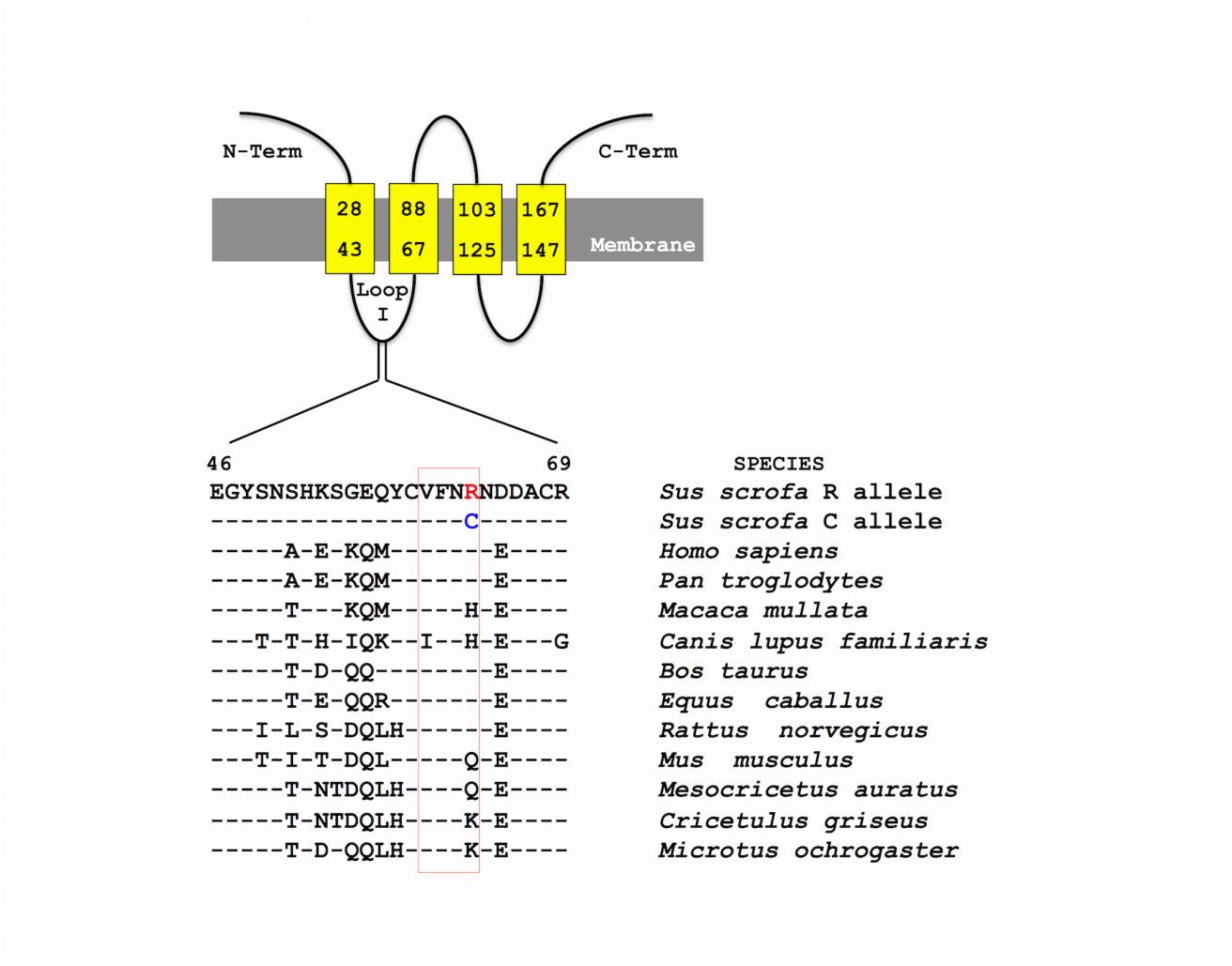


Figure 11. Hydrophobicity profile of the *SYNGR2 Arg63Cys* polypeptides based on the Kyte and Doolittle scale (the window consisted of 9 amino acid residues). An increase in the hydrophobicity score was observed in the predicted *SYNGR2 p.63Cys* polypeptide.

