# GENOMIC ORGANIZATION AND EXPRESSION OF THE WC1 HYBRID CORECEPTOR AND PATTERN RECOGNITION RECEPTOR ON PORCINE GAMMA DELTA T CELLS

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A Dissertation Presented

by

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Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 2022

Program in Animal Biotechnology and Biomedical Sciences

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## ACKNOWLEDGMENTS

I would like to thank my mentor Janice Telfer for her support, guidance, and patience during my time at Umass. I'd like to thank Cynthia Baldwin for her support throughout my time as a graduate student. I'd like to thank the Veterinary and Animal Sciences department and department head Rafael Fissore. I would also like to thank the other members of my committee, Yasu Morita and Sam Black for their support while I was pursuing this degree. I also wish to thank Sabine Hammer from the University of medicine in Vienna Austria.

This project was supported by the following Agriculture and Food Research Initiative Competitive Research Grants from the USDA National Institute of Agriculture

- Project #2011-67015-30736 NIFA-USDA/NIH Dual Purpose with Dual Benefit Research in Biomedicine and Agriculture Using Agriculturally Important Domestic Animals
- Project #2015-06970 NIFA-USDA
- Project #2017-67015-2663 NIFA-USDA

## ABSTRACT

## GENOMIC ORGANIZATION AND EXPRESSION OF THE WC1 HYBRID CORECEPTOR AND PATTERN RECOGNITION RECEPTOR ON PORCINE GAMMA DELTA T CELLS

FEBRUARY 2022

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γδ T cells are a crucial component of the immune response to a number of increasingly relevant and largely zoonotic pathogens to which efficacious vaccination is lacking. In ruminants and swine, γδ T cells represent a major population of peripheral blood and epithelial tissue-resident lymphocytes. γδT cells respond to both protein and non-protein antigens independently of MHC presentation and possess immunological memory. Upon activation, gamma delta T cells illicit a variety of effector functions and play an indispensable role of orchestrating the downstream immune response. These characteristics make gamma delta T cells a promising candidate for recruitment by vaccination, however, methods for effectively priming these cells remain to be elucidated. The type I transmembrane receptor Workshop Cluster One (WC1) is expressed as a multigenic array on γδ T cells in swine and ruminants. In cattle there are 13 unique WC1 genes (WC1-1 to WC1-13) each comprised of 6-11 SRCR domains that selectively bind unprocessed antigen in a manner that resembles a pattern recognition receptor (PRRs). WC1 functions as a hybrid PRR and co-receptor for the gamma delta TCR as it potentiates activation signals from the TCR and dictates antigen specificity of expressing γδ T cells. cDNA evidence suggests that porcine WC1 is expressed as a multigenic array consisting of 9 genes (WC1-1 to WC1-9) each encoding 6 SRCR domains with unique pathogen binding potential. The objective of this study is to characterize the multigenic array of porcine WC1, investigate its propensity for pathogen binding, and evaluate its expression on γδ T cells. Using the MAKER annotation pipeline, we annotated *Sscrofa11.1* for sequence derived from full-length cDNA transcripts representing the 9 porcine WC1 genes. We were able to map 8 of the 9 genes, leaving one ( WC1-8) unplaced in the current assembly. We defined three subpopulations of porcine γδ T cells based on expression of WC1 and CD2. Finally, we confirmed that porcine WC1 SRCR domains are capable of directly binding whole fixed bacteria including *Leptospira spp* and *Mycobacterium bovis*.

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# LITERATURE REVIEW

## 1.1 Introduction

Livestock production contributes significantly to the global economy and food security. Thus, preservation of this practice is a relevant concern for the human population. Factors including globalization and population growth have increased demand for animal-based protein and consequently has resulted in intensification of livestock production. As production intensifies, conditions conducive to the rapid spread of production-associated diseases and transboundary animal diseases are exacerbated [1–3]. Infectious diseases negatively impact livestock production through mortality, sterility, trade restrictions and reduced market value. Moreover, an estimated 60% of emerging infectious diseases of humans are zoonotic with livestock species serving as vector and reservoir hosts [2,4]. There is a significant overlap between pathogens that infect both domesticated livestock and wildlife and the increased instances of inter-species transmission leave unchecked opportunities for pathogens to acquire mutations that may increase virulence and lethality [5–9]. Vaccination is the most effective medical strategy we possess for disease prevention, but some current veterinary vaccines offer sub-optimal protection and for other diseases no effective vaccine exists [10,11]. There is a dire need to improve livestock vaccine efficacy, and increased understanding of the immune systems of livestock will facilitate this

γδ T cells have proven to be a component of the immune response to an increasing array of relevant pathogens to which efficacious vaccines are lacking (Table 1.1). In both ruminants, the livestock species in which they were first discovered, and swine, γδ T cells constitute a major portion of lymphocyte populations of the peripheral blood, in epithelial tissues and at sites of inflammation [12–17]. Innate-like properties of γδ T cells allow them to exist in a "pre-activated" state. Significantly higher percentages of these cells are found in young animals suggestive of their role in immune protection prior to the complete maturation of the immune system [12,18,19]. The propensity of γδ T cells to home to specific tissues, maintain barrier homeostasis and exert immune-modulating functions supports the potential for targeting them by vaccine constructs. Successful efforts to improve the efficacy of vaccines that are currently in use appear to be been correlated with recruitment of more γδ T cells when administered to animals earlier in life [20–25]. Therefore, a better understanding of γδ T cells in these species may allow us to improve vaccine regimes that are currently in use with minimal intervention or alteration. Livestock species possess an abundance of γδ T cells which makes them ideal candidates for research towards this goal.

Table 1.1 γδ T cell response to pathogens of swine

| Pathogen | Role in disease | γδ T cell Response | Ref |
|---|---|---|---|
| African swine fever virus (ASFV) | Protective | Higher counts of γδ T cells following infection are associated with increased survival. γδ T cells from ASFV-immune pigs present viral Ag to CD4+ T cells. | [26–28] |
| *Mycobacterium bovis* | Protective | Proliferate and produce IFN-γ following vaccination with BCG. γδ T cells isolated from BCG vaccinated animals show | [25] |

| | | | |
|---|---|---|---|
| | | enhanced proliferative capacity compared to those from unvaccinated animals when stimulated with *Mycobacterium tuberculosis*. | |
| Porcine reproductive and respiratory syndrome virus (PRRS) | Protective | Proliferate and produce IFN-γ starting from day 14- 50 post infection. Re-challenge met with antigen-specific memory response associated with increased IFN-γ and IL-12 production. Infection with PRRS type II is associated with upregulation of lymph node homing receptor CCR7 expression on γδ T cells. Response of γδ T cells to modified live virus vaccine is enhanced by mucosal exposure to *Mycobacterium tuberculosis* whole cell lysate. In vitro stimulation of PBMC isolated from animals recovered from experimental infection shows marked increase in number of $CD8^+$ γδ T cells and is correlated with increased cytoxic activity in culture. | [23,29–31] |
| Swine influenza virus (SIV) | Protective | Increased levels of γδ T cells in BAL and lower tonsils of pigs infected with H1N1. Vaccination with reverse genetics-derived H3N2 (TX98) confers partial cross-protection during heterosubtypic challenge with H1N1. Following vaccination, antigen specific γδ T cells associated with IFN-γ and IL-10 production before and after heterologous challenge. | [32,33] |
| Foot and mouth disease virus (FMDV) | Protective | Major responders to infection and in recall responses in vitro. Modulate recruitment and maturation of DCs and function as professional APCs. Purified naïve γδ T cells have increased mRNA expression of GM-CSF, IFN-α, IL-1, IL-6 and IL-8 following culture with high potency FMD vaccine antigen. | [19,34] |
| *Salmonella enterica* serovar typhimurium (STM) | Unknown | Purified γδ T cells cultured with STM show higher expression of PRRs including TLR2, TLR5, TLR9, TLR10, and NOD2, as well as increased expression of IL-8 when compared to unstimulated cells. | [35] |

| | | | |
|---|---|---|---|
| Classical swine fever virus (CSFV) | Unknown | Co-culture with CSFV and pDCs results in partial activation of γδ T cells and upregulation of MHC II surface expression. This suggests they that γδ T cells may serve as APCs for CSFV infection. | [36] |
| *Taenia solium* (helminth) | Unknown | Activated γδ T cells present from Stage I infection and are the predominant cell type surrounding cysterici lesions. Thought to contribute to predominantly Th1 cytokine pattern that is associated with symptomatic, viable and chronic infection. | [37] |
| Swine Dysentery | Unknown | Levels of γδ T cells increase following inoculation with *Brachyspira hydosenteriae,* the causative agent of swine dysentery. Higher numbers of γδ T cells in circulation prior to inoculation is correlated with increased susceptibility to infection with swine dysentery. | [38] |
| *Pasteurella multocida* | Unknown | $CD2^-$ γδ T cells increase in bronchoalveolar space after aerogenic immunization | [39] |

Livestock γδ T cells do not represent a homogeneous population of cells. Rather, subpopulations are found with variable representation in different tissues and organs. These subpopulations may also have different functions, thereby distinctively affecting the outcome of disease or inflammation when they are engaged in the immune response. Major γδ T cell subpopulations in ruminants are often distinguished based on differential expression of the family of transmembrane protein receptors called workshop cluster 1 (WC1) or T19 [12,40–42]. WC1 functions as a hybrid co-receptor and pattern recognition receptor (PRR) for the γδ T cell receptor (TCR) [43–50]. Defining the role of WC1 hybrid receptors in $WC1^+$ γδ T cell activation has been the subject of much of our work in

ruminants. Orthologues of WC1 have also been described in pigs and are found across a number of species including the chicken and duck-billed platypus further demonstrating value through their conservation over evolutionary time [51–54]. WC1 expression, or lack thereof, plays a direct role in antigen responsiveness and tissue homeostasis of γδ T cells in livestock, thus its potential as a target to enhance vaccine efficacy through increased γδ T cell participation cannot be overstated. The effort to define porcine γδ T cells and the molecular mechanisms employed by these cells is ongoing, and major discoveries are highlighted in Figure 1.1.

Figure 1.1

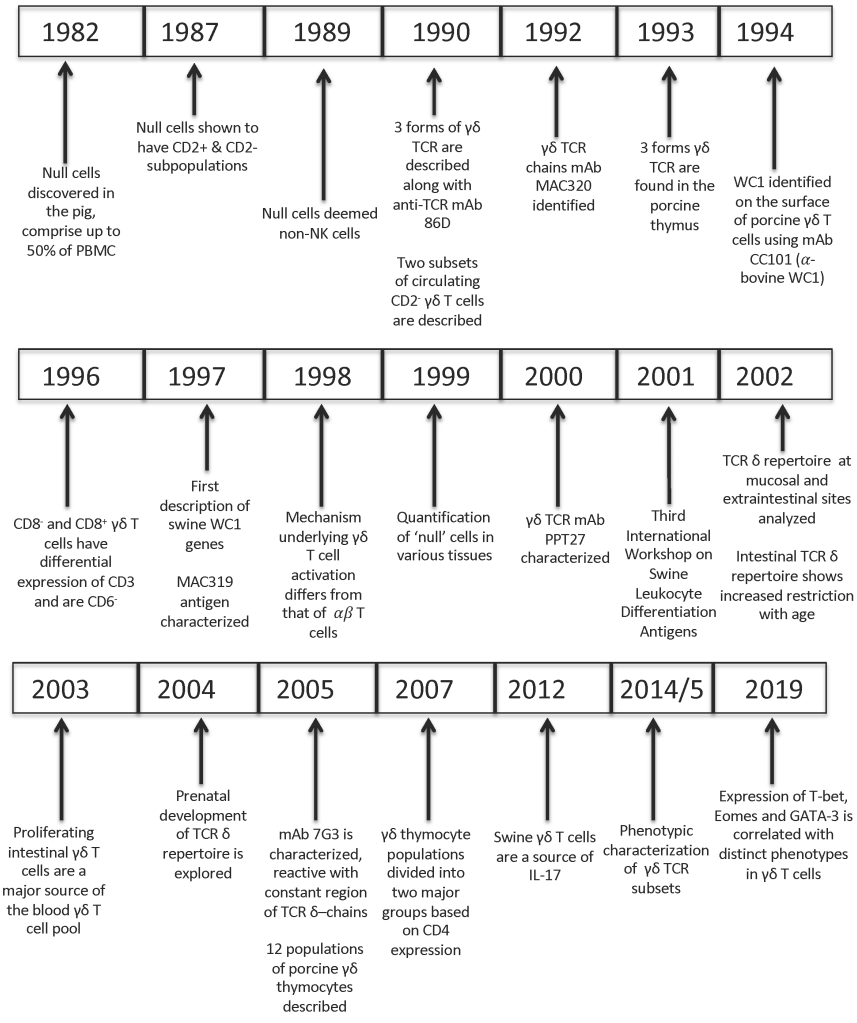



Figure 1.1 Timeline of porcine γδ T cell description.
Key discoveries for porcine γδ T cells since their first identification as null cells in 1982.

## 1.2 A role for γδ T cells in next generation vaccines

Most vaccines currently in use are designed to prime B lymphocytes and rely exclusively on the highly specific humoral immune response. Unfortunately, this approach has sometimes been ineffective against pathogens that exhibit extensive strain and serotype diversity [10,55–58]. A major goal of vaccine research is to design vaccines that offer heterologous protection against pathogens prone to mutation. For example, in humans,

this has focused on influenza vaccine construction to avoid the need for yearly vaccine renewal and immunization. Increased vaccine efficacy may often require engagement of both the humoral and cellular arms of the adaptive immune system. Thus, vaccines designed to achieve this will require successful engagement of both B and T lymphocytes in a manner that is cognizant of the ever-evolving immune evasion strategies employed by target pathogens.

While αβ T cells have been extensively studied across a multitude of species, evidence supporting the untapped potential of γδ T cells continues to increase. γδ T cells exhibit several innate-like antigen recognition properties including the ability to recognize antigen in the absence of presentation via the major histocompatibility complex (MHC) [59–62]. This property enables γδ T cells to recognize an array of peptide but also non-peptide antigens (reviewed in [63]) in a TCR-dependent manner. Perhaps because antigen processing is not required for γδ T cell recognition of antigens or because the γδ TCR is more broadly liganded, γδ T cells are often activated sooner than their αβ T cell counterparts [20]. Expression of various pattern recognition receptors (PRR) and other innate-like immune receptors further diversifies their antigen recognition potential. Moreover, γδ T cells in livestock are potent producers of critical "master regulator" cytokines like IFN-γ and IL-17, whose production orchestrates downstream cytokine and chemokine production by other cells, thereby shaping the immune response as a whole [64,65]. The propensity of γδ T cells to home to specific tissues, secrete pro-inflammatory and regulatory cytokines, exhibit memory or recall responses and even function as antigen-presenting cells for αβ T cells supports the concept that they have enormous

potential for priming by next generation vaccine constructs to contribute to protective immunity. As mentioned above, to do this understanding the biology of these cells in livestock species including pigs, an important food animal and potential reservoir for zoonotic pathogens, is needed.

## 1.3 Unique features of γδ T cell antigen recognition

### 1.3.1 TCR and CD3

T lymphocytes are a major component of the adaptive, memory-enabled immune system in higher vertebrates. Antigen recognition is generally attributed to their T cell receptor (TCR), a membrane-bound heterodimer composed of α with β or γ with δ chains. Rearrangement of varible (V) diversity (D) and joining (J) region sequence elements during T cell maturation generates diversity in antigen recognition, and collectively this area is referred to as the complementarity determining region 3 (CDR3) loop. The CDR3 loops of α and β TCR chains are equal in length, reflecting a strict requirement for both chains to physically contact the major histocompatibility complex (MHC) during antigen presentation [66]. In contrast, for the γδTCR which does not respond to MHC-presented peptides, the γ chain CDR3 loops are short with limited length variation, while the δ CDR3 loops vary extensively in length [66]**.** Analysis of porcine CDR3 regions of the δ chain suggests that the variable region of the porcine γδ TCR is polyclonal in young pigs and becomes oligoclonal with age [67]. The cause and effect of this reduced structural diversity observed in the porcine γδ TCR has not been studied.

Livestock γδ T cells recognize intrinsic pathogenic molecules that are unlikely to mutate because they are not bound by the requirement of MHC-presentation. An example of this is the *Mycobacterial* cell wall component mycolylarabinogalactan-peptidoglycan (mAGP), which is recognized by bovine γδ T cells but not αβ T cells [68,69]. Although γδ T cells are not MHC restricted, antigen-specific recognition of peptide molecules does occur and is TCR dependent [70–73]. Bovine γδ T cells respond to the peptide antigens major surface protein 2 (MSP2) from *Anaplasma marginale* and those derived from the mycobacterial protein complex ESAT6:CFP10 in *in vitro* recall responses [69,74–76]. Stimulation in the absence of APCs inhibits proliferation and IFN-γ secretion, and blocking of the TCR with specific mAb inhibits the response to these antigens [69,74,75]. Although porcine γδ T cells are involved in the immune response to a variety of pathogens, specific ligands and TCR involvement remain to be determined.

The TCR repertoire of porcine γδ T cells is highly diverse due to extensive duplication of genes that code for the variable regions of the delta chain (Vδ) and multiple constant regions of the gamma chain (Cγ) [77–80]. Pigs express four Cγ genes, but in some breeds one of these is a pseudogene [80,81]. This corroborates a much earlier study that found swine possess one α constant region, (possibly) two β constant regions, at least three γ and one δ TCR-constant region isotypes [79,80]. These porcine TCRγ constant regions are highly homologous on the amino acid level to corresponding TCR chains found in other species [79].

A TCR clonotype is defined as the unique nucleotide sequence which arises during the process of TCR gene rearrangement to increase the potential diversity of antigen recognition. As described in detail below, the majority of porcine γδ T cells are $CD4^-$ $CD8^-$. Biochemical analysis of porcine $CD4^-CD8^-$ T cells revealed three unique γδ TCR structures, which share a 40kDa δ chain, but differ in γ chain usage [79,80,82]. One γ chain has a molecular mass of 38kDa and is preferentially expressed on γδ lymphocytes present in the peripheral blood [79]. A second γ chain with a molecular mass of 37kDa is evenly distributed amongst γδ T cells in the blood and lymphoid tissues [79]. The third γ chain is 46kDa and is expressed on $CD2^+$ γδ T cells and is enriched in lymphoid tissues. Annotation of the swine TCRγ gene locus from several assemblies revealed 4 gene cassettes containing C, J and V genes [83]. Genomic sequence of the porcine TCR δ chain D segments revealed that there are 28 V α/δ segments including 4 TRDV1 and at least 6 Dδ segments, all of which are functionally utilized in expressed TCR δ chain genes [84]. It was also found that a single functional TCR δ chain was able to utilize more than three Dδ segments, further diversifying the repertoire of TCR δ chain molecules [84].

Porcine blood derived γδ T cells can be divided into two subsets based on CD2 expression (Figure 1.4) which is described in more detail below. Evaluation of TCRG expression on $CD2^-$ blood derived γδ T cells revealed that these cells transcribe all four TCRGC cassettes, a phenomenon that does not occur in ruminant counterparts [83]. These populations exhibit two distinct γ-chain clonotypes and share identical TCR-γ diversity [85]. With respect to the δ-chain, $CD2^+$ γδ T cells display higher TCR-δ diversity (~39 unique

clonotypes) but share a particular clonotype with $CD2^-$ cells (Vδ1DδxJδ4), and the two populations express this clonotype at similar frequencies [85]. These differences suggest the populations are stimulated or responding to different antigens.

The cluster of differentiation 3 (CD3) protein complex functions as a co-receptor for the TCR and is expressed on the surface of αβ and γδ T cells. The CD3-TCR complex is involved in antigen recognition, subsequent signal transduction and activation of immunocompetent T lymphocytes. CD3 molecules associated with γδ TCR are composed of six peptides which form three dimers (εγ, εδ and ζζ) [86,87]. Interestingly, CD3 molecules associated with the γδ TCR lack the CD3 δ chain [88,89]. One study utilized porcine CD3 as immunogen to raise a panel of mAbs (Table 2) which recognize various epitopes of γδ TCR-associated CD3. This study concluded that differences regarding antigenicity and signal transduction potentials of exist between CD3 molecules expressed on γδ T cells when compared to αβ T cells [90]. There are observable functional differences when total CD3 molecules and CD3 molecules that are restricted to the γδ TCR are ligated by antibodies. Unlike anti-CD3-ε mAbs, anti-γδ-T-cell-restricted CD3 mAbs do not induce antigenic modulation, lymphocyte proliferation, or CD3-redirected toxicity [90]. It has been shown that triggering different epitopes of CD3 illicit different cellular responses, therefore this failure to activate γδ T cells via CD3 may be due to fundamental differences in signaling characteristics, or simply that these γδ-T cell-restricted mAbs recognize non-mitogenic epitopes [91]. It is also plausible that triggering of the CD3-TCR complex alone is insufficient to induce porcine γδ T-cells, implying that additional signals are also required potentially in the form of co-receptors like WC1.