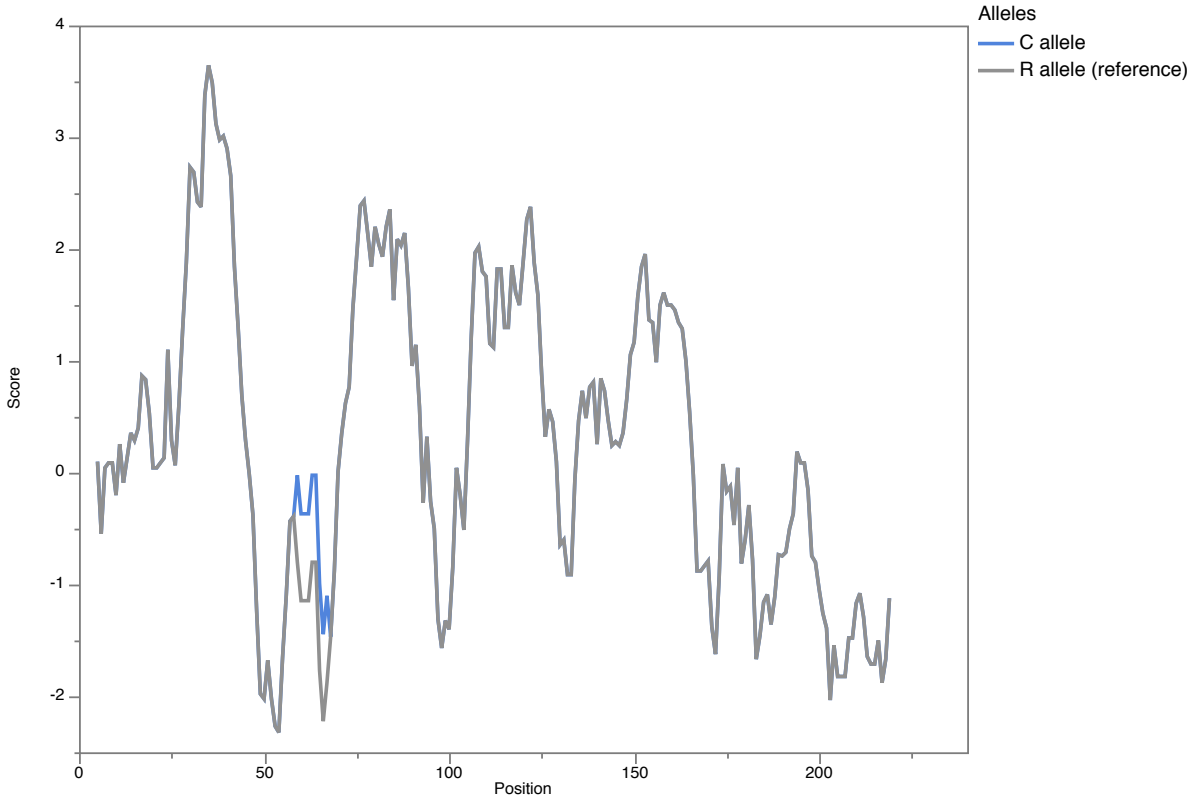
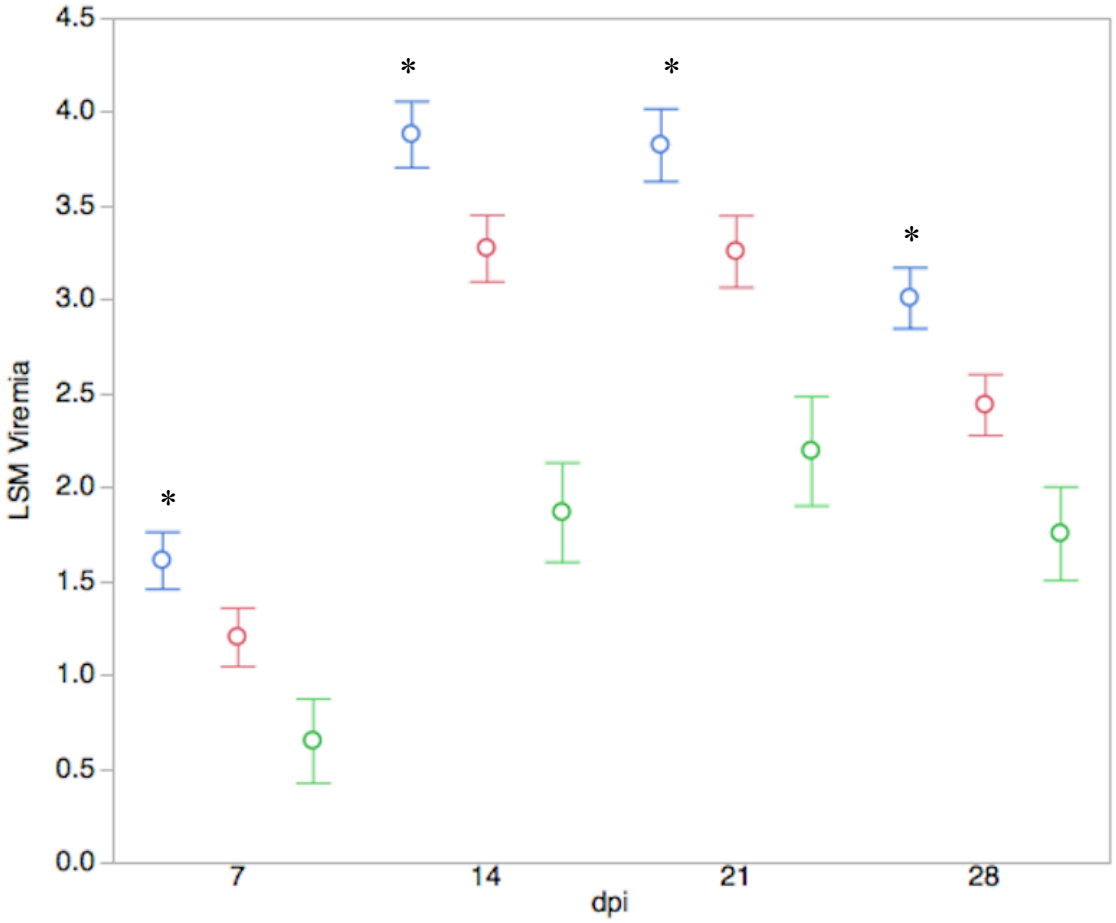
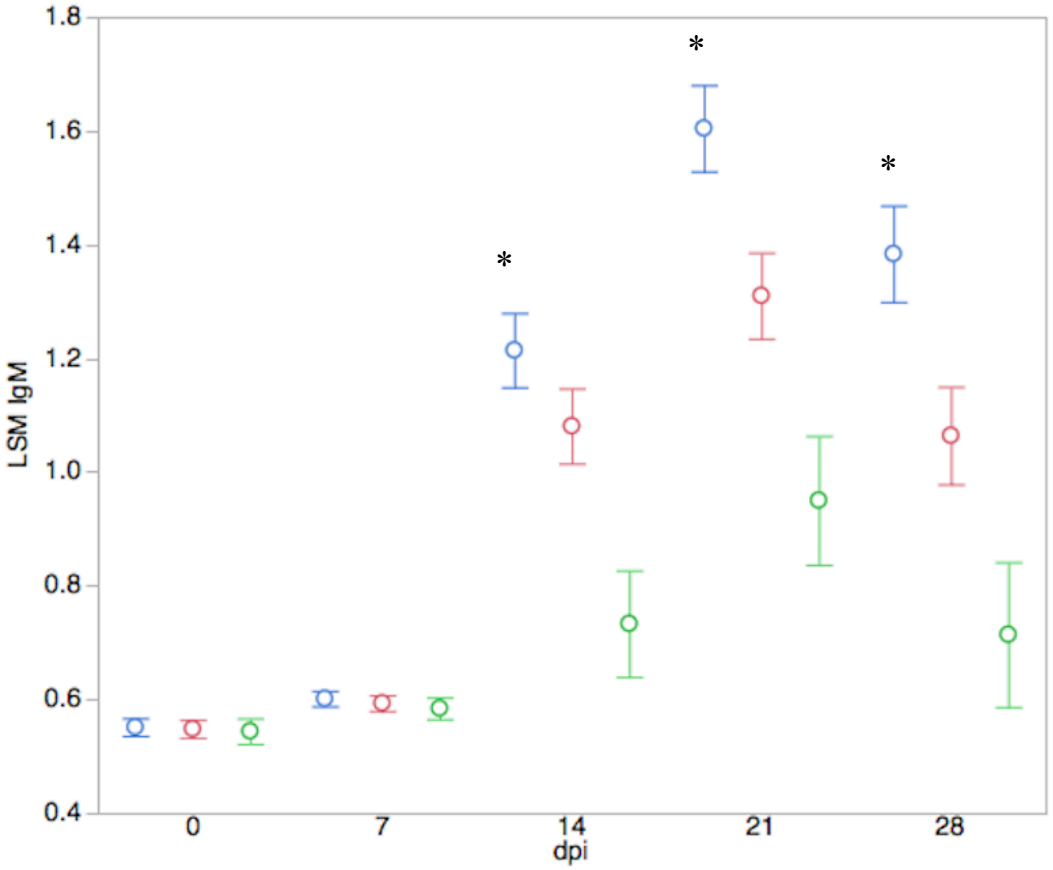


Figure 12. Least square means (LSM) and standard errors of the *SYNGR2 p.Arg63Cys* genotypes (*63Cys/63Cys* - green, *63Arg/63Cys* - red, *63Arg/63Arg* - blue) across weekly viremia measures following PCV2 challenge (n=307).



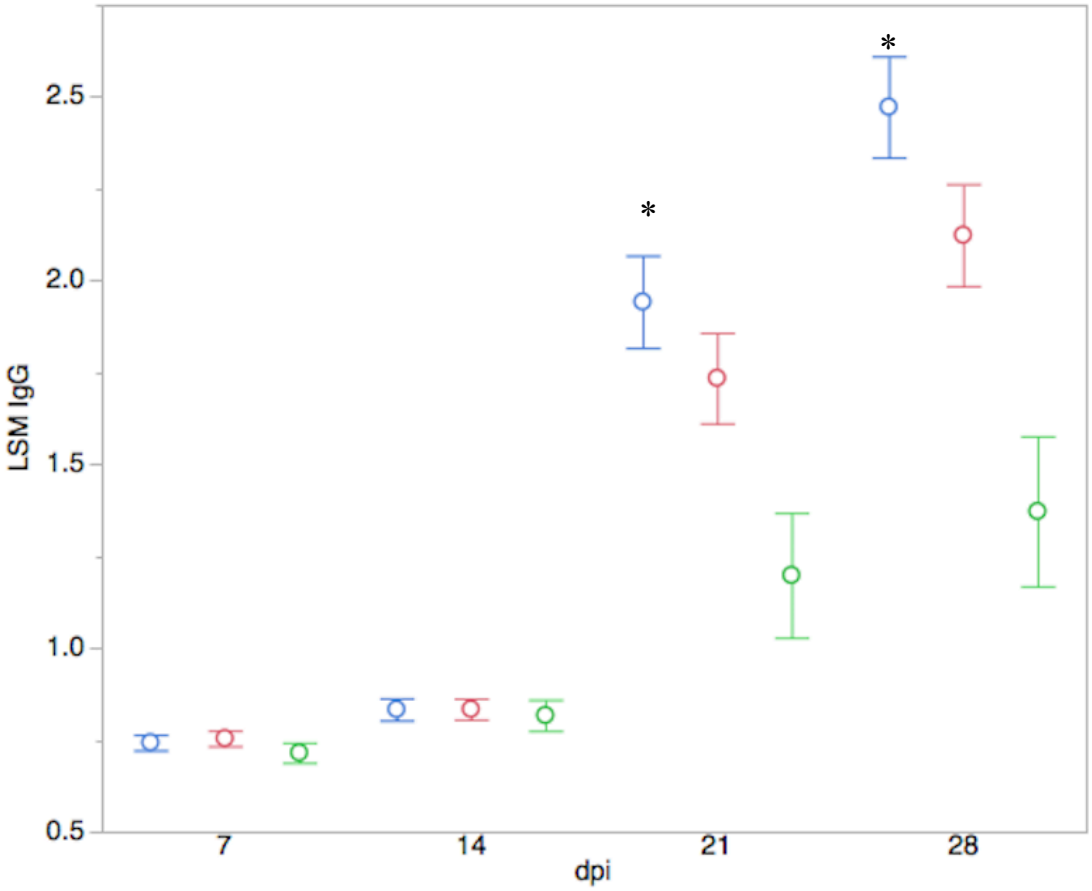
*Significant difference between alternate homozygotes (P <0.0001)