### 1.3.2 Pathogen recognition receptors

Often referred to as members of the "bridging" immune system, γδ T cells can retain immunological memory, making them adaptive-like T cells. They also exhibit several innate-like qualities including pattern recognition receptor (PRRs) expression [49,92]. PPRs are expressed on the surface of immune cells and exhibit broad reactivity to conserved molecules of pathogenic origin known as pathogen-associated molecular patterns (PAMPs), or molecules released by damaged and distressed cells called damage-associated molecular patterns (DAMPs) [93,94]. Engagement of PRRs expressed on innate immune cells induces production of co-stimulatory signals for cells of the adaptive immune system [95]. As a crucial component of the innate immune system, PRR recognition of PAMPs or DAMPs initiate microbicidal and pro-inflammatory responses, including induced cell death of infected cells, that function to eliminate or contain the infection long enough for the adaptive immune system to mount a sufficient response [96]. The four major subfamilies of PRRs include toll-like receptors (TLRs), nucleotide-binding oligomerization domain containing proteins (NODs) and NOD-like receptors (NLRs), retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLR) and C-type lectin receptors (CLRs)[97].

While the conventional paradigm is that innate immune system cells such as macrophages and dendritic cells are activated through PRR becoming activated and producing products that engage αβ T cells and B cells, γδ T cells also can be stimulated through PRR. Porcine γδ T cells respond to *Salmonella enterica* serovar Typhimurium infection through increased expression of TLRs, specifically TLR2, TLR5, TLR9,

TLR10, in addition to NOD2 [35]. Subsets of γδ T cells in the ileum, spleen and in circulation of gnotobiotic pigs increase expression of TLR2 and TLR3 following infection with human rotavirus [98]. Stimulation of prenatal bovine γδ T cells through TLR3 and TLR7 has been shown to induce differential expression of various cytokines and chemokines [99].

### 1.3.3 Workshop cluster 1 (WC1) proteins

In addition to the canonical PRRs, the majority of peripheral blood γδ T cells in livestock species express members of the workshop cluster 1 (WC1, aka T19 in sheep) multigenic array; these receptors have both PRR and TCR co-receptor activity, and may provide a direct route for antigen-specific engagement of γδ T cells by vaccines [12,100–102]. Originally discovered on γδ T cell subsets in cattle and sheep [12,42], WC1 is a type 1 integral membrane protein with up to eleven extracellular scavenger receptor cysteine rich (SRCR) domains in cattle, and exhibits significant homology to scavenger receptors CD5, CD6 and CD163 [51,103] found on T cells and macrophages.

As a member of the group B Scavenger Receptor Cysteine Rich (SRCR) superfamily, WC1 is composed of multiple extracellular (single-exon encoded) SRCR domains and is expressed exclusively on γδ T cells of swine and ruminants [51,104–107]. Group B SRCR domains possess 6-8 highly conserved cysteine residues which result in the formation of 3-4 disulfide bonds [108]. Other group B SRCR-containing scavenger receptors such as CD163A, CD5, CD6, and DMBT1 are capable of directly interacting with pathogens via extracellular SRCR domains [109–112]. WC1 is closest in sequence to CD163A, CD163b

and CD163c-α [51]. Each of these molecules share several highly homologous SRCR domains designated b, c, d, e and d' based on amino acid identity between domains of different genes and across species (Figure 1.2) [51,113]. Unlike CD163 molecules, WC1 possess an N-terminal SRCR domain, a1, which is the most variable domain in terms of amino acid identity [107].

Bovine WC1 molecules contain up to eleven extracellular SRCR domains, organized in the domain pattern of a1-[b2-c3-d4-e5-d6]-[b7-c8-d9-e10-d'11], where alphabet designations indicate SRCR domain clades between genes (Figure 1.2) [51,103]. Annotation of the bovine genome for WC1 revealed 13 genes encoding for WC1 in cattle *(WC1-1* to *WC1-13*) which are further classified based on deduced amino acid sequence of the a1 SRCR domain and cytoplasmic domain structure [107]. All but one bovine gene, WC1-11, encode a potential eleven extracellular SRCR domains. Like WC1 molecules observed in the pig, bovine WC1-11 has 6 extracellular SRCR domains instead of 11 [46,53]. Other non-SRCR immunoreceptors such as C-type lectin-like Ly49 and killer Ig-like receptor (KIR) are encoded by a large multi-gene families that are believed to have resulted from rapid repeated gene duplication guided by selective pressure of rapidly changing ligands [114–116]. The fact that there is little to no polymorphism among animals or between the species *Bos taurus* and *Bos indicus* suggests that selective pressure, likely from pathogens, works to conserve these sequences [107,117,118].

Figure 1.2

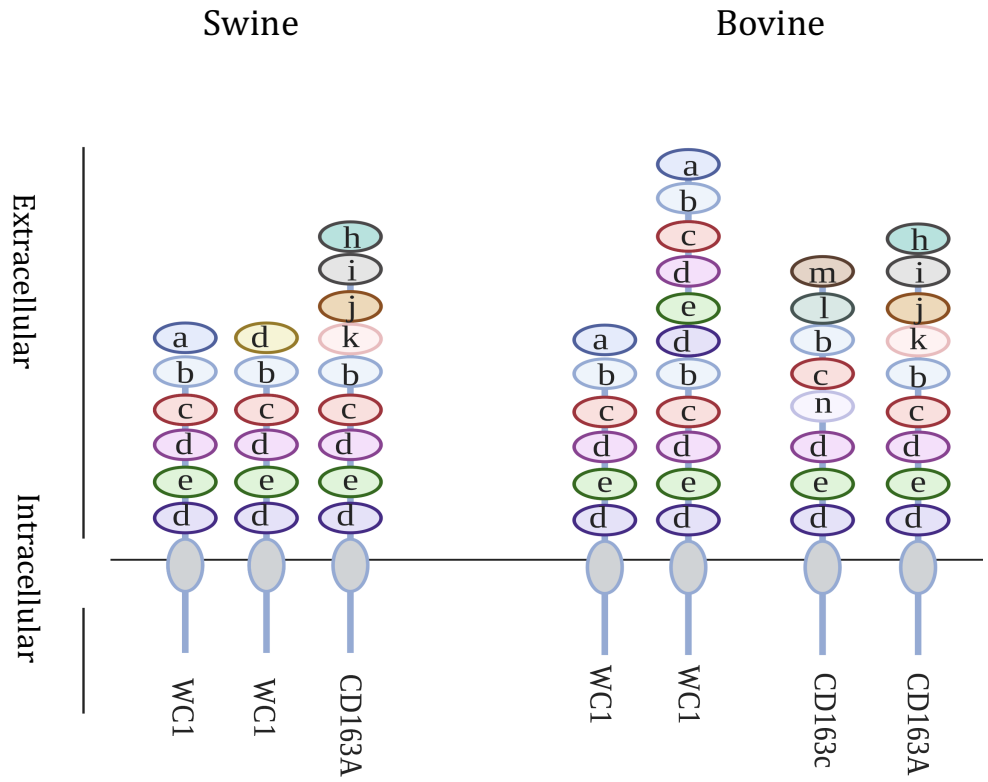



Figure 1.1 WC1 is a member of the Group B SRCR superfamily.
Extracellular domain architecture is shown Group B scavenger receptors WC1, CD163A and CD163cα. SRCR domains are represented by colored ovals and SRCR domain letters are indicated.

Bovine WC1 genes are further divided into two subtypes, 1.1 (WC1.1) and 1.2 (WC1.2), based on their reactivity with monoclonal antibodies (mAb) and amino acid composition of the first SRCR domain [119]. Different molecular forms of WC1 are found on functionally distinct subpopulations of bovine γδ T cells, and play an active role in what pathogens WC1$^+$ cells will respond to [13,49,120]. It was found that WC1.1$^+$ γδ T cells respond to the spirochete *Leptospira* while WC1.2$^+$ γδ T cell clones respond to the rickettsia *Anaplasma* [13,74]. Both WC1.1$^+$ and WC1.2$^+$ populations share the same γδ TCR

restriction using genes from only one TCRγ cassettes (Cγ5) while WC1$^-$ γδ T cells are not restricted in their gene usage [74,121]. This suggests that expression of individual WC1 receptors may encode antigen specificity. We have recently determined that SRCR domains of bovine WC1-4 and WC1-12 directly bind to *Mycobacterium bovis* BCG Danish and Pasteur strains (unpublished data). Because WC1$^+$ γδ T cell share a restricted set of TCR genes, yet respond to different pathogens, we hypothesize that WC1 functions as a hybrid co-receptor and pattern recognition receptor for the γδ TCR and gene expression plays the determining role in activation of WC1$^+$ γδ T cells.

Furthermore, knockdown experiments in cattle designed to reduce WC1.1-type molecules significantly reduced bovine γδ T cell activation by *Leptospira* [47]. WC1-3 was amongst the WC1.1-type molecules that was knocked down in these experiments. It was later shown that five of eleven WC1-3 extracellular SRCR domains bound to multiple serovars of *L. borgpetersenii* and *L. interrogans* [49]. Binding of WC1 to *Leptospira* is correlated with γδ T cell activation by *Leptospira*. WC1-4, expressed by WC1.2$^+$ *Leptospira*-nonresponsive cells, had no SRCR domains that bound to *Leptospira*. Mutational analysis of WC1 binding and non-binding SRCR b domains located the active site for *Leptospira* binding in 5 amino acid residues found in 3 regions of WC1-3 SRCR b2 [49]. This led us to our current working model, in which WC1 functions as a hybrid co-receptor and pattern recognition receptor for the γδ TCR through direct binding of individual SRCR domains with pathogen or pathogen-derived molecules (Figure 1.3).

Figure 1.3

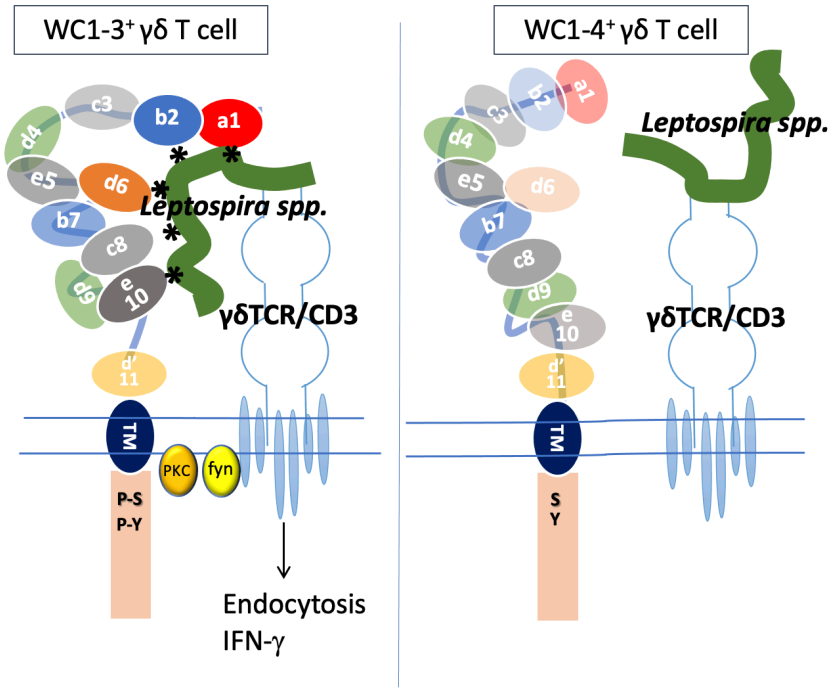



Figure 1.3 WC1 functions as a hybrid co-receptor and PRR through direct binding of SRCR domains to pathogen.
In our working model, WC1 molecules induce activation of expressing γδ T cells through directly binding to pathogens. WC1-3$^+$ γδ T cells are activated in response to *Leptospira spp.* and this is correlated with WC1-3 binding to *Leptospira spp.* via multiple SRCR domains (left). WC1-4 does not bind to *Leptospira spp.,* thus WC1-4$^+$ γδ T cells are not activated (right).

It has been shown with bovine γδ T cells that functional differences correlate with the WC1 genes expressed. That is, γδ T cell subpopulations in ruminants can be distinguished by differential expression of WC1 [40,42,49,103,105,122,123]. For example, while both WC1.1$^+$ and WC1$^-$ γδ T cells produce macrophage inflammatory protein-1α (MIP-1α) and granulocyte-macrophage colony-stimulating factor, WC1.2$^+$ γδ T cells produce IL-10 and TNF-β in response to stimulation through TLR3 and TLR7 [99]. Production of MIP-1α, a ligand for the chemokine receptor (CR) CCR5, reflects the ability of these cells to shape the immune response through recruitment of other cells including γδ T cells

since CCR5 and CXCR3 are both expressed on $WC1^+$ γδ T cells [124]. Stimulation of $WC1^+$ γδ T cells results in increased expression of CCR5 and CXCR3 and induces IFN-γ production demonstrating the proinflammatory nature of these cells [124].

While bovine WC1 functions as a pattern recognition receptor (PRR) it is also capable of exerting co-receptor activity via phosphorylation of tyrosine residues in its cytoplasmic domain [48,50,125]. There are three variations of cytoplasmic domains named Type I, II and III which are encoded for by 4, 5 or 6 exons respectively [107]. Type I and II cytoplasmic domains contain five tyrosine residues, while the Type III cytoplasmic domain contains eight [48]. Although bovine WC1 cytoplasmic domains contain multiple tyrosine phosphorylation motifs, the tyrosine that is required to be phosphorylated for co-receptor activity differs between type I and II and type III. Type I and II cytoplasmic domains are phosphorylated at the membrane proximal tyrosine (Y24) while Type III cytoplasmic domains are phosphorylated at the membrane distal tyrosine [48,125]. WC1 co-receptor activity is also governed by serine phosphorylation and a di-leucine endocytosis motif [50].

Swine belong to the same order as cattle, Artiodactyl, and maintain a large subset of $WC1^+$ γδ T cells. Like cattle, swine are susceptible to infection with Leptospira and mycobacterium, and serve as a reservoir host for these diseases. Because WC1 plays an active role in the γδ T-cell response to these diseases in cattle, it is important to define WC1 in swine as it holds the same potential. While the total number of WC1 gene members in swine, sheep and goats awaits finalization, cDNA evidence and genome

annotation results suggest the presence of 10 genes in swine, and up to 28 genes in sheep and goats [53,126,127].

### 1.4 Development and distribution of γδ T cells

#### 1.4.1 Thymic development

γδ T cells are the earliest detected T cell subset in the porcine thymus and subsequently populate the periphery where they are found in the fetal liver as early as 40 days of gestation [16,128]. This development underscores their importance in early immune development and protection [16,128,129]. Unlike αβ T cells, γδ T cells appear to develop without any $CD3\varepsilon^{lo}$ or $TCR\gamma\delta^{lo}$ transitional stage, highlighting the fact that they require less time to achieve maturation [16,128]. γδ thymocytes can be divided into two major families in the pig, the majority belong to $CD4^-$ γδ thymocytes which can be further subdivided according to CD2 and CD8αα expression, and a smaller portion of γδ thymocytes which express CD4 and the CD8αβ heterodimer, which are akin to double positive thymocytes of the αβ T cell lineage [130]. Maturation of the $CD4^-$ group begins with the $CD2^+CD8^-CD1^+CD45RC^-$ common precursor in the thymus, which diversifies further in the periphery to encompass $CD2^+CD8\alpha\alpha^+$, $CD2^+CD8^-$ and $CD2^-CD8^-$ subsets which lose CD1 expression and increase CD45RC expression as they mature [130]. The population of $CD4^+CD8\alpha\beta^+$ γδ thymocytes which always express CD1 and differentially express CD45RC have no counterpart in the periphery [130]. Comparison of MHC class II molecules (SLA-DR) expression on γδ T cells in the blood, spleen and thymus of pigs at different ages revealed that the majority of peripheral γδ T cells in fetal animals are MHC-II negative while a substantial proportion of peripheral γδ T cells in young animals express MHC-II [130]. Interestingly, thymocytes

from both fetal and young animals expressed MHC-II. Almost all of them displayed a $CD2^+CD8^+$ phenotype composed of $CD2^+CD4^-CD8\alpha\alpha^+$ in the periphery and both $CD2^+CD4^-CD8\alpha\alpha^+$ $CD2^+CD4^-CD8\alpha\alpha^+$ subsets in the thymus [130]. These observations suggests that all peripheral $CD8^+$ γδ T cells express CD8αα and that two subsets are differentiated based on MHC II expression. It has been proposed that one subset acquires CD8αα expression in the thymus, while the second acquires it resultant from stimulation in the periphery, but this remains to be confirmed.

It has been shown in sheep that thymic export of γδ T cells increases throughout fetal development and again after parturition with γδ T cells accounting for up to 38% of emigrating cells in 3 month old lambs [131]. Export of $WC1^+$ γδ $TCR^+$ thymocytes increases during the gestational transition period while export of $WC1^-$ γδ $TCR^+$ thymocytes remains constant throughout fetal life and increases during postnatal development [131]. This change correlated with changes of expression of CD2 on the γδ T cells: it is reduced from 40% to 15% of γδ thymic exports by 3 months of age, and as in the pig, CD2 expression is upregulated on γδ T cells after they are exported from the thymus [130,132–134]. CD2 expression also is known to differ on subpopulations of γδ T cells with $WC1^+$ γδ T cells generally being $CD2^-$ while $WC1^-$ γδ T cells are $CD2^+$. This occurs in cattle, sheep, goats, and pigs and may reflect the difference in γδ T cell subpopulations exported rather than the expression of CD2 by γδ T cells overall [12,40–42,82].

### 1.4.2 γδ T cells in blood

Calves are born immunologically naïve, meaning that the essential immune components are present at birth, but display suboptimal functionality until ~4-weeks of age. Given that the number of γδ T cells increases substantially prior to and immediately following birth in ruminants, it is reasonable to assume that they play an integral role through non-MHC-restricted cellular immunity prior to establishment of functional $CD4^+/CD8^+$ αβ T cell populations. It is known that γδ T cells comprise up to 60% of circulating lymphocytes in young cattle [12,52], and sheep [135,136] following birth. In general, the percentage of γδ T cells in ruminants is inversely correlated with age, decreasing to comprise ~5-30% of peripheral blood mononuclear cells (PBMC) in healthy adult ruminants [137]. However, the peripheral blood γδ T cells in sheep begin at a lower percentage, representing around 20% of total lymphocytes, and then increase to 50% at around 5-6 months of age and then taper off around 5-8 years of age ending at 5-10% [138]. Thus, the representation of γδ T cells is not simply from high to low in ruminants.

The absolute number of swine γδ T cells increases rapidly and consistently from birth until a plateau is reached around 19-25 weeks, when γδ T cells comprise up to 50% of circulating lymphocytes [139–142]. There is some discrepancy surrounding γδ T cell prevalence in adult pigs as early studies concluded that peripheral γδ T cells decline with age [132,143], while a recent synoptic comparison of published data regarding PBMC populations in healthy weaned piglets and non-gestating sows (~2.5 years of age) suggests that there is actually an increase in peripheral γδ T cells during the transition from adolescence to adulthood [144]. It is possible that these discrepancies are due to

varying definitions of the transition from adolescents to adulthood, a variable which is further complicated by the fact that different pig breeds reach sexual maturity and complete their skeletal growth at different ages. Lack of mAb which recognize porcine γδ T cell subsets (discussed below) may also be a contributing factor to the differences in findings.

### 1.4.3 γδ T cells in tissues

Despite this marked decrease with age in the blood, γδ T cells participate in immune surveillance, protection, and tissue homeostasis throughout the organism's lifetime. Restricted TCR gene usage observed in γδ T cell populations localized to specific compartments of the body denotes they are poised for recognition of tissue specific antigens [52,145,146]. Localization to the epithelial surfaces, particularly the dermal and epidermal layers of the skin and within the digestive tract, suggests these cells play a role in antigen sampling and tissue homeostasis [12,40].

Swine γδ T cells are a major component of porcine intraepithelial T cells (IETs), a specialized population of immune cells residing in the intestinal epithelium that serve as the first line of defense and are crucial for tissue barrier homeostasis (as reviewed in [147] ). IETs are among the first immune cells to populate intraepithelial tissues where they offer early protection and shape long-term intestinal health through interactions with epithelial cells and microbiota [147]. Porcine γδ T cells originating in the intestine contribute significantly to the migrating γδ T cell pool as a constant proportion of these cells are rapidly recirculated into the peripheral blood [148]. The majority of γδ IETs are found

within the large intestine and appear to be terminally differentiated while γδ IELs of the small intestine are less abundant, but more phenotypically diverse [149]. As pigs age, microbial populations of the distal intestine increase in abundance and diversity and γδ IETs located in these regions undergo more frequent activation than their proximally located counterparts demonstrating a role for microbial interactions and possibly tolerizing the gut to beneficial flora [149–153].

Porcine γδ T cells are also found in the dermis and are enriched in sites of skin inflammation [154,155]. γδ T cells are found in the nasal mucosa, and it has been reported that the proportion of γδ T cells in this tissue is higher than that of the peripheral blood [19]. Finally, during pregnancy, swine γδ T cells are a major component of the lymphocyte population in the uterine endometrium reflecting their importance in materno-fetal tolerance [156]. In ruminants, γδ T cells are also dominant in mucosal tissues of the reproductive tract and the number of γδ T cells in peripheral blood increases as cattle approach parturition [157,158].