Figure 13. Least square means (LSM) and standard errors of the *SYNGR2 p.Arg63Cys* genotypes (*63Cys/63Cys* -green, *63Arg/63Cys*-red, *63Arg/63Arg*-blue) across weekly IgM PCV2-specific antibody following PCV2 challenge (n=307).



*Significant difference between alternate homozygotes (P <0.0001)

Figure 14. Least square means (LSM) and standard errors of the *SYNGR2 p.Arg63Cys* genotypes (*63Cys/63Cys* -green, *63Arg/63Cys*-red, *63Arg/63Arg*-blue) across weekly IgG PCV2-specific antibody following PCV2 challenge (n=307).



*Significant difference between alternate homozygotes (P <0.0001)

Figure 15. PCV2 copy number from PK15 cells following inoculation with the UNL2014001 PCV2b strain. Number of viral copies is expressed as the mean log10 copies/well for 3 independent replicates with errors bars representing standard error of the mean.

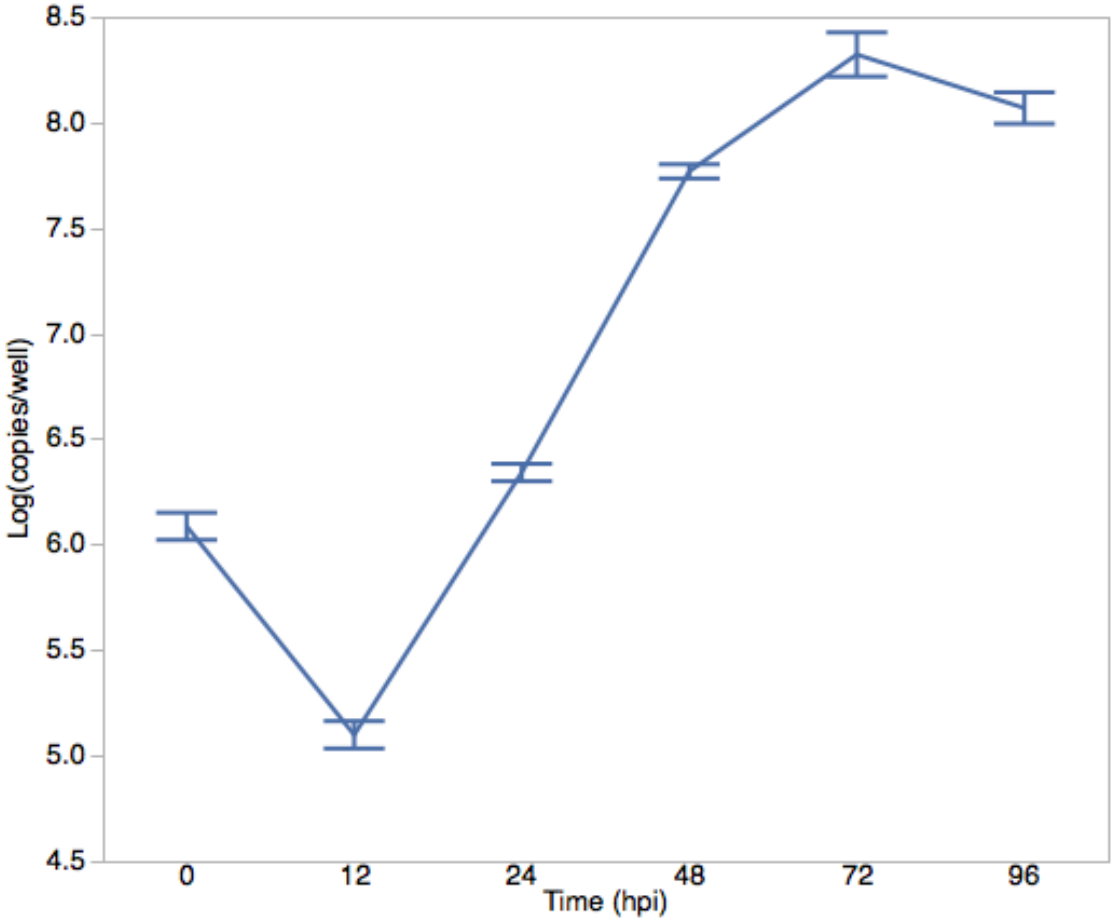


Figure 16. PCV2 copy number from supernatant samples following inoculation with the UNL2014001 PCV2b strain. Number of viral copies is expressed as the mean log10 copies/uL per 200 uL supernatant sample for 3 independent replicates with errors bars representing standard error of the mean.