### 1.5 Differentiation of swine γδ T cell populations

The ability to identify specific lymphocyte populations is an essential component of understanding the role of γδ T cells in immune responses. This then plays a role in improving vaccine efficacy as it allows us to reveal the best suited cellular target(s) for priming with next generation vaccines in a disease-specific context. The ongoing quest to define porcine γδ T cell subsets relies heavily upon interrogation with mAb designed to

recognize molecules found on the surface of porcine γδ T cells. Some of these are summarized in Table 1.2.

Table 1. 1 mAb used to define porcine γδ T cells in cited studies

| mAb | Target | Staining Pattern | Ref |
|---|---|---|---|
| 86D | γδ TCR subset (γ chain) | 8-12% PBL (3-4wk), 9-24% PBL (6-9mo)<br>31% PBL | 40,102,105,159 |
| B37C10 | SWC5 (WC1) | 10-20% PBL (3-4wk), 38-46% PBL (6-9mo)<br>17.9% PBL | 102,105 |
| CC101 | Bovine WC1, SWC5 (WC1) | 10-20% PBL (3-4wk) or 38-46% PBL (6-9mo)<br>39.8% PBL | 101,102,105 |
| GD3.5 | Bovine GD3.5 | 90% of the peripheral γδ T cell population | 160 |
| MAC318[a] | SWC4 | 28% PBL | 102 |
| MAC319[a] | SWC4 | 25.6 PBL<br>60% of MAC320$^+$ cells, 50% MAC320/86D$^+$ | 15,102 |
| MAC320[*] | SWC6 | 25-85% PBL, 100% of 'null' cells<br>47.1% PBL | 15,102,161 |
| MAC80 | CD2 | 44% PBL and ~45% of MAC320$^+$ | 15 |
| PG38A | γδ TCR subset (γ chain) | 25.6% PBL | 102,159 |
| PG92a | SWC5 (WC1) | 18.5% PBL | 102 |
| PPT16 | γδ TCR-associated CD3 | 65.7% CD3$^+$ PBL, 74% of PPT27$^+$ cells<br>22% of 86D$^+$ cells, 74% MAC320$^+$ cells | 90 |
| PPT27 | γδ TCR subsets | 30-40% (9.6% CD2$^+$) T cells in PBL and 34% T cells in spleen | 134 |
| PPT3 | Porcine CD3 ε-chain | Identifies total T cells | 162 |
| sIgM | B cells | 10% PBL | 15 |

It has been shown that individual γδ T cells can express multiple WC1 molecules on their surface in cattle [163]. Populations of bovine WC1$^+$ γδ T cells may be serologically defined based on the presence or lack of specific WC1 transcripts. Monoclonal antibodies that differentially recognize WC1.1-type and WC1.2-type gene products may be used to identify expressing γδ T cell populations. Furthermore, these mAb may also be used to identify pathogen-specific responsive subpopulations of γδ T cells [119]. For instance, mAb BAG25A positively stains γδ T cells expressing WC1.1-type transcripts, and stains *Leptospira* and *Mycobacteria* responsive γδ T cell populations in cattle [43,44,164]. Conversely, mAb CACTB32A positively stains γδ T cells that express WC1.2-type transcripts and those that respond to *Anaplasma* [43,44].

As in the thymus, porcine γδ T cells present in the periphery can be divided into three subsets based on the expression of the costimulatory molecules CD2 and CD8 into the three subpopulations CD2$^-$CD8$^-$, CD2$^+$CD8$^+$, and CD2$^+$CD8$^-$ [130,132–134]. CD8 expression on peripheral porcine γδ T cells is primarily in the form of CD8αα homodimers, but intestinal lymph γδ T cells express CD8αβ heterodimers [148]. A similar subset of large thymocytes, described in detail above, is also found to express the heterodimer [130,165]. While CD2$^+$CD8αα$^+$ and CD2$^+$CD8$^-$ subsets are predominantly found in the lymph nodes and spleen, CD2$^-$CD8$^-$ subsets dominate in the blood [16,82,132]. However, there is a small population of CD2$^+$CD8$^-$ γδ T cells present in circulation as well (Figure 1.4). In conventional models, CD2 is upregulated on memory T cells and plays a role in activation, while expression of CD8α is associated with non-MHC restricted cytolytic

activity and survival of effector memory subsets [166]. Their role on the porcine γδ T cells has not been specifically investigated.

Figure 1.4

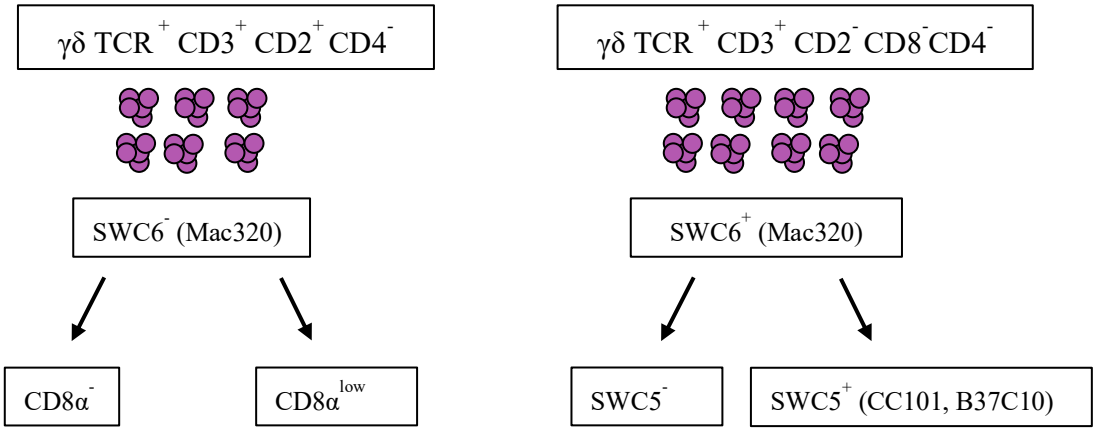



Figure 1.4 Major subpopulations of porcine γδ T cells in the blood.
Monoclonal antibodies (mAbs) have been used to characterize porcine γδ T cell subpopulations in various tissues and organs. The target ligand of the mAbs is shown when it is known (TCR, CD3, CD2, CD8) while in other cases the workshop name is given ('SWC') with the mAb name in parenthesis to the right. These categories result in 4 main subpopulations of γδ T cells within the PBMC of swine.

Peripheral porcine γδ T cells can be further subdivided based on differential expression of other cell surface antigens. For example, the $CD2^-CD8^-$ subset is devoid of CD6 and perforin expression [166,167]. The SWC6 mAb MAC320 was originally reported to stains 100% of the 'null' ($CD2^-$ $sIg^-$)cells in the peripheral blood (now known to be largely the γδ T cells) and recognizes a di-sulfide linked heterodimer that is present in two isoforms (270 and 280kDa) on porcine γδ T cells. [15] Under reducing conditions, mAb MAC320 immunoprecipitated two to three polypeptide chains between 130-160 kDa, of which the largest molecular weight band is also precipitated by the αSWC4 mAb MAC319 [15,159]. Antibodies MAC320, MAC319 and MAC318 exclusively bind porcine lymphocytes and

all 'null' lymphocytes are MAC320$^+$, of which 60% are MAC319$^+$ and ~45% are mAb 86D$^+$ [15]. Finally, the majority of SWC6$^+$CD2$^-$ cells in the blood co-express the WC1 orthologue known as SWC5 and identified by mAbs CC101, PG92a and B37C10 that differentially stain porcine PBL (Table 2). [102,105] We are currently in the process of evaluating WC1 recognition by several monoclonal antibodies (CC101, PG92a and B37C10) in the pig, and we hypothesize that these mAb recognize multiple porcine WC1 gene products (unpublished data).

There is some debate about whether the CD2$^+$ and CD2$^-$ γδ T cells represent two distinct lineages or represent the same lineage. Differential levels of TCR expression between CD2$^+$ (TCR γδ$^{med}$) and CD2$^-$ (TCRγδ$^{hi}$) subsets that are established in thymus with limited plasticity in the periphery have led some to conclude that they represent two distinct γδ T cell lineages [168]; however, *in vitro* analysis of CD2-sorted populations has demonstrated a high degree of plasticity in CD2 expression, and found that it can be induced in CD2$^-$ γδ T cells following stimulation with IL-2, IL-12, IL-18 and ConA [169]. The transcription factor (TF) GATA-3 is expressed in the majority of porcine γδ thymocytes irrespective of CD2/CD8α expression. While this is retained on extrathymic populations of CD2$^-$CD8$^-$CD27$^+$perforin$^-$ γδ T cells, the opposing CD2$^+$CD8α$^{hi}$/CD27-perforin$^+$ phenotype is associated with expression of transcription factors T-bet, Eomes or co-expression of both [151].

In the gut, γδ IETs are found in the large intestine and appear to be terminally differentiated based on their CD2$^+$CD8α$^+$ phenotype, while variable CD8α expression on

γδ IELs in the small intestine gives rise to the two phenotypically distinct populations: $CD2^+CD8\alpha^+$ and $CD2^+CD8\alpha^-$ [149]. As pigs age, microbial populations of the distal intestine increase in abundance and diversity and γδ IETs located in these regions appear to undergo more activation than their proximally located counterparts as demonstrated by decreased percentages of CD27 expression on distal γδ IETs [149–153].

### 1.6 Immune functions of γδ T cells

#### 1.6.1 Cytokine production

Differential expression of WC1 genes correlates with unique cytokine expression profiles of γδ T cells in cattle. $WC1^+$ γδ T cells are a significant source of interferon gamma (IFN-γ), a proinflammatory cytokine that is critical for pathogen clearance in a number of infections, and the ability of these cells to produce IFN-γ is based on the phenotype of expressed WC1 genes [43,74]. Tumor necrosis factor alpha (TNFα) is another inflammatory cytokine that is provided by $WC1^+$ γδ T cells in ruminants [170]. Subsets of bovine, caprine, and ovine γδ T cells are polarized in vivo to a Th17 phenotype (γδ17) and express IL-17A, a proinflammatory cytokine that plays a crucial role in host defense and is a major proponent of several inflammatory and autoimmune diseases [64,171]. The majority of IL-$17A^+$ γδ T cells in cattle, sheep and goats express WC1 while a small population of these cells are $WC1^-$ [64]. Production of IL-17 induces epithelial and stromal cells to produce CXCL1, CCL20, IL-6 and IL-8, which further shape the immune response through stimulation and recruitment of neutrophils and monocytes [172]. Naïve $WC1^+$ bovine γδ T cells isolated from peripheral blood and cultured with supernatant produced from fibroblasts infected with the parasite *Neospora caninum*, undergo

polarization to a Th17-like phenotype (γδ17) and demonstrate autonomous killing of infected cells in a manner that is dependent upon direct cell-to-cell contact [173]. Unlike classical Th17 cells, or IL-17$^+$ γδ T cells which home to the skin [174], Tγδ17 cells do not express CCR6 or IL-23R and are induced by IL-6 and IL-1β present in the supernatant in a TCR-independent manner [173].

Ruminant γδ T cells also function as immunoregulators through secretion of a variety of anti-inflammatory cytokines including IL-4, IL-10 and transforming growth factor beta (TGF-β) [68,175,176]. Spontaneous production of IL-10 by peripheral blood γδ T cells inhibits Ag-specific and nonspecific proliferation of CD4$^+$ and CD8$^+$ T cells further demonstrating their importance as an immunoregulatory subset [176]. Interestingly, IL-10 production is not dependent upon WC1 expression, but the propensity of WC1$^+$ γδ T cells to produce IL-10 does depend on the which WC1 gene is expressed [176].

Porcine γδ T cells also can produce a variety of cytokines in response to various stimuli (Table 1.3). For example, stimulation with mitogens Concanavalin A (Con A) or phorbol myristic acetate (PMA) induces mRNA expression of IL-1, IL-2, IL-6, IL-8, GM-CSF, TGF-β, IFN-α, IFN-γ and Lymphotactin (Ltn) in porcine γδ T cells. [19,177] Lymphotactin (Ltn) induces CD8$^+$ T cell and NK cell chemotaxis, demonstrating the ability of porcine γδ T cells to shape the immune response via recruitment of other cells [178]

Table 1. 2 Cytokines and chemokines produced by porcine γδ T cells

| Cytokine/Chemokine | Stimulation | Population | Ref |
|---|---|---|---|
| IL-17A | Mitogen | $CD8\alpha^+$ and $CD8^-$ | 64,171 |
| IL-10 | Mitogen | $Foxp3^+CD2^+CD8\alpha^+$ of the ileum, IEL and spleen | 98 |
| TGF-β | Mitogen<br>FMD vaccine antigen | $Foxp3^+$ $CD2^+CD8\alpha^+$ of the ileum, IEL and spleen | 98,19 |
| IFN-γ | Mitogen | $CD2^+CD8\alpha^-$<br>$CD2^-CD8\alpha^-$ | 29,98 |
| GM-CSF | FMD vaccine antigen | $CD2^+CD8\alpha^+$ | 19 |
| IFN-α | Mitogen,<br>FMD vaccine antigen | $CD2^+CD8\alpha^+$ | 19 |
| Lymphotactin (Ltn, XCL1) | FMD vaccine antigen | $CD2^+CD8\alpha^+$ | 19 |
| MIP-1α (CCL3) | FMD vaccine antigen | $CD2^+CD8\alpha^+$ | 19 |
| IL-1 | Mitogen,<br>FMD vaccine antigen | $CD2^+CD8\alpha^+$ | 19 |
| IL-6 | Mitogen,<br>FMD vaccine antigen | $CD2^+CD8\alpha^+$ | 19 |
| IL-8 | Mitogen,<br>FMD vaccine antigen,<br>STM | $CD2^+CD8\alpha^+$<br>$CD2^+CD8\alpha^{+/-}$ | 19,35 |

In addition to mitogenic stimulation, porcine γδ T cells produce cytokines in response to pathogens. Purified porcine γδ T cells stimulated with emergency FMD vaccine antigen show increased mRNA levels of Ltn, MIP-1α and TGF-β in some cases, while all cases result in increased mRNA expression of IL-1, IL-6, IL-8, GM-CSF and IFN-α [19]. Pigs vaccinated for the influenza A virus H1N1 acquire heterosubtypic cross-protection to swine influenza virus (SIV), which is associated with IFN-γ and IL-10 production by

antigen-specific γδ T cells [32,33]. In response to porcine reproductive and respiratory syndrome virus (PRRSV), γδ T cells proliferate and produce IFN-γ, and upon re-challenge increase production of IFN-γ and IL-12 [23,29–31]. Porcine γδ T cells play an integral role in shaping the immune response through cytokine production, like what is observed in cattle.

The influence or correlation of differential WC1 gene expression on porcine γδ T cells with cytokine production and response to pathogens remains to be defined. Studies evaluating cytokine production in the context of CD2 expression found that $CD2^+$ γδ T cell populations have a higher propensity for IFN-γ and TNF-α production, while IL-17A is produced exclusively by $CD2^-$ populations [64,179]. Such studies allow us to make predictions about the role of porcine WC1 gene expression and the potential influence it may have on cytokine production, as the majority of $WC1^+$ γδ T cells are $CD2^-$ [83].

### 1.6.2 Chemokine receptor and ligand expression

The propensity of lymphocytes to enter lymphoid tissues and localize to sites of infection is governed by expression of chemokine receptors (CR)[180]. Expression profiles of CRs can be used to reveal functional characteristics of lymphocytes including their homing capacity and potential migration patterns. As members of the large family of G-protein coupled receptors, CRs are classified according to their chemokine ligands (CL) which are divided into three groups: C, CX or CX3 based on the identity of amino acid residues between the first two N-terminal cysteines [181].

Bovine WC1$^+$ γδ T cells are induced to secrete IFN-γ following antigen stimulation, and transcript analysis has shown that this coincides with a substantial increase in the expression of CCR5 and CXCR3 [124]. CCR5 and CXCR3 expression on T lymphocytes coincides with a role in inflammatory responses and identifies effector memory cells with a Th1 phenotype [182,183]. Ligands for CCR5, which is expressed on activated T cells, are produced by dendritic cells at sites of inflammation thus resulting in an influx of effector T cells to the inflamed area [180,184]. CXCR3 is a marker for pre-Th1 and polarized central memory effector cells, and its ligands are also induced in response to inflammation [181,185]. In humans, CXCR3 expression on γδ T cells is associated with their ability to undergo transendothelial migration [186]. As mentioned above, WC1$^+$ γδ T cells from cattle are known to express the CCR5 ligand MIP-1α following stimulation by PAMPs [60,187]. Expression of MIP-1α likely results in the recruitment of additional WC1$^+$ γδ T cells as well as neutrophils to the inflamed tissue. The timely arrival of WC1$^+$ γδ T cells to inflammation sites, IFN-γ and CR ligand production along with their expression of CRs, including CCR5 and CXCR3 solidifies a role for these cells in shaping Th1 immune response.

Stimulation of circulating bovine γδ T cells with bacterial cell wall components LPS and peptidoglycan induces expression of chemokines CCL3 and CCL5 [60]. These chemokines function as potent chemoattractant for phagocytes of the innate immune system, further demonstrating the role that γδ T cells play in orchestrating both innate and adaptive immune responses to pathogens. *In vitro* studies have also shown that bovine γδ T cells also express CCL2, CCL8, CXCL1, CXCL2 and CXCL6, which are associated with

recruitment and activation of myeloid-derived cell types [188]. It was found that neonatal bovine γδ T cells produce CCL2, CCL3 and GM-CSF in response to viral TLR agonists and *in vivo* during bovine respiratory syncytial virus (BRSV) infection [99]. Priming of γδ T cells with pathogen associated molecular patterns (PAMPs) results in downstream production of chemotactic factors, including CCL3, CCL4, CCL5, CXCL8 and GM-CSF [60,189]. The chemokine expression by γδ T cells presumably influences local cellular traffic to promote the influx of lymphocytes and monocytes to infection sites [68,190].

While less is known about the expression of chemokines and chemokine ligands in porcine γδ T cells, a study evaluating differentiation receptor CD8α and lymph homing receptor CCR7 expression on porcine γδ T cells in the context of PRRSV-2 infection has established an interesting dichotomy for these markers. Naïve $CD8\alpha^-$ γδ T cells are $CCR7^-$ suggesting that they have the ability to migrate between the blood and tissue [30]. Following exposure to PRRSV, IFN-γ producing γδ T cells upregulate CD8α expression but stay $CCR7^-$, while TNF-α producing and proliferating γδ T cells upregulate CCR7 expression [30]. Expression of CCR7 allows these cells to drain into the lymphatic system during activation and differentiation [30].

### 1.6.3 Cytoxicity

γδ T cells are capable of cytoxic activity and may contribute to the response against intracellular pathogens by lysing infected cells or inhibiting bacterial cell growth [170,191,192]. Bovine $WC1^+$ γδ T cells exhibit cytolytic effector functions in response to cells infected with pathogens [170,191,193]. Cytolytic abilities are correlated with production of

granulysin, granzyme B and perforin, which counter intracellular and extracellular pathogens [65,190,194–196]. Populations of peripheral blood γδ T cells that are activated by cytokines IL-6 and IL-1β in the absence of TCR stimulation are capable of autonomously killing parasite-infected cells demonstrating their innate-like abilities [173]. NK-like cytoxicity that was not dependent upon MHC was observed in $WC1^+$ γδ T cells responding to *Babesia bovis* and in γδ T cells isolated from animals with foot-and-mouth disease (FMD) virus [65,170]. Additionally, TCR-dependent lysis of *Theileria parva*-infected autologous targets is carried out by WC1+ γδ T cell lines independently of MHC [191]. Currently, there is no evidence of porcine γδ T cells exhibiting cytolytic activity. This is not to say that these cells are unable to perform cytolytic functions, but the studies have not taken place. One barrier, discussed in detail earlier in this review, is the lack of mAbs and defined subsets of porcine γδ T cells.

### 1.6.4 Antigen Presentation

One way in which γδ T cells function as a bridge between the innate and adaptive immune systems is through antigen presentation via MHC class II. In cattle, γδ T cells take up antigen and present it to CD4 T cells to induce their activation and proliferation [197]. Following FMD infection, $WC1^+$ bovine γδ T cells upregulate MHC II and co-stimulatory molecules CD80 and CD86 [65]. These molecules are necessary for both antigen presentation and T cell activation, and $WC1^+$ γδ T cells directly induce CD4 T cell proliferation following FMD infection [65]. *In vitro* stimulation of bovine peripheral blood γδ T cells with *M bovis* Bacillus Calmette-Guerin (BCG) also induced upregulation of MHC II, CD80 and CD86 [198].