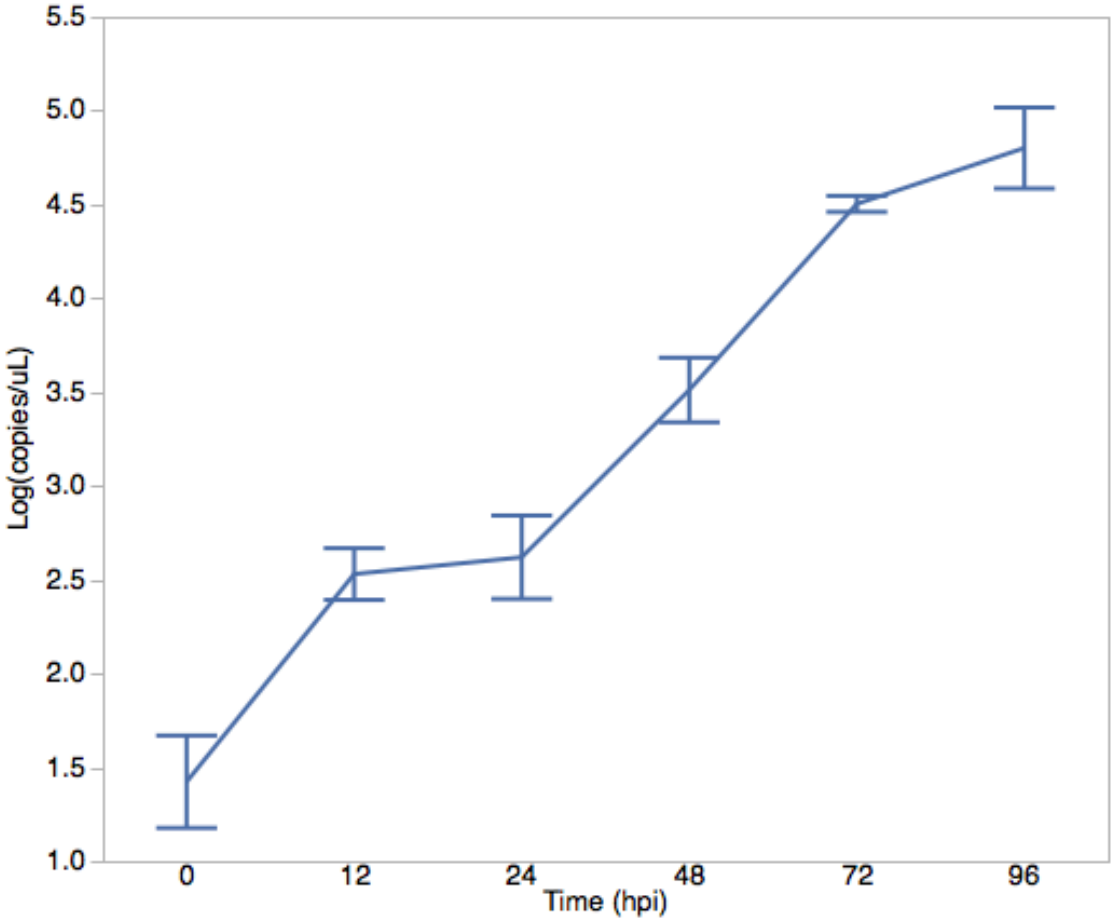


Figure 17. Expression of *SYNGR2* in PK15 infected and control cells following inoculation with UNL2014001 PCV2b strain. Expression represented as the mean log10 MNE across 3 independent replicates with error bars representing standard error of the mean.

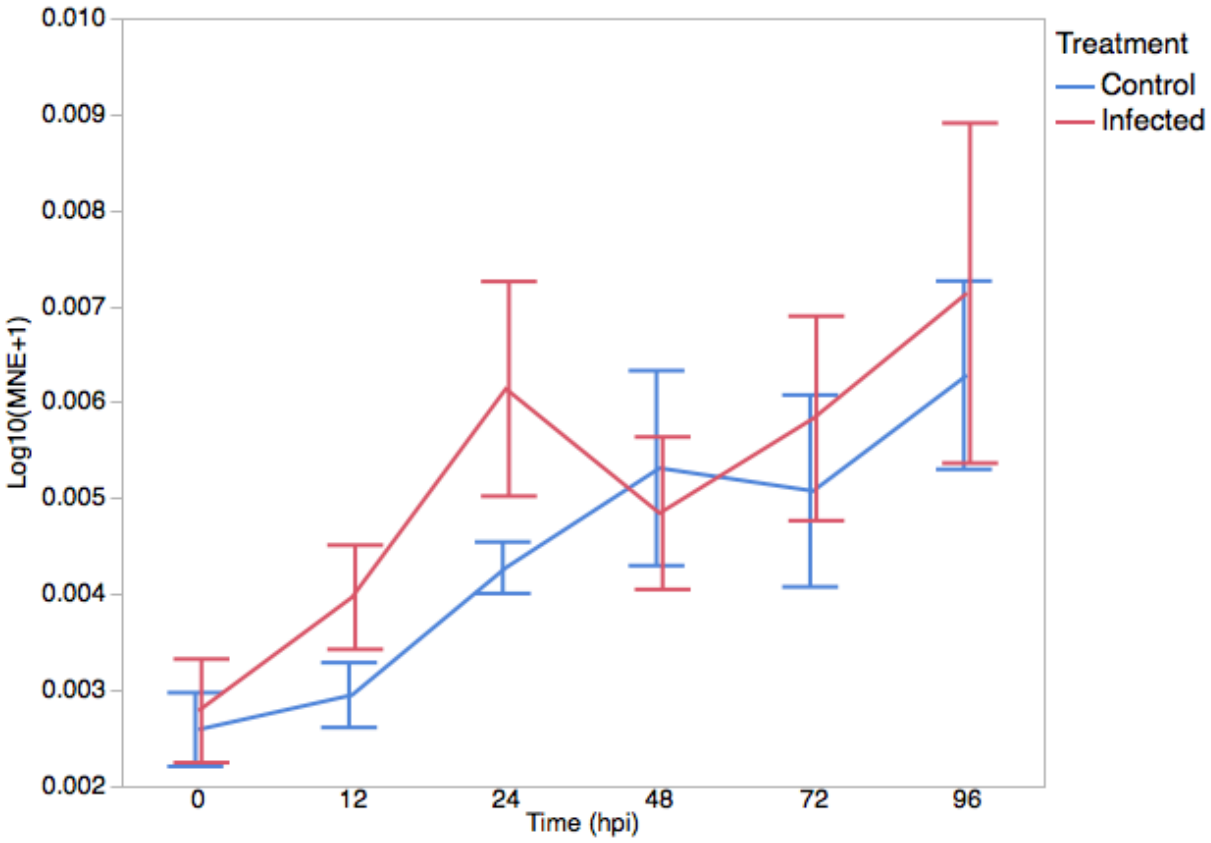


Figure 18. Expression of *BIRC5* in PK15 infected and control cells following inoculation with UNL2014001 PCV2b strain. Expression represented as the mean log10 MNE across 3 independent replicates with error bars representing standard error of the mean.

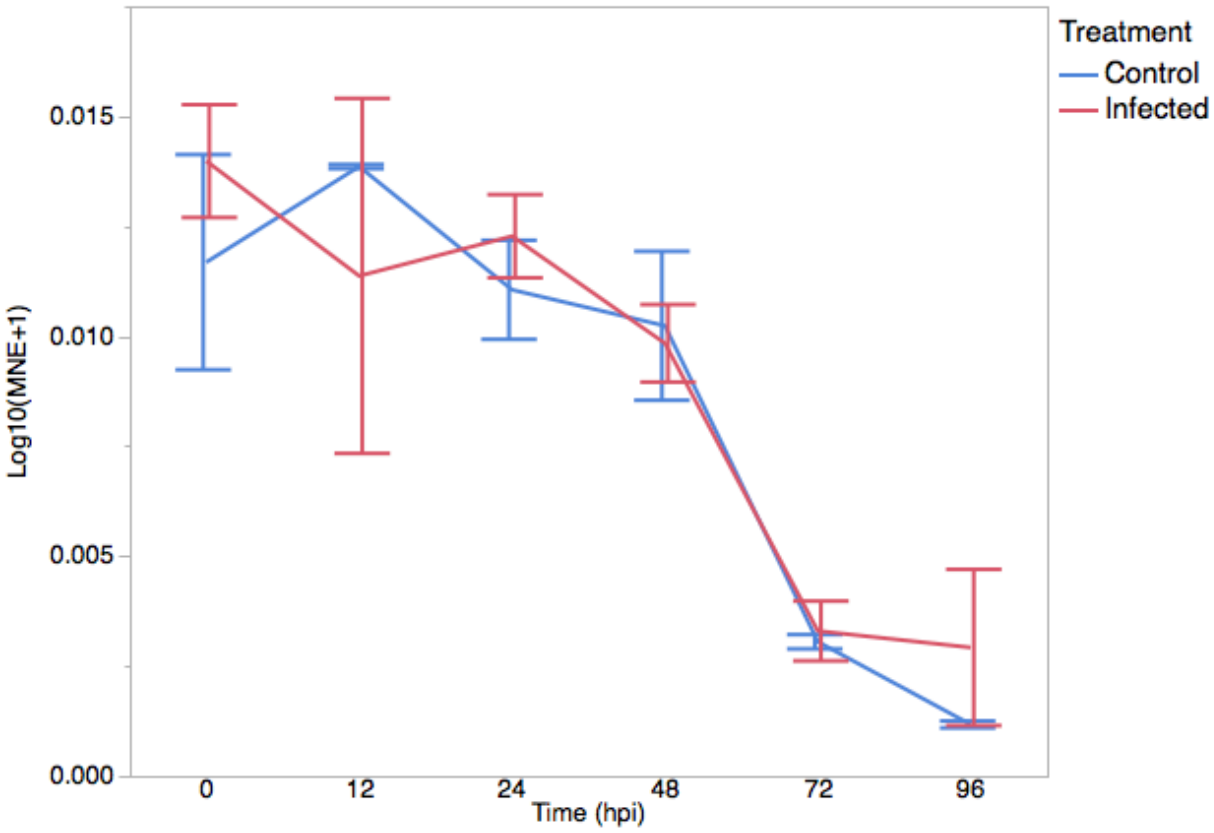


Figure 19. Expression of *SYNGR2* in PK15 cells transfected with *SYNGR2* specific siRNA-01, scramble siRNA, and non-transfected controls following inoculation with UNL2014001 PCV2b strain. Expression represented as mean log10 MNE relative to controls across 3 independent replicates with error bars representing standard error of the mean.