Stimulation of purified naïve porcine γδ T cells with high potency "emergency" FMD vaccine antigen induces a subset of circulating γδ T cells that display a phenotype like that of antigen presenting cells [19]. Additionally, these γδ T cells were confirmed to take up and present soluble antigen to $CD4^+$ T cells in a direct cell to cell interaction via MHC class II. This interaction likely has a significant influence on the downstream adaptive immune response to FMD [19]. Anti-viral immunity against African swine fever virus (ASFV) is attributed to a higher prevalence of γδ T cells in the peripheral blood, and these γδ T cells can present viral antigen to $CD4^+$ T cells [26–28]. Co-culture of peripheral porcine γδ T cells with classic swine fever virus (CSFV) and dendritic cells results in partial activation of γδ T cells as well as upregulation of MHC II surface expression. This upregulation in MHC II suggests that γδ T cells are serving as antigen presenting cells for dendritic cells during CSFV infection [36].

## 1.7 γδ T cell responses to infectious diseases of pigs

### 1.7.1 Protective response

*African swine fever virus (ASFV)*

Porcine γδ T cells play a protective role in the immune response to multiple pathogens that infect swine (Table 1.1). African swine fever virus (ASFV), a double-stranded DNA virus that infects domestic pigs and wild boar, is endemic in sub-Saharan Africa, and across parts of Europe and Asia [199,200]. There is currently no licensed vaccine available for ASFV; thus, methods to control the spread are extremely costly to producers. Monocytes and macrophages are the primary targets of infection; therefore, a

necessary component of vaccination involves stimulation of cytoxic cells to lyse infected cells. [201] Given that subsets of porcine γδ T cells are potent producers of cytokines necessary for viral clearance, γδ T cells could be an effective target for an ASFV vaccine [143,179]. Anti-viral immunity resultant from priming with live attenuated ASFV is dependent upon cellular immunity induced from CD8α$^+$ T cell subsets, including γδ T cells, but protection is limited to homologous viral strains. [202–204] A higher prevalence of γδ T cells in peripheral blood following virulent infection is associated with increased survival, and γδ T cells from ASFV-immune pigs are able to present viral antigens to CD4$^+$ T cells. [26–28]

*Mycobacterium bovis (M. bovis)*

*Mycobacterium bovis,* the causative agent of bovine tuberculosis, is a zoonotic pathogen that infects a range of wild and domestic mammals including swine [205,206]. In cattle, *M. bovis*-specific γδ T cells upregulate expression of tissue-homing receptors CXCR3 and CCR5 during infection, a phenomenon that presumably allows migration to inflamed tissue and is also observed in γδ T cell participants in recall responses against *Leptospira* antigens [195,207]. Antigen-specific γδ T cells produce IL-17A and IL-22, two key cytokines associated with detection and clearance of *M. bovis* infection [208]. While less is known about the porcine immune response to *M. bovis* infection, γδ T cells play an apparently protective role. Swine vaccinated with BCG mount a Th1-like immune response orchestrated by γδ T cell lymphoproliferation and IFN-γ production [25]. Additionally, BCG vaccination in young pigs was found to result in γδ T cell proliferation and IFN-γ production in response to stimulation with various mycobacterial antigens [25]. While the

population and other logistics of the porcine γδ T cell response to *M. bovis* and BCG remains to be determined, preliminary data from our lab suggests that $CD2^{-}WC1^{+}$ γδ T cells are likely involved. Immunoprecipitation assays using recombinant porcine WC1 SRCR domains and whole bacteria from various *M. bovis* BCG strains suggests that WC1 is capable of directly binding to multiple BCG strains (unpublished data).

*Porcine reproductive and respiratory syndrome (PRRS)*

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically devastating chronic viral diseases impacting global swine production to date. [209] There are several vaccines used to control PRRSV infection; however, the heterogeneity of the virus itself has significantly hindered the efficacy of commercially available vaccines. The primary means of infection for PRRSV includes viral entry into macrophages through the scavenger receptor CD163A, specifically through direct binding and interaction with the fifth SRCR domain, a b-type SRCR domain. [210–213] A significant portion of γδ T cells in the pig express WC1, which is a close relative to CD163A that shares similar amino acid identity through conserved SRCR domains. [51] Furthermore, γδ T cells have been shown to play an apparently protective role in the early immune response against PRRSV infection through increased secretion of Th1 cytokines such as IFN-γ and IL-12, and are capable of engaging in an antigen-specific, memory driven response upon re-challenge [23,29]. Infection with PRRSV has also been shown to induce the expression of the lymph node homing chemokine receptor CCR7, demonstrating a role for these cells within the lymphatic system during infection [30]. Interestingly, administration of *Mycobacterium tuberculosis* whole cell lysate (*Mtb* WCL) as a mucosal

adjuvant followed by administration of a modified live PRRS virus (PRRS-MLV) intranasal vaccine was correlated with increased Th1 cytokine production driven by γδ T cells, as well as a reduction in immunosuppressive cytokines IL-10 and TGF-β. [23]

*Swine influenza virus (SIV)*

Swine influenza virus (SIV) is a highly contagious acute respiratory disease of pigs caused by a strain of influenza virus A in the *Orthomyxoviridae* family. Epithelial cells lining the porcine respiratory tract express sialic acid receptors that are utilized by both avian (α-2,3 SA-galactose) and mammalian (α-2,6 SA-galactose) influenza viruses allowing pigs to serve as an ideal "mixing vessel" for the generation of new influenza A viruses that possess mammalian and avian RNA elements [214–216]. Reassorted viruses generated in the pig can cause pandemics in humans, as demonstrated by the 2009 H1N1 virus outbreak [214]. Increased levels of γδ T cells are located in the BAL and lower tonsils of pigs experimentally infected with H1N1 [32]. Vaccination of pigs with reverse genetics-derived H3N2 virus (NS1Δ126 TX98) confers partial cross-protection during heterosubtypic sub challenge with H1N1 and is associated with the production of IFN-γ and IL-10 by antigen-specific γδ T cells [32,33].

*Foot and mouth disease (FMD)*

FMD vaccines induce protection to aerosol infection within four days when administered to cattle [217] and pigs [218,219]. Non-adherent cells (NADC) isolated from the PBL of unvaccinated pigs proliferate in response to FMD emergency vaccine antigen and depletion of γδ T cells abrogate this response [19]. Purified γδ T cells from unvaccinated

animals produce GM-CSF, IFN-α and IL-8 when stimulated with FMD antigen alone and secrete IFN-γ when stimulated in the presence of the remaining NADC fraction [19]. Following vaccination, porcine γδ T cells acquire an APC phenotype and present soluble antigen to $CD4^+$ T cells [19,220].

### 1.7.2 Role of γδ T cells in disease progression unknown

*Salmonella enterica*

Nontyphoidal salmonellae (NTS) such as *Salmonella enterica* serovar typhimurium (STM) are a leading cause of foodborne zoonoses worldwide [221,222]. This particular serovar is also responsible for self-limiting gastroenteritis in humans [223]. Pigs are a key reservoir of infection for humans as they are asymptomatic carriers of many serovars of *Salmonella* [224]. The immune response to STM in pigs is characterized by intensified expression of pattern recognition receptors TLR2, TLR5, TLR9, TLR10, and NOD2 as indicated by increased levels of mRNA expression for these genes in γδ T cells. Also have increases expression of IL-8 [35].

*Classical swine fever virus (CSF)*

Classical swine fever virus (CSF) is a contagious, often fatal disease that infects domestic pigs and wild boar. Vaccination with live CSFV vaccines confers rapid protection in the absence of neutralizing antibodies, but not before virus-specific IFN-γ secreting cells appear in the blood, suggesting that protection is mediated by cellular mechanisms[225–228]. γδ T cells are refractory to infection with CSFV, but co-culture with CSFV and plasmacytoid dendritic cells (pDCs) results in partial activation of γδ T cells with

upregulation of MHC II surface expression [36]. *Ex vivo* challenge of γδT cells isolated from the tonsils and retropharyngeal lymph nodes from vaccinated pigs had upregulated MHC-II expression but did not contribute to cellular effector mechanisms induced by live attenuated CSFV [36]. Upregulation of MHC II on γδ T cells is associated with APC activity during ASFV infection, however the exact role of γδ T cells during CSFV infection remains to be determined.

*Taenia solium*

*Taenia solium* is a helminth that causes taeniasis, an intestinal infection with adult tapeworms, that occurs in humans after ingestion of contaminated pork [229]. Infected pigs pass eggs in their feces which can also infect humans, causing cysticercosis, via fecal-oral-ingestion of contaminated food or wastewater [229]. Cysticercosis can have devastating effects on human health as the larvae can develop in the muscles, skin, eyes, and central nervous system. Cyst development in the brain causes a condition called neurocysticercosis which can be deadly [229,230]. In 2015, WHO Foodborne Disease Burden Epidemiology Reference Group identified *T. solium* as a leading cause of death from food-borne diseases [229]. In humans, predominance of a Th1-type immune response to infection of the central nervous system can result in severely symptomatic individuals that experience chronic infection [230]. In contrast, granulomatous lesions comprised of Th1 and Th2 cells were associated with disintegrating parasites [230]. This implies that effective clearance of the parasite requires participation of Th1 and Th2 cellular immunity.

Histological studies revealed several similarities between human and porcine infections including the division of the immune response into four hypothetical progressive stages based on inflammatory infiltrates and associated accumulation of fibrosis [231–233]. Stage I consists of viable cysts surrounded by a thin layer of collagen. A mononuclear-rich inflammatory infiltrate is evident around the parasite in stage II. During stage III granulomas with associated inflammatory infiltrates and fibrosis are formed. In swine migration of eosinophils to the center of the lesion is also observed during stage III. By stage IV the center of the lesion contains disintegrated parasite and amorphous material reminiscent of necrosis. The granulomatous response observed in swine is like that in humans, but differs in the abundance of eosinophils, plasma cells and discrete deposition of collagen [37,233].

In the porcine model, activated γδ T cells are present from stage I of infection and are the predominant cell type surrounding cysterici lesions. They are thought to contribute to the predominant Th1-type cytokine pattern and potentially promote symptomatic, viable and chronic infection rendering γδ T cells likely pathological in this context [37,234]. It is likely that the appearance of γδ T cells early during infection impacts the type of immune response elicited overall around the cystericus. As the infection progresses from stage III to stage IV the number of CD4 and CD8 T lymphocytes increases [235]. In mouse models for cysticercosis, γδ T cells are also observed during the initial stages of infection [234,236]. In humans γδ T cells are not detected in the chronic granulomatous responses but may be present during initial stages of infection [230].

During stage II of the porcine model, the inflammatory infiltrate contains MHC-II positive areas that co-localized with monocytes/macrophages and B lymphocytes [37]. This data suggests that B lymphocytes may be presenting antigen in addition to macrophages, and if so, the antigen presentation activity by B cells could be influencing the strong Th2 phenotype suggested by the influx of eosinophils and plasma cells that occurs in stage III [37,237–239] . Many of the MHC II positive B lymphocytes were found distributed within the collagen layer located in the periphery of stage III and IV lesions, and this strategic location is thought to be important for the synthesis of antibodies that neutralize the antigens that have surpassed epithelioid and macrophage barriers [240].

*Swine dysentery*

Swine dysentery is an infectious disease characterized by mucohemorrhagic diarrhea and inflammation in the large intestine [241]. It is caused by the Gram-negative spirochaete *Brachyspira hydysenteriae,* and is associated with reduced growth, performance and variable mortality [241]. The disease is primarily controlled through the use of antimicrobial treatment and eradication programs, however, emerging antimicrobial resistance is an issue [242,243] Recovery from experimentally induced swine dysentery is associated with an increased percentage of circulating $CD8^+$ $CD4^-$ cells and *in vitro* proliferation of these cells against *B. hydysenteriae* antigens [244]. A study which sought to evaluate the levels of circulating lymphocyte subpopulations before and during experimentally induced swine dysentery found that pigs which developed swine dysentery following inoculation displayed a higher percentage of γδ T cells and lower percentages of $CD8^+$ and $CD4^+CD8^-$ T cells prior to inoculation than pigs who remained healthy throughout the

study [38]. In this study, the total number of lymphocytes was unchanged in both groups immediately following inoculation, but a shift in lymphocyte subpopulations was observed at onset of disease. At disease onset, the total number of T cells increased in both the healthy group and the group which subsequently developed swine dysentery. In the group that developed swine dysentery, this increase in total T cells was due to increased $CD4^+$ $CD8^+$ T cells [38]. In the group which remained healthy, there was an increase in γδ T cells [38]. Interestingly, despite the increase in γδ T cells within the healthy group, the total percentage of γδ T cells was still lower than that of the group which developed swine dysentery. This study presents an interesting dichotomy as pigs which initially had higher levels of γδ T cells and fewer $CD8^+$ T cells were more susceptible to develop swine dysentery following inoculation. A prior study in vitro showed that both $CD8^+$ and γδ T cells proliferate in response to *B. hydysenteriae* antigens [244]. A study done in mice found that γδ T cell-deficient mice were less susceptible to *Salmonella choleraesuius* than wild-type mice [245]. Additionally, γδ T cell-deficient mice showed a lower morbidity and mortality after Trypanpspma cruzi infection, as well as quicker recovery from *Pneumocystis carnii* infection [246,247]. These studies suggest that γδ T cells may not always be beneficial depending on the disease context. This phenomenon could be explained by ability of γδ T cells to down-regulate recruitment and function of $CD8^+$ T cells during infection, however, this remains to be determined.

*Pasteurella multocida*

*Pasteurella multocida* infections cause pneumonia in swine, with resultant economic losses from failure to reach market weight and antibiotic costs. Within a month, after

weekly aerosol immunizations of young pigs with a live temperature-sensitive mutant *Pasteurella multocida*, $CD2^{-}SWC1^{+}$ cells doubled in their percent representation in the bronchoalveolar lavage lymphocyte population. There was no significant change in any lymphocyte population in the blood [39] . The marker profile $CD2^{-}SWC1^{+}$ profile is consistent with $WC1^{+}$ γδ T cells, and the increase in this population in the bronchoalveolar space after immunization is especially remarkable because it is the only lymphocyte population to significantly increase. Although the role of $WC1^{+}$ γδ T cells in the immune response in the lung to *Pasteurella multocida* is unknown, this raises the intriguing possibility that WC1 and γδ TCR co-engagement of *Pasteurella multocida* leads to $WC1^{+}$ γδ T cell activation.

**1.8 Discussion**

γδ T cells have a demonstrated role in relevant infectious diseases which impact livestock production and human health. Engaging these cells through vaccination or immunomodulatory strategies may provide substantial benefits in the face of such diseases. Methods to prime γδ T cells with vaccine constructs might exploit features of γδ T cell activation, such as the utilization of PRR molecules. The WC1 hybrid coreceptor and PRR plays a multi-faceted role in the response of γδ T cells to bacterial pathogens, thus we hypothesize that it may serve as a target to recruit specific subpopulations of γδ T cells with vaccine constructs and potentially increase γδ T cell participation in livestock species. Our work with WC1 has demonstrated that it can bind to multiple strains of pathogens that infect livestock, including *Leptospira* spp*., M. bovis,* and *M. avium. WC1*

*molecules are likely* capable of binding to other pathogens that infect livestock, and the fact that WC1 is expressed as a multigenic array offers vast potential in this regard.

It was recently shown that WC1 receptors colocalize with the γδ TCR upon activation [248]. It was also found that *Leptospira* spirochetes bound specifically to WC1 on the surface of γδ T cells, supporting the concept that WC1, along with the TCR and ligand, form a signaling domain upon engagement [248]. These findings may indicate that both WC1 and the TCR bind the ligand together, or interaction between WC1 and the TCR occurs following WC1 ligand binding. The latter may represent a system similar to butyrophilin (BTN) molecules found on human γδ T cells, which bind to antigen and then interact directly with germline-encoded portions of the TCR [249,250]. BTNs are expressed as a multigene family, and it has been found that multiple BTN molecules (encoding different genes) are involved in the activation of γδ T cells [249,251]. Upon antigen binding, BTN2A1 associates with BTN3A1, and together they initiate activation of γδ T cells. First, BTN2A1 binds to the Vγ9 TCRγ chain, followed by the binding of a second ligand, possibly BTN3A1, to a separate TCR domain within Vδ2 [249]. The mode of γδ T cell activation employed by BTNs is especially interesting when considering the fact that individual bovine γδ T cells express up to 6 variants of WC1 [163]. Moreover, it was shown that WC1 variants remain separated on resting γδ T cells, clustering together only with homologous WC1 molecules, but upon activation, islands containing different variants coalesce and merge with the γδ TCR islands [248]. It is also possible that WC1 functions more closely to coreceptors like CD4 and CD8 but through direct engagement with the same ligand recognized by the γδ TCR instead of MHC molecules. To evaluate this

possibility, more work is needed to identify the exact ligands of WC1 molecules. While no specific ligands have been identified to date, experiments have been carried out to characterize the nature of the WC1 ligand in the *Leptospira* spp. model. Multiple SRCR domains derived from bovine WC1 bind to *Leptospira* spp., and pre-treatment of the bacteria with proteinase K does not diminish binding activity [49]. From this, we can conclude that the WC1 ligand found on *Leptospira* spp. is not a protein. Additionally, experiments using polymyxin B to block LPS signaling could rule out LPS as a potential ligand [49]. Because WC1 is expressed as a multigene array, and each molecule encodes six to eleven pathogen binding SRCR domains, WC1 likely engages with a large number of diverse ligands.

Awareness of specific ligands recognized by the γδ TCR is equally essential to understanding the mechanisms behind γδ T cell activation. Antigen recognition by γδ T cells is not bound by the requirement of MHC presentation, allowing them to recognize intrinsic pathogenic molecules that are unlikely to mutate. This feature of γδ T cells makes them an attractive target for next-generation vaccines, especially when considering protection strategies against pathogens that exhibit broad strain and serotype diversity. Several ligands have been identified for bovine γδ T cells, including the *Mycobacterial* cell wall component mycolylarabinogalactan-peptidoglycan (mAGP) [68,69]. Bovine γδ T cells were also found to respond to the peptide antigens major surface protein 2 (MSP2) from *Anaplasma marginale* and those derived from the mycobacterial protein complex ESAT6:CFP10 in *in vitro* recall responses [69,74–76]. There is a gap in the current knowledge concerning specific ligands of porcine γδ T cells. While they may recognize some of the

same ligands engaged by bovine γδ T cells, more work is needed to confirm this. γδ T cells have been shown to interact with unconventional antigens, including MHC-related T22 in mice, MHC class I polypeptide-related sequence (MIC) in humans, and the lipid-presenting MHC CD1d molecules [59,62,252]. Human γδ T cells also respond indirectly to phosphoantigens (pAgs) like endogenous isopentenyl pyrophosphate (IPP) and (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) [253,254].

Another feature of γδ T cells which demonstrates their influence in the adaptive immune response is their ability to function as APCs. Activated bovine γδ T cells have been shown to present antigen to αβ T cells [197]. $WC1^+$ bovine γδ T cells upregulate MHC II, and co-stimulatory molecules CD80 and CD86 and induce $CD4^+$ T cell proliferation following FMD infection [65]. Stimulation of bovine peripheral blood γδ T cells with *M bovis* Bacillus Calmette-Guerin (BCG) is followed by upregulation of MHC II, CD80, and CD86 [198]. Additionally, bovine γδ T cells have been shown to respond to *M.* bovis-infected DCs [198]. During this interaction, both cell types were found to influence each other through the secretion of cytokines, with γδ T cells producing IFN-γ and DCs producing IL-12 [198]. A small population of $CD2^+CD8^+$ porcine γδ T cells express professional APC-associated molecules, including MHC class II, CD80/CD40, and CD31 [220]. Moreover, this population of γδ T cells is capable of antigen uptake and presentation to $CD4^+$ T cells in a direct cell to cell interaction via MHC class II [220]. Porcine γδ T cells were also found to present viral antigen from ASFV to $CD4^+$ T cells [26–28]. Additionally, co-culture of porcine γδ T cells with CSFV and dendritic cells is followed by the upregulation of MHC II surface expression by γδ T cells [36]. The upregulation of MHC II

may indicate that the γδ T cells serve as APCs for dendritic cells in this context [36]. The ability of livestock γδ T cells to function as antigen-presenting cells may provide an opportunity to fine-tune the adaptive immune response when engaging these cells with next-generation vaccines.

# CHAPTER 2

# THE WC1 MULTIGENIC ARRAY IN SUS SCROFA

## 2.1 Introduction

In ruminants and swine, γδ T cells constitute a significant portion of lymphocyte populations within the peripheral blood, epithelial tissues, and at sites of inflammation [12–17]. Moreover, γδ T cells in livestock are potent producers of critical "master regulator" cytokines like IFN-γ and IL-17 [24,64,75,168,174,176,255]. Production of these cytokines orchestrates downstream cytokine and chemokine production by other immune cells, thereby shaping the immune response as a whole [64,65]. γδ T cells have demonstrated the ability to retain immunological memory, making them an important asset of the adaptive immune response [207,256,257]. Despite their propensity for immunological memory, γδ T cells exhibit innate-like antigen recognition properties, including the ability to recognize antigen in the absence of presentation via the major histocompatibility complex (MHC) [59–62,195,256–259]. This property enables γδ T cells to recognize peptide and non-peptide antigens (reviewed in [63]) in a TCR-dependent manner [70–73]. For example, the *Mycobacterial* cell wall component mycolic arabinogalactan-peptidoglycan (mAGP) is recognized by bovine γδ T cells, but not αβ T cells [68,69]. Bovine γδ T cells respond to the peptide antigen major surface protein 2 (MSP2) of *Anaplasma marginale,* and those derived from the *Mycobacterial* protein complex ESAT6:CFP10 during *in vitro* recall responses [69,74–76].