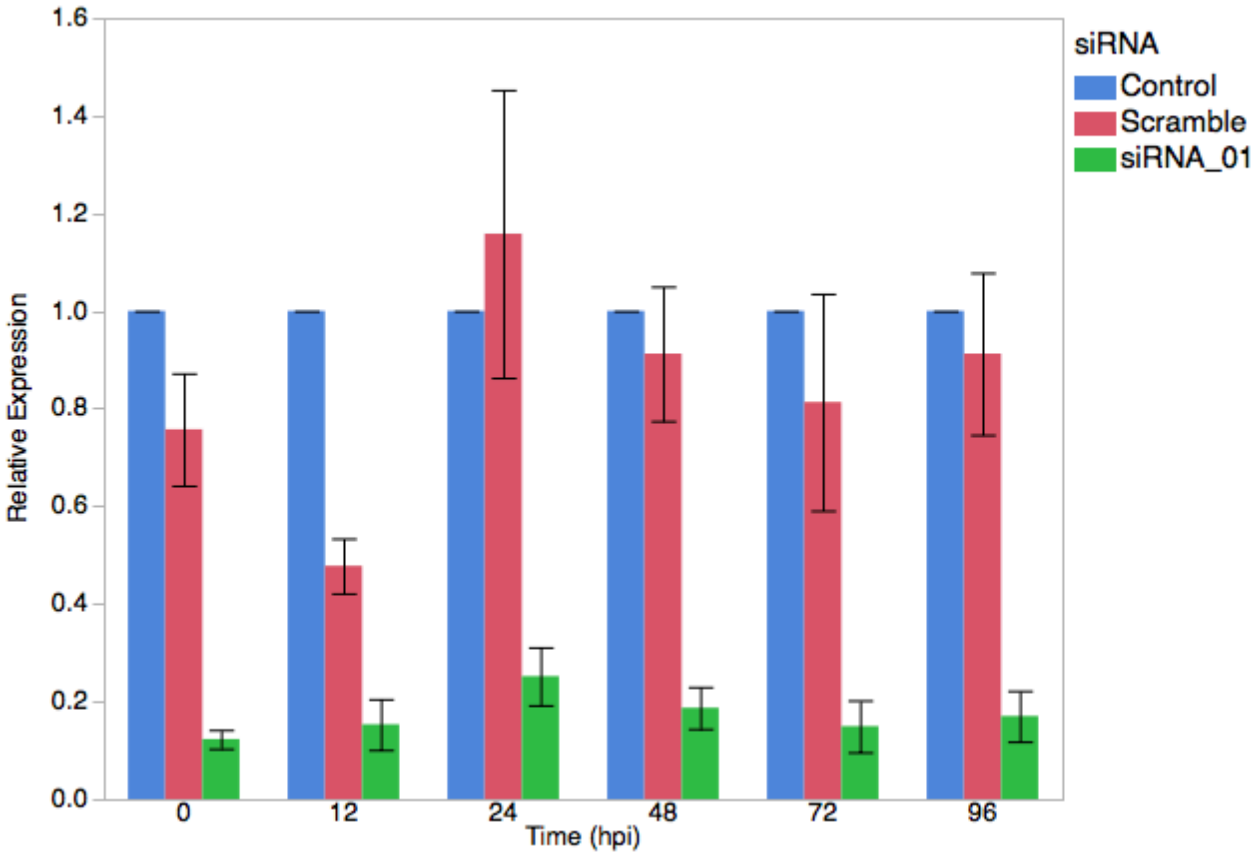
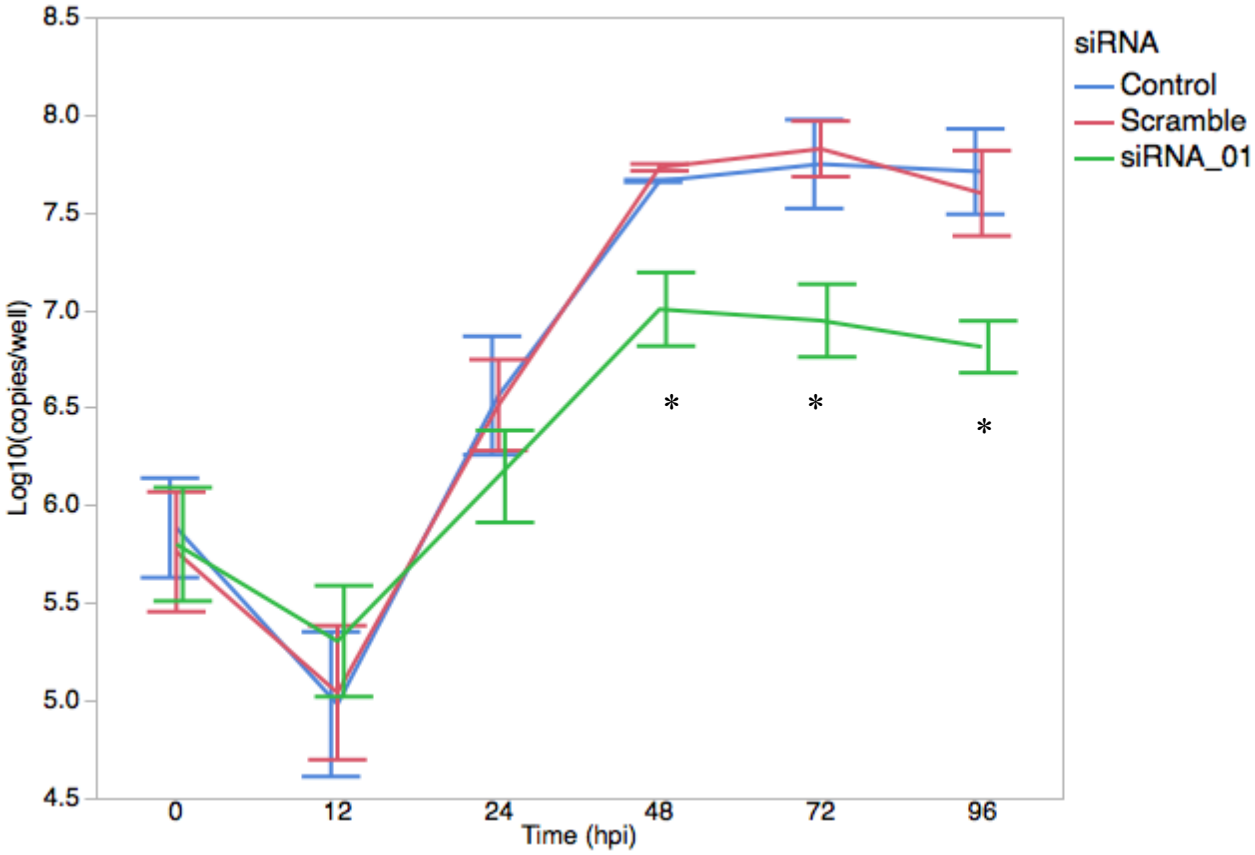