γδ T cell subpopulations in swine and ruminants are distinguished by differential expression of the scavenger receptor cystine-rich (SRCR) superfamily member Workshop Cluster 1 (WC1) [12,40–42,83]. Glycoproteins belonging to the SRCR superfamily, including CD163A, CD5, CD6, and DMBT1, are widely expressed on immune cells. SRCR superfamily members are composed of multiple, single exon encoded, extracellular SRCR domains that directly bind to pathogenic molecules and illicit activation of expressing cells [109–112]. WC1 molecules function as hybrid pattern recognition receptors (PRRs) and co-receptors for the γδ TCR through direct binding of their extracellular SRCR domains with pathogenic molecules [47,49]. The PRR and co-receptor activity of WC1 molecules requires phosphorylation of specific serine and tyrosine residues found within the cytoplasmic domain, which occurs following the engagement of extracellular SRCR domains with pathogenic molecules [48,50,125].

WC1 is expressed as a multigenic array with 13 unique genes found in cattle (*WC1-1* to *WC1-13).* Each gene encodes six to eleven extracellular SRCR domains, a type I transmembrane domain, and cytoplasmic signaling domain [107,117]. Variable expression of these gene products divides $WC1^+$ γδ T cells into the serologically defined subpopulations: $WC1.1^+$ and $WC1.2^+$ [44,107,117,119]. Interestingly, bovine $WC1^+$ γδ T cell subpopulations differentially respond to pathogens based upon which WC1 gene products are expressed [13,49,120]. For example, it was found that $WC1.1^+$ γδ T cells produce IFN-γ in response to *Lepstopira borgpetersenii* while $WC1.2^+$ γδ T cell clones produce IFN-γ in response *Anaplasma marginale* [13,43,47,74,164]. Both $WC1.1^+$ and $WC1.2^+$ γδ T cell populations share the same TCR restriction, utilizing genes from only one TCRγ cassette

(Cγ5), while WC1$^{-}$ γδ T cells are not restricted in TCR gene usage [74,121]. Despite their shared restriction in TCR gene usage, differences in pathogen reactivity observed between WC1.1 and WC1.2-type subsets suggest that differential expression of WC1 molecules may encode antigen specificity.

Bovine WC1 plays a role in pathogen recognition and subsequent activation of expressing γδ T cells; thus, it is reasonable to postulate that WC1 homologs in swine function in a similar manner. While cDNA evidence representing partial WC1 gene transcripts has been reported in swine, the total number of functional porcine WC1 genes remains to be determined [53]. The ability of bovine WC1 molecules to bind to pathogens is altered by changes to single amino acid residues within pathogen-binding SRCR domains [49]. Obtaining complete sequences for porcine WC1 genes may allow us to predict the role of individual WC1 molecules regarding the ability of their SRCR domains to bind pathogens. Additionally, little is known about the structure of porcine WC1 genes in terms of extracellular domain architecture, cytoplasmic domain composition, and exon structure. This gap in knowledge makes it impossible to distinguish cDNA sequences derived from unique genes as opposed to allelic polymorphisms.

Characterization of the extracellular SRCR domains in porcine WC1 may help isolate potential ligands and aid in understanding the role of WC1 in the γδ T cell response. Published cDNA evidence for porcine WC1 consists of one full-length cDNA clone (ppWC1) and four partial cDNA clones (p19e25, p23e3, p24e1, p29e1) potentially representing several unique genes [53]. Between published cDNA sequences and predicted

gene models in the assembly (*Sscrofa11.*1), there is no evidence of an eleven extracellular SRCR-containing WC1 gene in swine. The eleven SRCR-containing WC1 genes found in cattle are the result of an internal SRCR domain duplication [53,103]. If swine lack an eleven SRCR-containing WC1 molecule, it will imply that porcine WC1 diverged before bovine WC1. This knowledge will enhance our understanding of the evolutionary relationship between swine and cattle. Before our work of defining the multigenic array of WC1 in the porcine model, the genetic diversity of WC1 molecules expressed on porcine γδ T cells had not been explored. It is now known that the WC1 gene family in swine is polygenic [126]. It remains to be determined if differential WC1 gene expression on porcine γδ T cells influences cytokine production and pathogen responsiveness like what is observed in the bovine model. By defining the WC1 gene family in swine, we have provided a basis for future work to assess the influence of WC1 gene expression on γδ T cell function in swine.

## 2.2 Methods

### 2.2.1 Animals and cells

Blood was be collected from Yorkshire Duroc F1 cross adults and piglets (Parson's Farm in Amherst Massachusetts) by a state veterinarian through the anterior vena cava into heparin. Peripheral blood mononuclear cells (PBMC) from individual animals were isolated by centrifugation over Ficoll-Plaque PLUS columns (GE Healthcare Bio-Sciences, Piscataway, NJ). Total RNA from individual animals was prepared using QIAzol Lysis Reagent (QIAGEN, Germantown, MD) and purified using RNeasy spin columns (RNeasy Mini Kit, QIAGEN).

### 2.2.2 PCR amplification and cloning

Gene-specific reverse primers for 5'RACE PCR were designed to anneal in SRCR domain two to amplify SRCR domain one, signal sequence, and 5'UTR (Table 2.1). Gene-specific forward primers for the 3'RACE were designed to anneal in the intracytoplasmic domain (ICD) and amplify everything downstream through the polyA tail (Table 2.1). cDNA for 5' and 3' RACE PCR was generated from 1 μg of total RNA. SMARTScribe reverse transcriptase was primed with 5' or 3' CDS Primer A, respectively (SMARTer RACE 5'3' Kit, Takara Bio Mountain View, CA). As per instructions, 1 μl of SMARTer II A Oligonucleotide was added to 5'RACE reverse transcription reactions. The universal primer mix (UPM-L) was paired with 5' and 3' Gene Specific Primers (GSP). All GSPs contain an additional fifteen base pair (bp) sequence: 5 - GAT TAC GCC AAG CTT-3', necessary for in-fusion cloning with the pRACE vector. A nested PCR reaction was used to generate 3'RACE products: primary amplification was carried out with primer four, and secondary amplification was carried out with primer 5 (Table 2.1). Cycling parameters of 5' and 3' RACE PCR reactions were as follows: 1 minute 30 seconds at 94°C, 20-40 cycles of 30 seconds at 94°C, 30 seconds at 55-62°C, and 2-3 minutes at 72°, followed by 10 minutes at 68°C. All RACE PCR products were visualized on a 1.5 % agarose DNA gel with ethidium bromide. Bands corresponding to 750-1.2 kb were excised and purified using the NucleoSpin Gel and PCR Clean-up Kit (Takara Bio Mountain View, CA). Products were cloned into the pRACE vector using the In-Fusion HD Cloning Kit (Takara Bio Mountain View, CA) and transformed into commercially prepared DH5α Stellar Competent Cells (Takara Bio

Mountain View, CA). A total of 24 5'RACE clones and 19 3'RACE clones were sequenced commercially via Sanger sequencing (GeneWiz South Plainfield, NJ).

Forward and reverse primers to amplify full-length WC1 cDNA transcripts were designed using the 5'RACE and 3'RACE sequences as a template (Table 2.2). cDNA for RT-PCR was prepared with 700 ng-1 μg of total RNA and oligo dT primed AMV reverse transcriptase (AMV RT kit; Promega, Madison, WI) or random hexamer primed iScript reverse transcriptase (iScript cDNA Synthesis Kit; Biorad, Hercules, CA). All RT-PCR reactions were carried out using Taq Polymerase or DreamTaq Polymerase (ThermoFisher Scientific Waltham, MA). Cycling parameters were as follows; 5 minutes at 95°C, followed by 30 seconds at 95°C, 30 seconds at 55°C and 2 minutes at 68°C for 35 cycles, with an additional 10 minutes at 68°C and 1 minute at 25°C, or 2-minute hot start at 95°C, followed by 30 seconds at 95°C, 30 seconds at 60°C, 4 minutes at 72°C for 35 cycles. All products were visualized on a 1-1.5 % agarose DNA gel with ethidium bromide. Bands between 2.5 and 3kb were excised and purified using QIAEX II Gel Extraction Kit (QIAGEN), ligated into the pCR2.1 or pCR4 topo-vectors with the topo-ta cloning kit (Invitrogen) and transformed into non-commercial DH10β or commercially prepared DH5α *Escherichia coli* (Invitrogen). Thirteen cDNA clones were sequenced commercially by Sanger sequencing (GeneWiz, South Plainfield, NJ).

### 2.2.3 Sequence analysis

Plasmid DNA was sent to Genewiz (South Plainfield, NJ) for sanger sequencing. 5'RACE clones were sequenced with M13 (-21) forward primer, and 3'RACE clones were sequenced with the M13 reverse primer. Sequencing of plasmids containing full-

length WC1 was initially performed using the T7 forward and M13 reverse primers. Subsequent sequencing with sequence-specific internal primers was also performed (Table 2.3). Sequencing trace files were processed in 4Peaks (Nucleobytes). Nucleotide sequences were aligned in Bioedit [260], and consensus sequences were generated manually. WC1 sequences reported here that were derived from cloned 5'RACE and 3'RACE products were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/, see Table 2.4 for accession numbers). WC1 sequences reported here that were derived from standard PCR amplification were also submitted to GenBank (see Table 2.5 for accession numbers).

Phylogenetic trees were generated using the Maximum Likelihood method and JTT matrix-based model in MEGAX [261–263]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model and then selecting the topology with a superior log-likelihood value. Gene versus allelic variation was determined by performing a pairwise analysis on the first SRCR domain of all unique clones using MEGAX with the complete deletion option. [262,263] Amino acid sequences were aligned in Bioedit and converted to .aln files in ClustalX. A minimum pairwise score of 0.03 (or 3 AA differences per 103 AA of the first SRCR domain) was required for classification as a unique gene. A minimum pairwise score of 0.01 (or 1 AA per 103 AA of the first SRCR domain) was required for classification as allelic variation.

Signal and transmembrane sequences were verified using SignalIP-5.0 Server (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), respectively. Other sequences used in this analysis include bovine genes WC1-3, WC1-4, WC1-9, and WC1-11,[107] cDNA clone ppWC1, [53] CD163 (NCIB Gene ID: 397031), and predicted genes CD163L1(NCIB Gene ID:100144477) and LOC100627089 (NCIB Gene ID: 100627089) from the porcine genomic assembly *Sscrofa11.1* (Genbank GCA_000003025.6).

### 2.2.4 Genome annotation

Assembly *Suscrofa11.1* (Genbank GCA_000003025.6), provided by the Swine Genome Sequencing Consortium, was annotated for WC1 using cDNA evidence. Amino acid sequences derived from the acquired cDNA evidence and sequences derived from the thirteen bovine WC1 genes were blasted (NCBI blastp) against *Sscrofa 11.1.* This initial blast yielded hits on eight contigs (102, 965, 1034, 1446, 1733, 2257, 2307) along with chromosome 5 (Table 2.6). The contigs and chromosome 5 were annotated for cDNA transcript evidence using MAKER version 2.31 with soft masking and extra steps to find alternative splicing [264]. Sequences encompassing the eight contigs and chromosome 5 were programmed into the MAKER control file under *Genome* before the initial run. FASTA files containing nucleotide and amino acid sequences derived from cDNA clones were imported into the MAKER control file under *EST evidence* and *protein,* respectively. A file containing the nucleotide sequences of the thirteen WC1 genes found in cattle was used as an alternative test [107].

Ab Initio gene prediction was performed using the SNAP gene finder with an AED threshold of 0.25. SNAP was trained by running MAKER several times with bootstrapping [265]. Briefly: gene predictions were first inferred directly from EST evidence. The accessory script *maker2zff* was used to generate a ZFF-formatted file and the FASTA files needed to train SNAP. Next, a SNAP species parameter/HMM file was generated by running the *hmm-assembler.pl* script. The HMM file was then used in the subsequent MAKER runs, and gene predictions were no longer inferred from cDNA evidence. This process was repeated three times, generating a new HMM file with each MAKER run until a final set of predicted genes was generated. Predicted gene models were visualized using JBrowse and refined manually based on cDNA evidence of UTR, exon boundaries, and ORF's [266]. Manual refinement of predicted gene models included (i) checking the models for the correct exon/intron structure, (ii) initiation and termination codons were identified when present, and (iii) exons were added or removed if it was determined that the coding region in the predicted model was incorrect (Table 2.7). Following manual curation, adjustments were made to the GFF3 file produced by MAKER (outlined in Table 2.8).

## 2.3 Results

### 2.3.1 cDNA evidence for nine porcine WC1 genes

First, we wanted to establish a cDNA library of any WC1 genes that had not been accounted for in the genomic assembly *Sscrofa11.1*. Using 5'RACE PCR, we obtained twenty-four cDNA clones spanning the 5'UTR through SRCR domain two. We set out to determine how many of the 5'RACE clones represented unique WC1 genes. Deduced

amino acid sequences derived from the first SRCR domain of the twenty-four cDNA clones were aligned as described above. Comparison of amino acid sequences revealed nine unique cDNA clones (Figure 2.1A), with the remaining clones representing redundant transcripts. Redundant transcripts were removed from downstream analyses.

Next, a pairwise analysis (described above) was performed on the amino acid sequences of the first SRCR domain of all non-redundant cDNA clones generated using the 5'RACE and previously published cDNA clone ppWC1 (Table 2.9) [53]. Of the nine 5'RACE clones, eight represented unique genes possessing a pairwise score of 0.03 or above, and one clone (5RA_2) represented an allele (removed from downstream analysis). Pairwise analysis of this alignment revealed nine unique genes encoding WC1, which were then designated *ssWC1-1* to *ssWC1-9* (Tabe 2.10). One of these genes corresponded to the ppWC1 clone described by Kanan et al. (Gene ID 100144477) [53].

### 2.3.2 Swine WC1 genes can be subdivided into three types based on SRCR 1

The current porcine genomic assembly *Sscrofa11.1* contains a predicted WC1 gene model (NCIB Gene ID: 100627089), which possesses a d1 SRCR domain at the N-terminus. We initially hypothesized that this was an error because 1) no supporting cDNA evidence existed for this gene, and 2) WC1 genes in cattle all begin with an a1 SRCR domain at the N-terminus [107]. Members of the group B scavenger receptor family possess SRCR domains which contain 6-8 conserved cysteine residues that join to form disulfide bonds [51]. In cattle, N-terminal a1 SRCR domains lack cysteine residues 2 and 7 and thus contain cysteine residues 1, 3, 4, 5, 6, and 8. The remaining SRCR domains, which are not located at the N-terminus (b2, c3, d4, e5, d6, b7, c8, d9, e10, d11) contain

eight conserved cysteine residues [51]. Analysis of the eight unique clones generated using 5'RACE revealed that several clones (5RA_2, 5RA_22, 5RA_26, and 5RA_30, designated ssWC1-1, ssWC-3, ssWC1-4, and ssWC1-2, respectively) had eight cysteine residues present in the first SRCR domain (Figure 2.1A). The presence of cysteines 2 and 7 in the first SRCR domain indicates that they do not represent a1 SRCR domains. It was also hypothesized that the predicted WC1 gene containing an N-terminal d1 domain might represent an eleven SRCR domain-containing WC1 molecule that was missing sequence at the N-terminus. We were able to confirm that this was not the case. Each clone bearing an N-terminal d1 was immediately preceded by the signal sequence, thus confirming that d1 was the most N-terminal SRCR domain present.

Bovine WC1 genes can be subdivided into one of two subtypes, WC1.1 or WC1.2, based on their reactivity with mAbs and amino acid composition of the first SRCR domain (WC1.2-type genes have an additional four amino acids in the N-terminal a1 SRCR domain) [119]. Different molecular forms of WC1 are found on functionally distinct subpopulations of bovine γδ T cells and play an active role in what pathogens $WC1^+$ cells will respond to [13,49,120]. Therefore, it was of interest to us to identify which porcine WC1 genes represented WC1.1-type molecules and which represented WC1.2-type molecules. To classify porcine WC1 genes as WC1.1 or WC1.2 and investigate the potential of an N-terminal d1 SRCR domain, we performed a phylogenetic analysis of the first SRCR domain of each unique 5'RACE clone and cDNA clone ppWC1 (ssWC1-9) [53]. The first SRCR domain of unique 5'RACE clones and cDNA clone ppWC1 were aligned with sequences derived from individual SRCR domains of a WC1.1-type gene, bovine WC1-3

(btWC1-3), and a WC1.2-type gene, bovine WC1-4 (btWC1-4). In the resulting phylogram (Figure 2.1B), the first SRCR domain of clones 5RA_2 (ssWC1-1), 5RA_22 (ssWC1-3), 5RA_26 (ssWC1-4), and 5RA_30 (ssWC1-2) clustered with bovine SRCR domains d4, d6, d9, and d11, but remained on a separate clade, confirming that they represented N-terminal d1 SRCR domains. In contrast, SRCR domain one of clones 5RA_12 (ssWC1-8) and ppWC1 (ssWC1-9) clustered with btWC1-3 SRCR a1, indicating that they represented WC1.1-type genes. SRCR domain one from clones 5RA_1 (ssWC1-6), 5RA_3 (ssWC1-7) and 5RA_35 (ssWC1-5) clustered with btWC1-4 SRCR a1, indicating that they represented WC1.2-type genes. Thus, we had found cDNA evidence to support nine unique WC1 genes in swine (ssWC1-1 to ssWC1-9). Of the nine porcine WC1 genes, four represented d1-WC1 genes, two represented WC1.1-type genes, and three represented WC1.2-type genes.

### 2.3.3 Analysis of swine WC1 gene signal sequence and 5'UTR

Next, we compared the signal sequences and 5'UTR of the three swine WC1 gene types; d1-WC1, WC1.1, and WC1.2. Amino acid sequences representing ssWC1-1 through ssWC1-9 were analyzed using the SignalIP-5.0 server to confirm the presence of a signal sequence and cleavage peptide. Deduced amino acid sequences representing the signal sequences of each WC1 gene were aligned and compared. Comparison of the three gene types revealed that d1-WC1 genes display a unique signal sequence motif, "MQC," as opposed to "MAL," which is seen in WC1.1 and WC1.2 genes (Figure 2.1A). Nucleotide alignment of 5'UTR revealed variation in length and sequence content of d1-WC1 genes compared to WC1.1 or WC1.2-type genes (Figure 2.1C). The signal and 5'UTR sequences were largely conserved within gene types (Table 2.11).

### 2.3.4 Full-length cDNA evidence for porcine WC1

Next, we sought to obtain cDNA evidence representing full-length WC1 genes. We first utilized 3'RACE PCR to obtain sequences spanning the intracytoplasmic domains of porcine WC1. With 3'RACE PCR, we obtained nineteen cDNA clones spanning the intracytoplasmic domain to the polyA tail. Amino acid alignments were performed to eliminate redundant transcripts. Of the nineteen clones, eight represented unique transcripts and were used for further analysis (3RA_5, 3RA_6, 3RA_14, 3RA_17, 3RA_18, 3RA_22, 3RA_40, 3RA_45). Sequences from the eight unique cDNA clones were aligned with sequences derived from the cytoplasmic domain of the predicted d1-WC1 gene in *Sscrofa11.1* (Gene ID: 100627089) and cDNA clone ppWC1 (Figure 2.2A). Based on the resulting phylogram (Figure 2.2B), it was apparent that four of the clones represented d1-WC1 genes (3RA_5, 3RA_17, 3RA_18, and 3RA_22) as they clustered on the same clade as the predicted d1-WC1 gene (Gene ID: 100627089) (Figure 2.2B). Three clones represented WC1.1-type genes (3RA_40, 3RA_6, 3RA_14) as confirmed by their clustering with ppWC1, a WC1.1-type gene (Figure 2.2B). The eighth and final 3'RACE clone (3RA_45) was on its own branch but shared a clade with WC1.1-type genes (Figure 2.2B). Therefore, we hypothesized that this clone represented a WC1.2-type intracytoplasmic domain. Nucleotide alignment of 3' untranslated regions revealed variation in length and sequence content of d1-WC1 genes compared to WC1.1 (Figure 2.2C). We did not have a 3'UTR sequence derived from a WC1.2-type gene, but we hypothesize that the 3'UTR of WC1.2-type genes would vary compared to d1-WC1 and WC1.1-type 3'UTR sequences. Sequences obtained from the 3'RACE were used to design reverse primers for amplifying full-length WC1. These primers were paired with

forward primers designed using sequences derived from the 5'RACE results (Table 2.2). Through standard PCR amplification, we obtained thirteen cDNA clones representing full-length WC1 genes (Table 2.5). We had successfully amplified ssWC1-1, ssWC1-2, ssWC1-3, ssWC1-5, ssWC1-6, ssWC1-7, ssWC1-8 and ssWC1-9. Interestingly, the cDNA clone which represented full-length ssWC1-8 was lacking a transmembrane domain (Figure 2.3A). There are also examples of cDNA-derived transcripts which lack a transmembrane domain in bovine WC1 [107]. These clones may represent a secreted form of the WC1 molecule. Amplification of full-length ssWC1-4 was not successful; thus, we utilized sequence derived from the 5'RACE clone (5RA_26) in downstream analysis. Deduced amino acid sequences derived from the full-length cDNA clones were aligned with sequences representing two eleven SRCR domain-containing bovine WC1 genes (btWC1-3 and btWC1-4) and a six SRCR domain-containing bovine gene (btWC1-11) and phylogenetic analysis were performed (Figure 2.3A and Figure 2.3B). This analysis confirmed that none of the cDNA evidence acquired here represented an eleven SRCR domain-containing molecule. Thus, we had full-length cDNA evidence spanning from the 5'UTR to the 3'UTR of ssWC1-1, ssWC1-2, ssWC1-3, and ssWC1-9 (Figure 2.4). cDNA evidence representing ssWC1-5, ssWC1-6, ssWC1-7, and ssWC1-8 spanned from the 5'UTR through the stop codon (Figure 2.4.).

### 2.3.5 Annotation of WC1 genes in *Sscrofa11.1*

To confirm that we had cDNA evidence for all existing WC1 genes in swine and to determine gene structures, we annotated the genomic assembly *Sscrofa 11.1* using the MAKER annotation pipeline with manual curation [267]. Initially, MAKER generated twelve gene models based on the genomic sequence. Manual polishing of the MAKER

results yielded a total of fifteen gene models based on the genomic sequence (Table 2.12 and Figure 2.5). Deduced amino acid sequences derived from the gene models were aligned with cDNA-derived sequence representing ssWC1-1 to confirm which gene models represented full-length WC1 genes (spanning the initiation methionine to the stop codon), an alignment with cDNA-derived sequence representing ssWC1-1 was generated (Supplemental Figure 2.1). Of the fifteen gene models, four represented full-length genes (C1034-2, C1733-2, C2565-2, and Chr5-1). The remaining models varied in length and content (Figure 2.5). Model C1446-2 was determined to be a pseudogene as analysis of the sequence revealed numerous indels, which induced multiple premature stop codons. Therefore, model C1446-2 was removed from downstream analysis. To confirm the presence of a functional signal peptide in each gene model, which appeared to have a signal peptide, we utilized SignalIP-5.0 Server. The presence of functional signal peptide was confirmed in gene models C965-1, C1446-1, C2257-1, C2565-1, C2565-2 and Chr5-1 (Figure 2.6). The signal peptide of two gene models, C1034-2 and C1733-2 appeared non-functional (Figure 2.6). However, C1034-2 and C1733-2 possessed an open reading frame that encompassed a six SRCR domain WC1 molecule with a transmembrane domain and intracytoplasmic domain. Therefore, we chose to keep these models for downstream analysis to evaluate the possibility of an assembly error.

We next sought to determine which gene models were supported by cDNA evidence. We began this analysis by comparing SRCR domain one, the most unique SRCR domain [107]. Amino acid sequences representing the first SRCR domain of cDNA derived WC1 genes were aligned with SRCR domain one derived from the gene models, and a pairwise

analysis was performed (Figure 2.7A and Table 2.13). A pairwise score of zero corresponds to a 100% match in amino acid identities between the two sequences being compared. Based on the results, we were able to confirm an exact match for ssWC1-2 (C2257-1), ssWC1-4 (C1733-2), ssWC1-5 (C965-1), and ssWC1-6 (C2307-1) (Table 2.13). Interestingly, ssWC1-1 had an identical pairwise score with two gene models (C1034-2 and C2565-1); therefore, further interrogation was needed to confirm a match. While ssWC1-9 was not an exact match for gene model Chr5-1, the low pairwise score of 0.0396 indicated a high level of shared amino acid identity between the two sequences (Table 2.13).

Next, we compared full-length gene models with full-length cDNA evidence. There were two instances in which partial gene models were split across contigs. In the first instance, gene model C2257-1 (comprised of 5'UTR through the end of SRCR domain one) could be contigged with gene model 2565-3 (which spanned from SRCR domain two through the 3'UTR). In the second instance, gene model C965-1 (comprised of 5'UTR through SRCR domain five) could be contigged with gene model C1733-1 (which consisted of SRCR domain six through the 3'UTR). The models were combined to generate full-length WC1 gene models (C2257-1_C2565-3 and C965-1_C1733-1), and the resulting sequences were used in downstream analysis. Amino acid sequences representing full-length WC1 genes derived from cDNA was aligned with sequence representing Chr5-1, C2565-2, C2257-1_C2565-3, and C965-1_C1733-1, and a pairwise analysis was performed (Figure 2.8A and Table 2.14). The resulting phylogram and (Figure 2.8B) and pairwise scores confirmed the presence of ssWC1-9 (Chr5-1). While model C2565-2

shared a clade with ssWC1-5, we determined this did not represent a match because SRCR domain one of C965-1 shared 100% identity with SRCR domain one of ssWC1-5 in the previous analysis (Figure 2.8B). Finally, we compared the intracytoplasmic domain (ICD) sequences. Amino acid sequence derived from the ICD of cDNA derived WC1 genes were aligned with ICD sequence derived from the gene models (Figure 2.9A). The resulting phylogram (Figure 2.9B) confirmed the presence of ssWC1-3 (C102-2).

After this analysis, we confirmed the presence of eight of the nine cDNA-supported genes, ssWC1-1, ssWC1-2, ssWC1-3, ssWC1-4, ssWC1-5, ssWC1-6, ssWC1-7, and ssWC1-9 in the current assembly (Figure 2.10A, B, C and Table 2.15). While cDNA evidence for ssWC1-8 exists, this gene remains unplaced in this assembly. We generated four full-length gene models (Chr5-1, C2565-2, C965-1_C1733-1, and C2257-1_C2565-3), four incomplete models that were supported by cDNA evidence (C2565-1, C102-1, C102-2 and C2307-1), and one confirmed pseudogene (C1446-2). Despite the lack of cDNA evidence, full-length gene model C2565-2 possessed a viable open reading frame and was determined to represent an additional WC1 gene (ssWC1-10). Gene model 2565-1 represented a d1 WC1 gene spanning from the 5'UTR through ICD exon three. Because this model contained functional signal peptide and a clean open reading frame, we determined that it represented an additional WC1 gene that had not been accounted for by cDNA evidence (ssWC1-11). There was also an incomplete gene model that lacked cDNA evidence (C1446-1), but it was not classified as a pseudogene because it possessed a functional signal peptide. However, because gene model 1446-1 only spanned the
5'UTR through SRCR domain three, it was not wincluded in our analysis. Additionally,

there was one gene model that lacked supporting cDNA evidence and a functional signal peptide. Gene model 1034-1 represented a WC1.2-type molecule that contained a partial d6 SRCR domain and spanned through the end of ICD exon number 5.

### 2.3.6 Porcine WC1 gene structure

We anticipated at least three unique gene structures in swine, corresponding to the three types of genes that we had confirmed through analysis of cDNA sequences: d1-WC1, WC1.1, and WC1.2. Following the annotation, we identified four unique exon-intron structures for swine WC1 genes (Type I, Type II, Type III and Type IV) (Figure 2.11A, B, C, and D). Consistent with previous findings in cattle, SRCR domains were encoded by a single exon. Primary distinguishing features between the gene structures include the number of exons and total coding region length. Type I and Type IV gene structures are encoded by 14 exons but vary in coding region length: 16,456 bp and 28,792 bp, respectively. Type II and Type III gene structures are encoded by 15 exons and are 22,724 bp and 25,770 bp, respectively.

While d1-WC1 genes ssWC1-1 and ssWC1-4 possess Type I gene structures, ssWC1-2 and ssWC1-11 are Type II. In addition to coding region length and exon number, Type I and Type II gene structures differ in the contents of their first and second exons. In the Type, I structure, the 5'UTR, signal sequence, and SRCR domain one is all contained within the first exon. In contrast, the signal sequence of the Type II gene structure is split between exons one and two. Additionally, SRCR domain one is found within exon two in the Type II structure. Genomic evidence representing the d1-WC1 gene ssWC1-3 lacks 5'UTR, signal sequence, and SRCR domain one. Therefore, the gene structure of ssWC1-

3 could not be determined. The WC1.2-type genes, ssWC1-5, ssWC1-6, ssWC1-7, and ssWC1-10, possess the Type IV gene structure. Finally, the WC1.1-type gene ssWC1-9 represents a Type III gene structure. The gene structure of ssWC1-8 could not be determined because this gene was not placed in the current genomic assembly.

### 2.3.7 Intracytoplasmic domains

Signaling through transmembrane proteins involves the binding of an extracellular signal which is then translated to intracellular signals. Colligation of bovine WC1 with γδ TCR/CD3 transduces a positive signal dependent upon phosphorylation of a tyrosine residue in the cytoplasmic domain of WC1 [48,120,125]. The cytoplasmic domains of bovine WC1 can be divided into three groups (Type I, Type II, and Type III) encoded by 4, 5, or 6 exons, respectively [107]. Type I and II cytoplasmic domains contain five tyrosine residues ($Y_{15}$EDA, $Y_{24}$EEL, $Y_{29}$LLT, $Y_{70}$DDA, and $Y_{138}$DDV), and phosphorylation of the second tyrosine is required for WC1 potentiation of T cell activation through the TCR [48,125]. The bovine Type III cytoplasmic domain contains eight tyrosine residues total, three of which are conserved with those found in Type I and Type II cytoplasmic domains ($Y_{24}$EEL, $Y_{70}$DDA, and $Y_{138}$DDV) but diverge by 1-2 amino acids in Type III ($Y_{24}$EEL is $Y_{24}$QEI and $Y_{70}$DDA is $Y_{70}$DDV) [48,107]. In addition to the three conserved tyrosine residues, the Type III cytoplasmic domain possesses five unique tyrosine residues ($Y_{55}$YTG, $Y_{56}$TGD, $Y_{84}$DDV, $Y_{116}$SQT, $Y_{199}$DDV) [48,107]. In Type III cytoplasmic domains, phosphorylation occurs on the last tyrosine residue [48].

To characterize porcine WC1 cytoplasmic domains, we compared them with bovine WC1 cytoplasmic domains. Non-redundant cytoplasmic domains derived from porcine WC1

genes were aligned with bovine WC1 cytoplasmic domains Type I, II, and III (Figure 2.12A). The resulting phylogram showed that the Type III porcine cytoplasmic domain clustered with bovine Type III (Figure 2.12B). From this, we concluded that there are four types of cytoplasmic domains found in porcine WC1 (Type III, IV, V, and VI), which are defined by amino acid sequence, length, and the number of tyrosine residues (Table 2.16). Based on the available genomic evidence, we determined that five exons encode cytoplasmic domains Type III, IV, and V. Cytoplasmic domain VI is derived from the porcine WC1 gene ssWC1-8. This gene was unplaced in the current assembly; thus, genomic sequence to determine the number of exons was unavailable. Like bovine WC1 cytoplasmic domains, which possess tyrosine residues that are phosphorylated to evoke co-receptor activity, porcine WC1 cytoplasmic domains contain multiple tyrosine residues which are potentially phosphorylated (Figure 2.12A). The porcine Type III intracytoplasmic domain has eight tyrosine residues, six of which are organized into phosphorylation motifs. The Type IV intracytoplasmic domain has six tyrosine residues, five of which are organized into phosphorylation motifs. The Type V intracytoplasmic domain has six tyrosine residues, four of which are organized into phosphorylation motifs. Finally, the Type VI intracytoplasmic domain has five tyrosine residues, four of which are organized into phosphorylation motifs. While it remains to be determined if these residues are phosphorylated upon activation of porcine WC1, we predict that this is the case as it is what we observe in cattle. To get an idea of potential phosphorylation sites and theoretical kinases that may phosphorylate these sites, we utilized the NetPhos 3.1 server. NetPhos 3.1 is a server that predicts tyrosine phosphorylation sites in eukaryotic proteins. The results from this analysis are summarized in Table 2.17.

## 2.4 Discussion

Prior to this work, there was limited cDNA evidence for porcine WC1 genes; thus, we were unsure of how many genes comprised this family. Published evidence consisted of one full-length cDNA clone, ppWC1, and several partial cDNA clones which had not been assigned gene numbers. Through 5'RACE, 3'RACE, and standard PCR analysis, we obtained a plethora of cDNA evidence that was used to define the multigenic array of porcine WC1. Through phylogenic and pairwise analysis, we concluded that our cDNA evidence supported the existence of nine unique WC1 genes in swine. One of these genes, ssWC1-8, did not contain a transmembrane sequence. This could potentially represent a secreted form of porcine WC1. However, we only had one full-length cDNA clone representing ssWC1-8, and this gene was not placed in the genomic assembly; therefore, we cannot say if the lack of a transmembrane domain is due to the amplification of a splice isoform or if ssWC1-8 is strictly expressed as a secreted molecule. There is evidence for bovine WC1 splice isoforms which lack a transmembrane domain [107]. Secreted forms of WC1 molecules may exhibit antimicrobial properties like the related SRCR family member DMBT1. Moreover, it has been shown that soluble bovine WC1 SRCR domains inhibit *Leptospira* growth *in vitro* [49].

Through comparison with bovine WC1, it was determined that four of the nine genes possess an N-terminal d1-SRCR domain (ssWC1-1, ssWC1-2, ssWC1-3, and ssWC1-4), which is not present in bovine WC1. Of the remaining genes, two were classified as WC1.1-type (ssWC1-8 and ssWC1-9), and three were classified as WC1.2-type (ssWC1-5, ssWC-6, and ssWC1-7). While gene duplication is natural process that occurs

throughout evolutionary time, it is expected that duplicated genes will acquire missense mutations rendering them pseudogenes, unless they provide some advantage to the organism. We know that WC1 molecules differentially bind to pathogenic molecules based on discontinuous amino acid residues dispersed throughout their SRCR domains [49]. We hypothesize that WC1 molecules have coevolved with different pathogens, thus providing the organism with γδ T cells that are responsive to a broad array of pathogens. The absence of a d1 SRCR domain containing WC1 molecule in cattle may indicate that WC1 molecules containing a d1 SRCR domain respond to a pathogen, or set of pathogens, that do not infect cattle.

Through annotation of *Sscrofa11.1,* we confirmed the presence of seven of the nine cDNA-supported genes, ssWC1-1, ssWC1-2, ssWC1-3, ssWC1-4, ssWC1-5, ssWC1-6, and ssWC1-7, in the current assembly. While cDNA evidence for ssWC1-8 exists, this gene remains unplaced in *Sscrofa11.1*. Genome annotation also revealed genomic evidence for two additional genes, ssWC1-10 (C2565-2) and ssWC1-11 (C2565-1), which were not accounted for by cDNA evidence. We identified one pseudogene, C1446-2. In addition, genomic evidence for one incomplete WC1 gene was found, C1446-1, and it remains to be determined if this represents a pseudogene or an expressed transcript. One way to confirm this would be to design PCR primers based on the genomic sequence and attempt to amplify the transcript in question.

Analysis of exon/intron structures derived from genome annotation revealed four unique gene structures for porcine WC1. The gene structures vary in length and exon/intron

architecture, but all possess six SRCR domains encoded by single exons. We did not find any evidence to support the existence of an eleven SRCR domain molecule in swine. This aligns with the hypothesis that porcine WC1 represents a more ancient molecular form from which bovine WC1 diverged. This divergence is evidenced by the duplication event, which occurred in bovine WC1 only.

We determined that there are four types of cytoplasmic domains found in porcine WC1: Type III, IV, V, and VI. We had cDNA evidence supporting all cytoplasmic domain types and genomic evidence for Type III, IV, and V. Through comparisons with the genomic evidence, it was determined that five exons encode cytoplasmic domain Type III, IV, and V. While the cytoplasmic domain Type VI is supported by cDNA evidence, there was no available genomic evidence. Therefore, the number of exons encoding the Type VI cytoplasmic domain remains to be determined.

In cattle, WC1 co-receptor activity is dependent on phosphorylation of a tyrosine residue within the cytoplasmic domain [125]. We are interested in the ability of porcine WC1 to function as a co-receptor. As a prelude to investigating co-receptor activity, we wanted to identify potential sites of tyrosine phosphorylation in the intracytoplasmic domains of porcine WC1. We found that porcine WC1 cytoplasmic domains possess multiple tyrosine residues that may be phosphorylated during an activation event. The porcine Type III intracytoplasmic domain possesses eight tyrosine residues, and of these six are organized into tyrosine phosphorylation motifs ($Y_{24}$QEID, $Y_{68}$APEP, $Y_{84}$DDVE, $Y_{105}$FSTE, $Y_{116}$SQTG, and $Y_{150}$DDVE). Four of the six porcine Type III phosphorylation

motifs ($Y_{24}$QEID, $Y_{84}$DDVE, $Y_{116}$SQTG, and $Y_{150}$DDVE) are also found within bovine WC1 cytoplasmic domain Type III molecules. The Type IV intracytoplasmic domain has six tyrosine residues, five of which are organized into phosphorylation motifs ($Y_{24}$EEID, $Y_{29}$LVTP, $Y_{54}$YTGE, $Y_{84}$DDAE, and $Y_{151}$DDVE). Of the five phosphorylation motifs found within porcine Type IV intracytoplasmic domains, four are found within bovine Type I and II intracytoplasmic domains ($Y_{24}$EEID, $Y_{29}$LVTP, $Y_{84}$DDAE, and $Y_{151}$DDVE), while the fifth ($Y_{54}$YTGE) is found in the bovine Type III intracytoplasmic domain. The porcine Type V intracytoplasmic domain has six tyrosine residues, four of which are organized into phosphorylation motifs ($Y_{30}$EEID, $Y_{60}$YTGE, $Y_{90}$DDAE, and $Y_{157}$DDVE). Of the four tyrosine phosphorylation motifs found in the porcine Type V intracytoplasmic domain, three are found within bovine Type I and II intracytoplasmic domains ($Y_{30}$EEID, $Y_{90}$DDAE, and $Y_{157}$DDVE) while the fourth ($Y_{60}$YTGE) is found in the bovine Type III intracytoplasmic domain. Finally, the Type VI intracytoplasmic domain has five tyrosine residues, four of which are organized into phosphorylation motifs ($Y_{24}$EEID, $Y_{29}$LVTP, $Y_{54}$TGE and $Y_{84}$DDAE). Of the four tyrosine phosphorylation motifs found in the porcine Type VI intracytoplasmic domain, three are found within bovine Type I and II intracytoplasmic domains ($Y_{24}$EEID, $Y_{29}$LVTP, and $Y_{84}$DDAE) while the fourth ($Y_{54}$TGE) is found in the bovine Type III intracytoplasmic domain.

Phosphorylation by src kinase family members is required for intracellular signaling events following ligation of cell accessory molecules in T cells. In bovine intracytoplasmic domains, specific tyrosine residues are preferentially phosphorylated

upon co-ligation of extracellular SRCR domains with the γδ TCR [48,125]. In bovine Type I and II intracytoplasmic domains, the second tyrosine residue is the main target of phosphorylation [48,125]. In contrast, the last tyrosine residue of the bovine Type III intracytoplasmic domain is phosphorylated. In COS-7 and HEK-293 cotransfection systems, as well as in Jurkat T cells, the second tyrosine residue of bovine Type I and II intracytoplasmic domains was phosphorylated by members of the src family of tyrosine kinases [125,268]. Furthermore, an association between WC1 and src family kinase members was demonstrated by coimmunoprecipitation of the two components using either anti-WC1 or anti-src Ab [125]. It was later shown that src tyrosine kinases were constitutively expressed in HEK-293 cells and sorted $WC1^{+}$ γδ T cells and cotransfection of members of the src family tyrosine kinases enhanced phosphorylation levels of WC1 intracytoplasmic domains [125,268]. Phosphorylation of the last tyrosine residue in the bovine Type III intracytoplasmic domain ($Y_{199}$DDV) functionally substitutes for the phosphorylation of the second tyrosine residue ($Y_{24}$EEL) in type I and Type II intracytoplasmic domains [48]. The second tyrosine residue found in bovine Type III intracytoplasmic domains differs from that in Type I and Type II intracytoplasmic domains by amino acid substitutions of glutamic acid to glutamine and leucine to isoleucine within the $Y_{24}$EEL. The same substitutions are also observed in the porcine Type III intracytoplasmic domain. Upon mutation of the last tyrosine residue ($Y_{199}$DDV) in bovine Type III intracytoplasmic domains, the $Y_{24}$QEI motif is as readily phosphorylated by src tyrosine kinases as the $Y_{24}$EEL motif in Type I and II intracytoplasmic domains [48]. Once phosphorylated, the $Y_{24}$EEL is bound by the SH2 domain of lck [269]. The failure of $Y_{24}$QEI to be phosphorylated under normal

circumstances, and the preferential phosphorylation $Y_{199}$DDV of in the bovine Type III intracytoplasmic domain is most likely due to conformational changes induced by the additional 80 amino acids found within the Type III intracytoplasmic domain [48].