Figure 20. PCV2 copy number from PK15 cells transfected with SYNGR2 specific siRNA-01, Scramble siRNA, and non-transfected controls following inoculation with UNL2014001 PCV2b strain. Number of viral copies from PK15 cells is expressed as mean log10 copies/well across 3 independent replicates with error bars representing standard error of the mean.



*Significant difference between siRNA_01 and Scramble or Control ($P < 0.05$)

Figure 21. Secondary structure of *SYNGR2 p.63Cys* allele predicted by PSIPRED

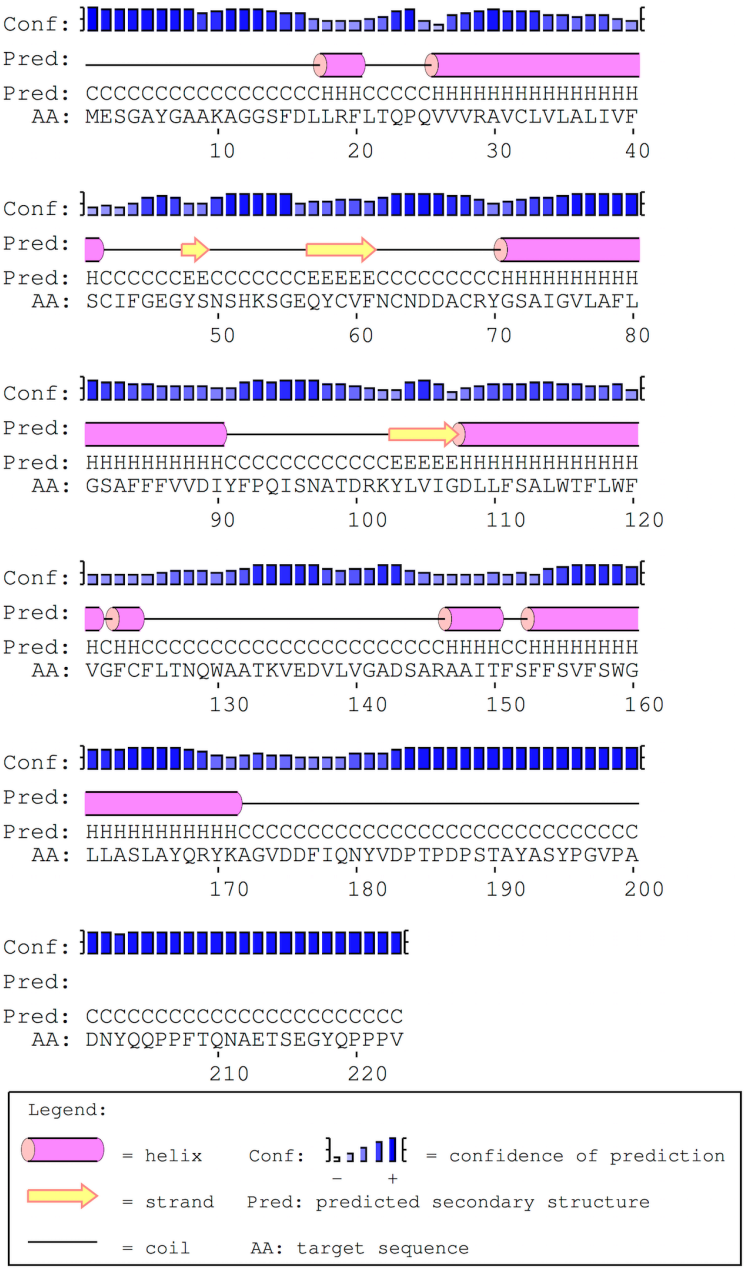


Figure 22. Secondary structure of *SYNGR2 p.63Arg* allele predicted by PSIPRED