Src tyrosine kinase SH2 domains and the adaptor protein Shc likely bind phosphorylated $Y_{24}$EEL in bovine Type I and II intracytoplasmic domains [270]. In contrast, phosphorylated $Y_{199}$DDV in the type III intracytoplasmic domain likely binds the SH2 domain of the adaptor proteins SLP-76 or Nck [271,272]. A similar dichotomy is observed in porcine intracytoplasmic domains, where all amino acid residues encompassed within each phosphorylation motif likely contribute to adaptor protein recruitment and binding. Adaptor proteins possess multiple protein-binding sites which bring respective binding partners wiihin physical proximity to one another. The function of adaptor proteins is to facilitate the generation of larger signaling complexes. Therefore, recruitment of specific adaptor proteins influences downstream signaling events, which dictate the result of activation. Furhter interrogation is needed to determine which tyrosine residues are phosphorylated in porcine WC1 intracytoplasmic domains, and which adaptor proteins are recruited as a result.

Table 2.1 Primers used in 5'RACE and 3'RACE

| Primer | Orientation | Sequence (5'→3') |
|---|---|---|
| 1 | rev | GATTACGCCAAGCTTCGCCCTGAACAGGGTCCATTCCCTTGA |
| 2 | rev | GATTACGCCAAGCTTCCTCACATCGGTGGCGTCCATCCTTCA |
| 3 | rev | GATTACGCAAGCTTCGCCCTTGAGCCGGACTTCTGAGTATGC |
| 4 | fwd | GATTACGCCAAGCTTGCATGAGGAGGACGCTGGAGTGAGGT |
| 5 | fwd | GATTACGCCAAGCTTCTCCTGGAGCCTGGCAGAGGCCGAGGTG |

Table 2.2 Primers used to amplify full length WC1 genes

| Primer[1] | Orientation | Sequence (5'→3') | Gene |
|---|---|---|---|
| 1 | fwd | AATTATGAATTCATGCAGTGCTCTCTCCAAGGAC | pan-d1WC1 |
| | rev | AATTATCTCGAGTCAAAGTGTCTCTTGCTTCAAAAAG | |
| 2 | fwd | GAACCGGAATTCACCTGAGGCTGAAGGATGGACGC | ssWC1-6 |
| | rev | AATTATCTCGAGTCATGGGAAAGCCACCGTGGAGGC | |
| 3 | fwd | GAACCGGAATTCACCTGAGGCTGAAGGATGGACG | ssWC1-5 |
| | rev | GATCTCGAGGTATCTGAATCTTCGTCTCATATCATTACAACA | |
| 4 | fwd | GCAATACAGAGCCAGAATCACCTTTCACTATCC | ssWC1-7 |
| | rev | AATTATCTCGAGTCATGGGAAAGCCACCGTGGAGGC | |
| 5 | fwd | CTGAGGCTGAAGGATGGAAGGCACCGCC | ssWC1-8 |
| | rev | TCATGGGAAAGCTACGGTGAATGTTCCAGGAGCAC | |
| 6 | fwd | TATGAATTCACAATGGGGGCAGTCGATGCACAG | ssWC1-4 |
| | rev | AATTATCTCGAGTCAAAGTGTCTCTTGCTTCAAAAAG | |
| 7 | fwd | ATGGCTCTGGACAGACATCTCTCT | ssWC1-9 |
| | rev | TCATGGGAAAGCTACGGTGAAT | |

[1] Refers to primer sets which include the listed forward and reverse primers; fwd = forward primer; rev = reverse primer

Table 2.3 Primers used for internal sequencing reactions

| Primer | Orientation | Sequence (5'→3') | Type[1] | Target |
|---|---|---|---|---|
| 1 | fwd | CTCAGGTCATCTGTGTAGAG | d1-WC1 | SRCR b2 |
| 2 | fwd | CTCAGGTCATCTGTGTAGAG | d1-WC1 | SRCR b2 |
| 3 | fwd | CTGAAGAGTTCAGGTGTAAG | d1-WC1 | SRCR b2 |
| 4 | fwd | CATAGAAGCCAAGGTGTTAG | d1-WC1 | SRCR b2 |
| 5 | rev | TCCAGGAGTCATCACACA | d1-WC1 | SRCR d6 |
| 6 | rev | ATAACGAGGACCAGGAAG | d1-WC1 | TM[2] |
| 7 | fwd | GACTCGGCTGCCATATTA | d1-WC1 | ICD[3] |
| 8 | fwd | GATGGAGAACACTCTGTG | WC1.1 | SRCR d4 |
| 9 | rev | CACTCTCCCTGAGCAATAA | WC1.1 | SRCR d4 |
| 10 | fwd | GAGTTATTGCTCAGGGAGAG | WC1.1 | SRCR d4 |
| 11 | fwd | TGAAGAGTTCAGGTGTAAGG | WC1.2 | SRCR b2 |
| 12 | fwd | TTCTGGAGGATGGAGAAC | WC1.2 | SRCR c3 |
| 13 | fwd | GTGAACTGCACAGGAAAG | WC1.2 | SRCR d4 |
| 14 | fwd | CCTGGGAAAGGAGAAGATA | WC1.2 | TM[2] |
| 15 | rev | ATCCCACTCTCCTCTTCT | WC1.2 | TM[2] |

[1] Refers to the gene's most distal SRCR domain (d1-WC1, WC1.1-type and WC1.2-type) that the primer anneals to. Some primers are pan-reactive between two or more gene types due to the conserved nature of WC1 SRCR domains.

[2] TM = transmembrane domain

[3] ICD = intracytoplasmic domain

Table 2.4 cDNA clones derived from 5'RACE and 3'RACE PCR[1]

| GenBank Accession # | cDNA Clone | Gene Description[2] |
|---|---|---|
| MZ686971 | 5RA_1 | WC1-6 |
| MZ686973 | 5RA_2 | WC1-2 |
| MZ686972 | 5RA_3 | WC1-7 |
| MZ686974 | 5RA_4 | WC1-2 |
| MZ686975 | 5RA_8 | WC1-1 |
| MZ686976 | 5RA_9 | WC1-1 |
| MZ686977 | 5RA_13 | WC1-8 |
| MZ686978 | 5RA_14 | WC1-1 |
| MZ686979 | 5RA_17 | WC1-8 |
| MZ686980 | 5RA_12 | WC1-8 |
| MZ686981 | 5RA_18 | WC1-5 |
| MZ686982 | 5RA_21 | WC1-1 |
| MZ686983 | 5RA_22 | WC1-3 |
| MZ686984 | 5RA_24 | WC1-1 |
| MZ686985 | 5RA_26 | WC1-4 |
| MZ686986 | 5RA_27 | WC1-1 |
| MZ686987 | 5RA_28 | WC1-1 |
| MZ686988 | 5RA_29 | WC1-5 |
| MZ686989 | 5RA_30 | WC1-2 |
| MZ686990 | 5RA_31 | WC1-1 |
| MZ686991 | 5RA_35 | WC1-5 |
| MZ686992 | 5RA_36 | WC1-6 |
| MZ686993 | 5RA_34 | WC1-6 |
| MZ686994 | 5RA_6B | WC1-5 |
| MZ686995 | 3RA_1 | d1-type |
| MZ686996 | 3RA_2 | d1-type |
| MZ686997 | 3RA_4 | WC1.1-like |
| MZ686998 | 3RA_5 | d1-type |
| MZ686999 | 3RA_8 | d1-type |
| MZ687000 | 3RA_12 | d1-type |
| MZ687001 | 3RA_13 | d1-type |
| MZ687002 | 3RA_40 | WC1.1-like |
| MZ687003 | 3RA_6 | WC1.1-like |
| MZ687004 | 3RA_24 | d1-type |
| MZ687005 | 3RA_23 | d1-type |
| MZ687006 | 3RA_22 | d1-type |
| MZ687007 | 3RA_18 | d1-type |
| MZ687008 | 3RA_17 | d1-type |
| MZ687009 | 3RA_15 | d1-type |
| MZ687010 | 3RA_14 | WC1.1-like |
| MZ687011 | 3RA_28 | WC1.2-like |

[1] 5RA = derived from 5'RACE; 3RA = derived from 3'RACE

[2] Either the gene name as assigned, or if unnumbered the type of gene structure represented

Table 2.5 cDNA clones derived from standard PCR

| GenBank Accession # | cDNA Clone | Gene Description[1] | Primer Set[2] |
|---|---|---|---|
| MZ687012 | 1.1FL_1 | WC1-8 | 5 |
| MZ687013 | 1.1FL_4 | WC1-8 | 5 |
| MZ687014 | 1.2FL_4 | WC1-6 | 2 |
| MZ687014 | 1.2FL_22 | WC1-5 | 3 |
| MZ687015 | 1.2FL_23 | WC1-5 | 3 |
| MZ687000 | FL_38 | WC1-2 | 1 |
| MZ687018 | FL_43 | WC1-1 | 1 |
| MZ687019 | FL_46 | WC1-3 | 1 |
| MZ687020 | FL_55 | WC1-3 | 1 |
| MZ687021 | FL_59 | WC1-3 | 1 |
| MZ687022 | FL_60 | WC1-2 | 1 |
| MZ687023 | FL_70 | WC1-9 | 7 |
| MZ687024 | G7_FL7 | WC1-7 | 4 |

Table 2.6 *Sscrofa11.1* scaffolds annotated for WC1

| Scaffold | Contig | Size (bp) |
|---|---|---|
| NW_18084797.1 | 1034 | 37,252 |
| NW_018085127.1 | 2565 | 83,331 |
| NW_018084793.1 | 102 | 36,784 |
| NW_018085361.1 | 965 | 33,397 |
| NW_018084937.1 | 1733 | 35,698 |
| NW_018085069.1 | 2307 | 34,187 |
| NW_018085057.1 | 2257 | 22,707 |
| NW_018084880.1 | 1446 | 39,765 |
| NC_010447.5 | Chr5 | 104,526,007 |

[1] Gene name as assigned, or if unnumbered, the type of gene structure
[2] Refers to primer sets defined in Table 2.2

Table 2.7 Mutations observed in genomic sequence

| Annotated Gene | Exon | Mutation | Position (bp) |
|---|---|---|---|
| C102-1 | 1 | 1 bp deletion | 2769 |
| C102-1 | 1 | G > C | 2921 |
| C102-1 | 2 | 2 single bp deletions | 3085, 3159 |
| C102-2 | 7 | C > T | 26260 |
| C1034-1 | 1 | 5 single bp deletions | 395, 435, 442, 464,502, 512, 520 |
| C1034-1 | 2 | 1 bp deletion | 1455 |
| C1034-2 | 4 | 1 bp deletion | 21361 |
| C1034-2 | 5 | 2 bp deletion | 21115, 21114 |
| C1034-2 | 5 | 6 single bp deletions | 21068, 21006, 20900, 20985, 20976, 20956 |
| C1034-2 | 6 | 1 bp deletion | 19895 |
| C1034-2 | 8 | 1 bp deletion | 17892 |
| C1034-2 | 10 | 1 bp deletion | 17096 |
| C1446-1 | 1 | 1 bp deletion | 23748 |
| C1446-1 | 2 | 2 single bp deletions | 30703, 30747 |
| C1446-1 | 4 | 2 single bp deletions | 37124, 37198 |
| C1733-1 | 2 | 1 bp deletion | 634 |
| C1733-1 | 5 | 1 bp deletion | 2466 |
| C1733-1 | 7 | 2 single bp deletions | 4725, 4877 |
| C1733-2 | 2 | 1 bp deletion | 23650 |
| C2565-1 | 3 | 1 bp deletion | 7639 |
| C2565-1 | 4 | 1 bp deletion | 7325 |
| C2565-1 | 6 | 1 bp deletion | 5440 |
| C2565-1 | 7 | 1 bp deletion | 4197 |
| C2565-1 | 11 | 1 bp deletion | 1539 |
| C2565-2 | 1 | 1 bp deletion | 35570 |
| C2565-2 | 2 | 1 bp deletion | 42535 |
| C2565-2 | 5 | 1 bp deletion | 54078 |
| C2565-3 | 2 | 6 single bp deletions | 82496, 82518, 82536, 82559, 82574, 82654 |
| C2565-3 | 2 | G > A | 82502 |

Table 2.8 Modifications to MAKER GFF3 File

| **Gene Model C102-1** | | | | |
|---|---|---|---|---|
| | Polished Coordinates | | Original GFF3 File Coordinates | |
| Exon | Start | Stop | Start | Stop |
| 1 | 2753 | 2845 | 2764 | 3091 |
| 2 | 2927 | 3239 | 3159 | 3239 |
| 3 | 4019 | 4332 | 4019 | 4375 |
| 4 | 8176 | 8215 | 8274 | 8285 |
| 5 | 8307 | 8630 | 8307 | 8630 |
| 6 | 9475 | 9593 | - | - |
| 7 | 10010 | 10102 | 10009 | 10101 |
| 8 | 10369 | 10453 | 10369 | 10419 |
| 9 | 11353 | 11498 | - | - |
| 10 | 12589 | 12849 | - | - |
| **Gene Model C102-2** | | | | |
| | Polished Coordinates | | Original GFF3 File Coordinates | |
| Exon | Start | Stop | Start | Stop |
| 1 | 32573 | 32882 | 32573 | 32886 |
| 2 | 32176 | 32483 | 32176 | 32483 |
| 3 | 30360 | 30452 | 30360 | 30452 |
| 4 | 29956 | 30271 | 29957 | 30271 |
| 5 | 28018 | 28334 | 28020 | 28334 |
| 6 | 26667 | 26708 | 26667 | 26708 |
| 7 | 26256 | 26575 | 26261 | 26575 |
| 8 | 26001 | 26029 | 26001 | 26034 |
| 9 | 25471 | 25607 | 25471 | 25608 |
| 10 | 24965 | 25060 | 24965 | 25060 |
| 11 | 24676 | 24676 | 24676 | 24676 |
| 12 | 23483 | 23629 | 23483 | 23629 |
| 13 | 22205 | 22462 | 22267 | 22462 |
| **Gene Model C965-1** | | | | |
| | Polished Coordinates | | Original GFF3 File Coordinates | |
| Exon | Start | Stop | Start | Stop |
| 1 | 24113 | 24239 | - | - |
| 2 | 17106 | 17435 | 17106 | 17437 |
| 3 | 11084 | 11392 | 11084 | 11392 |
| 4 | 10685 | 10991 | 10685 | 10993 |
| 5 | 5070 | 5162 | 5070 | 5162 |
| 6 | 4673 | 4988 | 4674 | 4988 |
| 7 | 3578 | 3891 | 3578 | 3891 |
| **Gene Model C1034-1** | | | | |
| | Polished Coordinates | | Original GFF3 File Coordinates | |
| Exon | Start | Stop | Start | Stop |
| 1 | 353 | 591 | - | - |

| | | | | |
|---|---|---|---|---|
| 2 | 1402 | 1520 | - | - |
| 3 | 1932 | 2024 | - | - |
| 4 | 2293 | 2379 | - | - |
| 5 | 3210 | 3357 | - | - |
| 6 | 4464 | 4610 | - | - |
| **Gene Model 1034-2** | | | | |
| | Polished Coordinates | | Original GFF3 File Coordinates | |
| Exon | Start | Stop | Start | Stop |
| 1 | 29905 | 30340 | 29905 | 30340 |
| 2 | 23420 | 23728 | 23420 | 23728 |
| 3 | 23022 | 23330 | 23022 | 23330 |
| 4 | 21327 | 21418 | 21326 | 21418 |
| 5 | 20938 | 21245 | 20938 | 21244 |
| 6 | 19600 | 19919 | 19600 | 19913 |
| 7 | 18253 | 18253 | 18253 | 18253 |
| 8 | 17848 | 18161 | 17848 | 18161 |
| 9 | 17590 | 17623 | 17591 | 17623 |
| 10 | 17062 | 17197 | 17062 | 17198 |
| 11 | 16558 | 16653 | 16558 | 16653 |
| 12 | 16269 | 16352 | 16269 | 16352 |
| 13 | 15105 | 15251 | 15105 | 15251 |
| 14 | 13829 | 14085 | 13890 | 14085 |
| **Gene Model 1446-1** | | | | |
| | Polished Coordinates | | Original GFF3 File Coordinates | |
| Exon | Start | Stop | Start | Stop |
| 1 | 23696 | 23818 | - | - |
| 2 | 30492 | 30819 | 30493 | 30680 |
| 3 | 36558 | 36867 | 36559 | 36867 |
| 4 | 36958 | 37263 | 36958 | 37262 |
| **Gene Model 1446-2** | | | | |
| | Polished Coordinates | | Original GFF3 File Coordinates | |
| Exon | Start | Stop | Start | Stop |
| 1 | 8330 | 8413 | - | - |
| **Gene Model 1733-1** | | | | |
| | Polished Coordinates | | Original GFF3 File Coordinates | |
| Exon | Start | Stop | Start | Stop |
| 1 | 237 | 279 | - | - |
| 2 | 371 | 694 | - | - |
| 3 | 1527 | 1656 | - | - |
| 4 | 2060 | 2157 | - | - |
| 5 | 2424 | 2506 | - | - |
| 6 | 3398 | 3500 | - | - |
| 7 | 4645 | 4904 | - | - |
| **Gene 1733-2** | | | | |
| | Polished Coordinates | | Original GFF3 File Coordinates | |

| Exon | Start | Stop | Start | Stop |
|---|---|---|---|---|
| 1 | 30081 | 30516 | 30081 | 30439 |
| 2 | 23500 | 23907 | 23600 | 23907 |
| 3 | 23201 | 23509 | 23201 | 23509 |
| 4 | 21788 | 21880 | 21788 | 21880 |
| 5 | 21392 | 21706 | 21392 | 21706 |
| 6 | 20009 | 20323 | 20009 | 20323 |
| 7 | 18659 | 18700 | 18659 | 18700 |
| 8 | 18253 | 18567 | 18253 | 18567 |
| 9 | 17993 | 18026 | 17994 | 18026 |
| 10 | 17463 | 17599 | 17463 | 17600 |
| 11 | 16958 | 17053 | 16958 | 17053 |
| 12 | 16669 | 16752 | 16669 | 16752 |
| 13 | 15301 | 15447 | 15301 | 15447 |
| 14 | 14023 | 14085 | 14085 | 14280 |

**Gene Model C2257-1**

| | Polished Coordinates | | Original GFF3 File Coordinates | |
|---|---|---|---|---|
| Exon | Start | Stop | Start | Stop |
| 1 | 9382 | 9526 | 9382 | 9442 |
| 2 | 1685 | 1998 | 1655 | 1998 |

**Gene Model 2307-1**

| | Polished Coordinates | | Original GFF3 File Coordinates | |
|---|---|---|---|---|
| Exon | Start | Stop | Start | Stop |
| 1 | 3474 | 3804 | 3473 | 3804 |
| 2 | 9550 | 9858 | 9550 | 9858 |
| 3 | 9949 | 10248 | 9949 | 10248 |
| 4 | 15449 | 15541 | 15449 | 15541 |
| 5 | 15622 | 15937 | 15623 | 15937 |
| 6 | 16718 | 17032 | 16718 | 17032 |
| 7 | 20911 | 20952 | 20911 | 20952 |
| 8 | 21045 | 21368 | 21044 | 21367 |
| 9 | 22202 | 22332 | 22171 | 22332 |
| 10 | 22747 | 22840 | 22747 | 22839 |
| 11 | 23109 | 23194 | 23108 | 23194 |
| 12 | 24089 | 24235 | 24089 | 24141 |
| 13 | 25327 | 25587 | 25327 | 25472 |

**Gene Model C2565-**1

| | Polished Coordinates | | Original GFF3 File Coordinates | |
|---|---|---|---|---|
| Exon | Start | Stop | Start | Stop |
| 1 | 20650 | 20794 | 20650 | 20795 |
| 2 | 12996 | 13309 | 12996 | 13309 |
| 3 | 7579 | 7886 | 7579 | 7886 |
| 4 | 7183 | 7490 | 7183 | 7490 |
| 5 | 5776 | 5868 | 5776 | 5868 |

| | | | | |
|---|---|---|---|---|
| 6 | 5384 | 5697 | 5384 | 5697 |
| 7 | 4012 | 4325 | 4012 | 4325 |
| 8 | 2666 | 2707 | 2666 | 2707 |
| 9 | 2260 | 2574 | 2260 | 2574 |
| 10 | 2000 | 2033 | 2001 | 2033 |
| 11 | 1474 | 1609 | 1474 | 1610 |
| 12 | 976 | 1071 | 976 | 1071 |
| 13 | 688 | 770 | 497 | 770 |
| **Gene Model C2565-2** | | | | |
| | Polished Coordinates | | Original GFF3 File Coordinates | |
| Exon | Start | Stop | Start | Stop |
| 1 | 35518 | 35640 | 35518 | 35460 |
| 2 | 42314 | 42642 | 42314 | 42642 |
| 3 | 48151 | 48459 | 48151 | 48459 |
| 4 | 48550 | 48858 | 48550 | 48858 |
| 5 | 54065 | 54156 | 54065 | 54156 |
| 6 | 54238 | 54552 | 54238 | 54552 |
| 7 | 55335 | 55649 | 55335 | 55649 |
| 8 | 59475 | 59516 | 59475 | 59519 |
| 9 | 59609 | 59932 | 59608 | 59931 |
| 10 | 60764 | 60897 | 60765 | 60896 |
| 11 | 61329 | 61404 | 61392 | 61403 |
| 12 | 61673 | 61758 | 61672 | 61758 |
| 13 | 62656 | 62802 | 62656 | 62802 |
| 14 | 63909 | 64170 | 63910 | 64055 |
| **Gene Model C2565-3** | | | | |
| | Polished Coordinates | | Original GFF3 File Coordinates | |
| Exon | Start | Stop | Start | Stop |
| 1 | 82927 | 82976 | - | - |
| 2 | 82435 | 82738 | 82435 | 82965 |
| 3 | 81069 | 81161 | 81069 | 81161 |
| 4 | 80673 | 80988 | 80673 | 80987 |
| 5 | 79284 | 79598 | 79284 | 79598 |
| 6 | 77934 | 77975 | 77934 | 77975 |
| 7 | 77528 | 77842 | 77528 | 77842 |
| 8 | 77268 | 77301 | 77269 | 77301 |
| 9 | 76738 | 76874 | 76738 | 76875 |
| 10 | 76232 | 76327 | 76232 | 76327 |
| 11 | 75943 | 76026 | 75943 | 76026 |
| 12 | 74575 | 74721 | 74575 | 74721 |
| 13 | 73297 | 73554 | 73359 | 73554 |
| **Gene Model Chr5-1** | | | | |
| | Polished Coordinates | | Original GFF3 File Coordinates | |
| Exon | Start | Stop | Start | Stop |

| | | | | |
|---|---|---|---|---|
| 1 | 63514409 | 63514478 | 63514409 | 63514478 |
| 2 | 63508015 | 63508332 | 63508015 | 63508334 |
| 3 | 63500570 | 63500878 | 63500570 | 63500878 |
| 4 | 63500171 | 63500479 | 63500171 | 63500479 |
| 5 | 63495151 | 63495243 | 63495151 | 63495243 |
| 6 | 63494759 | 63495073 | 63494759 | 63495073 |
| 7 | 63493357 | 63493671 | 63493357 | 63493671 |
| 8 | 63492981 | 63493022 | 63492981 | 63493022 |
| 9 | 63492575 | 63492889 | 63492575 | 63492889 |
| 10 | 63492297 | 63492329 | 63492297 | 63492329 |
| 11 | 63491597 | 63491734 | 63491597 | 63491734 |
| 12 | 63491098 | 63491190 | 63491098 | 63491190 |
| 13 | 63490744 | 63490830 | 63490744 | 63490830 |
| 14 | 63490083 | 63490229 | 63490083 | 63490229 |
| 15 | 63488680 | 63488963 | 63488680 | 63488963 |

Table 2.9 Pairwise analysis of SRCR domain 1 derived from non-redundant 5'RACE cDNA clones

| Clone | 5RA_2 | 5RA_8 | 5RA_22 | 5RA_26 | 5RA_30 | 5RA_1 | 5RA_3 | 5RA_35 | 5RA_12 | ppWC1 |
|---|---|---|---|---|---|---|---|---|---|---|
| 5RA_2 | 0 | | | | | | | | | |
| 5RA_8 | 0.0101 | 0 | | | | | | | | |
| 5RA_22 | 0.2231 | 0.2231 | 0 | | | | | | | |
| 5RA_26 | 0.0619 | 0.0513 | 0.2744 | 0 | | | | | | |
| 5RA_30 | 0.0408 | 0.0305 | 0.2485 | 0.0834 | 0 | | | | | |
| 5RA_1 | 0.9163 | 0.9163 | 0.9163 | 0.9163 | 0.9163 | 0 | | | | |
| 5RA_3 | 0.9416 | 0.9416 | 0.9416 | 0.9163 | 0.9416 | 0.1165 | 0 | | | |
| 5RA_35 | 0.9676 | 0.9676 | 0.9676 | 0.9676 | 0.9676 | 0.0943 | 0.1863 | 0 | | |
| 5RA_12 | 0.7340 | 0.7340 | 0.7340 | 0.7550 | 0.7340 | 0.5978 | 0.6349 | 0.6162 | 0 | |
| ppWC1 | 0.6931 | 0.6931 | 0.7133 | 0.7133 | 0.6931 | 0.5621 | 0.6162 | 0.5798 | 0.0834 | 0 |

Table 2.10 Assignment of unique 5'RACE PCR clones and cDNA clone ppWC1

| Clone | Gene | Type | Allele | Redundancy |
|---|---|---|---|---|
| 5RA_2 | ssWC1-1 | d1 | 2 | 4 |
| 5RA_8 | ssWC1-1 | d1 | 1 | 6 |
| 5RA_30 | ssWC1-2 | d1 | 1 | 1 |
| 5RA_22 | ssWC1-3 | d1 | 1 | 1 |
| 5RA_26 | ssWC1-4 | d1 | 1 | 1 |
| 5RA_35 | ssWC1-5 | WC1.2 | 1 | 4 |
| 5RA_1 | ssWC1-6 | WC1.2 | 1 | 3 |
| 5RA_3 | ssWC1-7 | WC1.2 | 1 | 1 |
| 5RA_12 | ssWC1-8 | WC1.1 | 1 | 3 |
| ppWC1 | ssWC1-9 | WC1.1 | 1 | 1 |

Table 2.11 Swine WC1 genes signal peptides

| Gene | Type | Signal Sequence | Start[1] | Stop[1] | Cleavage[2] |
|---|---|---|---|---|---|
| ssWC1-1 | d1 | MQCFLQGLVFLLLGVLSSA | 1 | 19 | 20 |
| ssWC1-2 | d1 | MQCSLQGLVFLLLGVLSSA | 1 | 19 | 20 |
| ssWC1-3 | d1 | MQCSLQGLVFLLLGVLSSA | 1 | 19 | 20 |
| ssWC1-4 | d1 | MQCFLQGLVFLLPGVLSSA | 1 | 19 | 20 |
| ssWC1-5 | WC1.2 | MALNRHLSLQRLGFLLLIMVGGQ | 1 | 24 | 25 |
| ssWC1-6 | WC1.2 | MALNRHLSLQRLGFLLLIMVGGQ | 1 | 24 | 25 |
| ssWC1-7 | WC1.2 | MSLDRHLSLQGLCFLLLIMVGAQ | 1 | 24 | 25 |
| ssWC1-8 | WC1.1 | MALDRHLSLQGLCFLLLIVVGGQ | 1 | 24 | 25 |
| ssWC1-9 | WC1.1 | MALDRHLSLQGLCFLLLIVVGGQ | 1 | 24 | 25 |
| ssWC1-10 | WC1.2 | MALNRHLSLQRLGFLLLIMVGGQ | 1 | 24 | 25 |
| ssWC1-11 | d1 | MQCFLQGLVFLLPGVLSSA | 1 | 19 | 20 |



[1] Refers to amino acid number

[2] Refers to the amino acid number which corresponds to the site of cleavage within the signal peptide.

Table 2.12 Gene models generated by MAKER with manual curation

| | Designation | Scaffold | Type | Start | Stop | Orientation | Contents |
|---|---|---|---|---|---|---|---|
| 1 | C102-1 | 102 | WC1.2 | 2753 | 12849 | + | SRCR 4 → 3'UTR |
| 2 | C102-2 | 102 | d1 | 22251 | 32882 | - | SRCR 2 → 3'UTR |
| 3 | C965-1 | 965 | WC1.2 | 3578 | 24239 | - | 5'UTR → SRCR 5 |
| 4 | C1034-1 | 1034 | WC1.2 | 353 | 4610 | + | SRCR 6 → ICD Ex 5[1] |
| 5 | C1034-2 | 1034 | d1 | 13829 | 30340 | - | 5'UTR → 3'UTR |
| 6 | C1446-1 | 1446 | WC1.2 | 23696 | 37263 | + | 5'UTR → SRCR 3 |
| 7 | C1446-2 | 1446 | d1 | 394 | 8431 | - | 5'UTR → SRCR 1 |
| 8 | C1733-1 | 1733 | WC1.2 | 237 | 4904 | + | SRCR6 → 3'UTR |
| 9 | C1733-2 | 1733 | d1 | 15300 | 30516 | - | 5'UTR → 3'UTR |
| 10 | C2307-1 | 2307 | WC1.2 | 3474 | 25587 | + | SRCR 1 → 3'UTR |
| 11 | C2565-1 | 2565 | d1 | 688 | 20650 | - | 5'UTR → ICD Ex 5[1] |
| 12 | C2565-2 | 2565 | WC1.2 | 35518 | 64170 | + | 5'UTR → 3'UTR |
| 13 | C2565-3 | 2565 | d1 | 73444 | 82976 | - | SRCR 2 → 3'UTR |
| 14 | C2257-1 | 2257 | d1 | 1685 | 9526 | - | 5'UTR → SRCR1 |
| 15 | Chr5-1 | Chr5 | WC1.1 | 63488963 | 63514409 | - | Signal Sequence → 3'UTR |



[1] Intracytoplasmic domain exon

Table 2.13 Pairwise analysis of SRCR domain 1 sequences derived from MAKER gene models and cDNA

| | ssWC1-1 | ssWC1-2 | ssWC1-3 | ssWC1-4 | ssWC1-5 | ssWC1-6 | ssWC1-7 | ssWC1-8 | ssWC1-9 |
|---|---|---|---|---|---|---|---|---|---|
| C965-1 | 0.9775 | 1.0042 | 0.9775 | 0.9775 | 0 | 0.1295 | 0.2184 | 0.6176 | 0.6176 |
| C1034-2 | 0.0099 | 0.0404 | 0.2206 | 0.0612 | 0.9775 | 0.9015 | 0.9262 | 0.7133 | 0.6931 |
| C1446-1 | 0.9515 | 0.9775 | 0.9515 | 0.9515 | 0.0188 | 0.1189 | 0.2069 | 0.6176 | 0.5996 |
| C1733-2 | 0.0507 | 0.0825 | 0.2713 | 0 | 0.9775 | 0.9015 | 0.9015 | 0.7339 | 0.7133 |
| C2307-1 | 0.9015 | 0.9262 | 0.9015 | 0.9015 | 0.1295 | 0 | 0.1084 | 0.5996 | 0.5996 |
| C2257-1 | 0.0301 | 0 | 0.2456 | 0.0825 | 1.0042 | 0.9262 | 0.9515 | 0.7133 | 0.6931 |
| C2565-1 | 0.0099 | 0.0200 | 0.2330 | 0.0612 | 0.9775 | 0.9015 | 0.9262 | 0.7133 | 0.6931 |
| C2565-2 | 0.9515 | 0.9775 | 0.9515 | 0.9515 | 0.0284 | 0.1295 | 0.2184 | 0.6176 | 0.5996 |
| Chr5-1 | 0.7133 | 0.7133 | 0.7133 | 0.7339 | 0.6176 | 0.5996 | 0.6359 | 0.0295 | 0.0396 |

Table 2.14 Pairwise analysis of full length WC1 gene models derived from MAKER and full-length cDNA

| | ssWC1-1 | ssWC1-2 | ssWC1-3 | ssWC1-5 | ssWC1-6 | ssWC1-7 | ssWC1-8 | ssWC1-9 |
|---|---|---|---|---|---|---|---|---|
| C965-1_1733-1 | 0.2881 | 0.2911 | 0.2820 | 0.0171 | 0.0524 | 0.0669 | 0.2032 | 0.1830 |
| C1034-2 | 0.0291 | 0.0337 | 0.0394 | 0.2843 | 0.2862 | 0.2832 | 0.3012 | 0.2873 |
| C1733-2 | 0.0078 | 0.0122 | 0.0685 | 0.2903 | 0.2907 | 0.2862 | 0.3031 | 0.2918 |
| C2257-1_C2565-3 | 0.0178 | 0.0111 | 0.0697 | 0.3009 | 0.2968 | 0.2937 | 0.2992 | 0.2903 |
| C2565-2 | 0.2922 | 0.2953 | 0.2937 | 0.0135 | 0.0375 | 0.0503 | 0.1997 | 0.1767 |
| Chr5-1 | 0.3022 | 0.3022 | 0.2903 | 0.1864 | 0.1821 | 0.1794 | 0.0377 | 0.0200 |

Table 2.15 Location of WC1 genes in *Sscrofa11.1*

| Gene | Genomic | Gene ID | Scaffold | Start | End | Orientation |
|---|---|---|---|---|---|---|
| ssWC1-1 | 1034-2 | 11025982 | NW_018084797.1 | 13829 | 30340 | - |
| ssWC1-2 | C2257-1 | | NW_018085057.1 | 1685 | 9526 | - |
| ssWC1-2 | C2565-3 | 110255322 | NW_018085127.1 | 73444 | 82976 | - |
| ssWC1-3 | C102-2 | 110257980 | NW_018084793.1 | 22251 | 32882 | - |
| ssWC1-4 | C1733-2 | 100627089 | NW_018084937.1 | 14084 | 30482 | |
| ssWC1-5 | C965-1 | 110259181 | NW_018085361.1 | 3578 | 24239 | - |
| ssWC1-5 | C1733-1 | | NW_018085127.1 | 237 | 4904 | + |
| ssWC1-6 | C2307-1 | 110258535 | NW_018085069.1 | 3474 | 25587 | + |
| ssWC1-7 | C102-1 | 110257981 | NW_018084793.1 | 2753 | 12849 | + |
| ssWC1-8 | not placed | | | | | |
| ssWC1-9 | Chr5-1 | 100144477 | Chr5 NC_010447.5 | 63488963 | 63514409 | - |
| ssWC1-10 | C2565-2 | 110258625 | NW_018085127.1 | 35518 | 64170 | + |
| ssWC1-11 | 2565-1 | 110258624 | NW_018085127.1 | 956 | 20954 | - |

Table 2.16 Porcine WC1 intracytoplasmic domains

| Gene | Gene Type | ICD Type | Exons[1] | # Tyrosine Residues |
|---|---|---|---|---|
| ssWC1-1 | d1 | III | 5 | 7 |
| ssWC1-2 | d1 | III | 5 | 7 |
| ssWC1-3 | d1 | III | 5 | 7 |
| ssWC1-4 | d1 | III | 5 | 7 |
| ssWC1-5 | WC1.2 | V | 5 | 6 |
| ssWC1-6 | WC1.2 | V | 5 | 6 |
| ssWC1-7 | WC1.2 | V | 5 | 6 |
| ssWC1-8 | WC1.1 | VI | - | 4 |
| ssWC1-9 | WC1.1 | IV | 5 | 5 |
| ssWC1-10 | WC1.2 | V | 5 | 5 |
| ssWC1-11 | d1 | III | 3 | 4 |

[1] Number of exons that encompass the cytoplasmic domain

Table 2.17 Tyrosine residues found in porcine WC1 intracytoplasmic domains

| Tyrosine | ICD[1] | Exon[2] | AA # | Context | Theoretical Kinase[3] |
|---|---|---|---|---|---|
| 1 | III | 2 | 24 | YQEID | SRC |
| 2 | III | 3 | 55 | YYTGD | - |
| 3 | III | 3 | 56 | YYTGD | - |
| 4 | III | 3 | 68 | YAPEP | SRC |
| 5 | III | 4 | 84 | YDDVE | unspecified |
| 6 | III | 4 | 105 | YFSTE | INSR |
| 7 | III | 4 | 116 | YSQTG | unspecified |
| 8 | III | 5 | 150 | YDDVD | SRC |
| 1 | IV | 2 | 24 | YEEID | SRC |
| 2 | IV | 2 | 29 | YLVTP | unspecified |
| 3 | IV | 3 | 54 | YYTGE | INSR |
| 4 | IV | 3 | 55 | YYTGE | - |
| 5 | IV | 4 | 84 | YDDAE | unspecified |
| 6 | IV | 5 | 151 | YDDVE | SRC |
| 1 | V | 2 | 21 | YKGAV | - |
| 2 | V | 2 | 30 | YEEID | SRC |
| 3 | V | 3 | 60 | YYTGE | INSR |
| 4 | V | 3 | 61 | YYTGE | - |
| 5 | V | 4 | 90 | YDDAE | unspecified |
| 6 | V | 5 | 157 | YDDVE | SRC |
| 1 | VI | unknown[4] | 24 | YEEID | SRC |
| 2 | VI | unknown\ | 29 | YLVTP | unspecified |
| 3 | VI | unknown | 54 | YYTGE | INSR |
| 4 | VI | unknown | 55 | YYTGE | - |
| 5 | VI | unknown | 84 | YDDAE | unspecified |

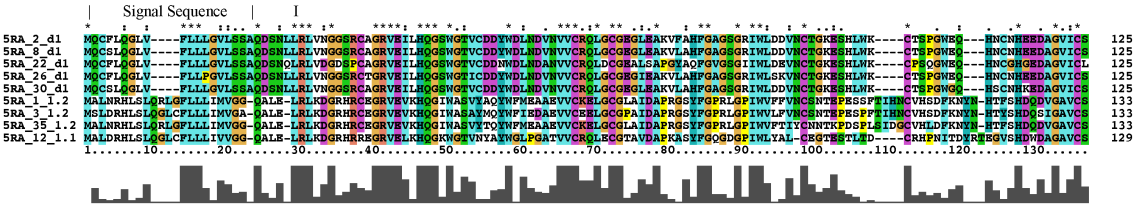
[1] ICD = intracytoplasmic domain

[2] Refers to the cytoplasmic domain exon number

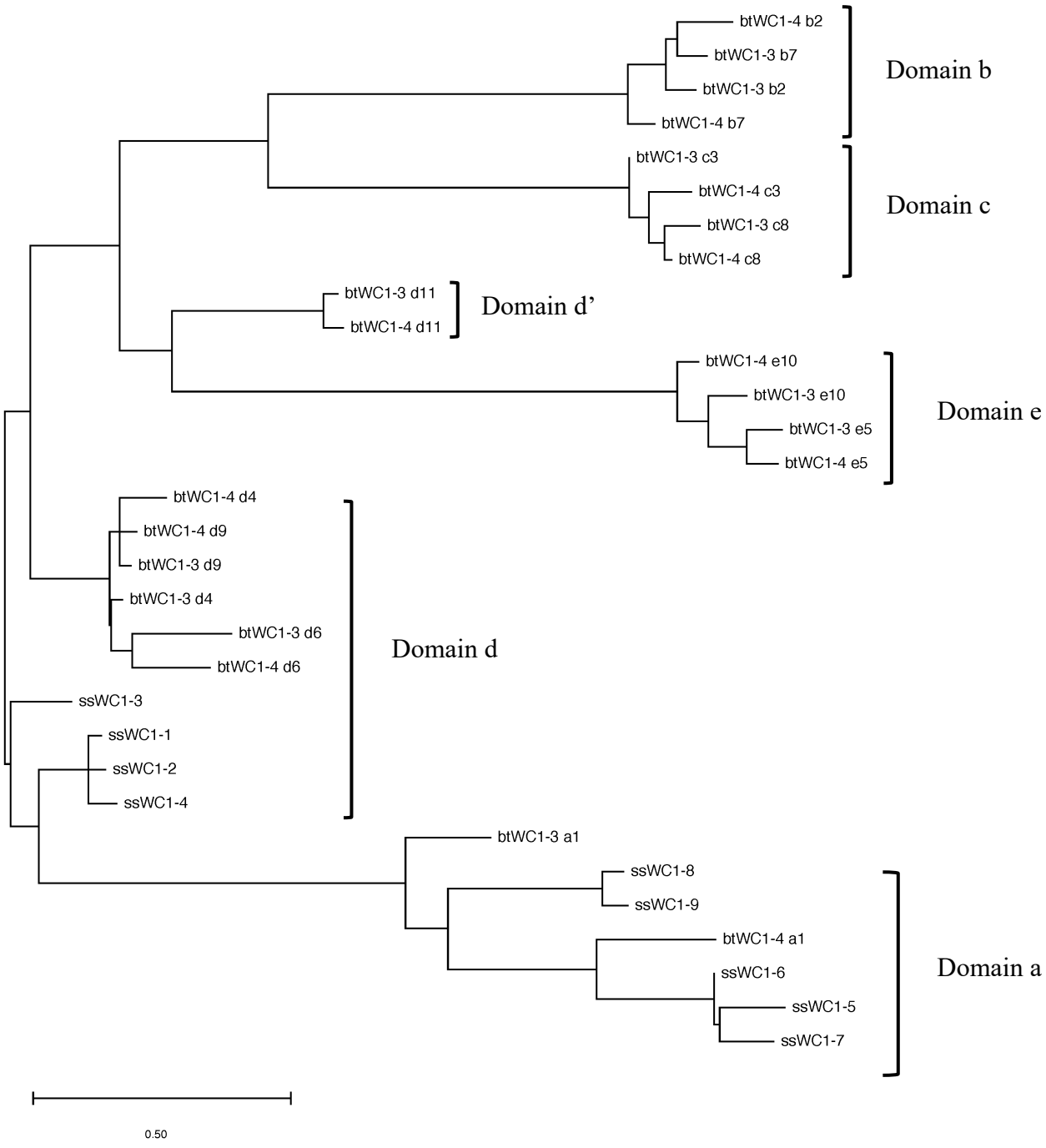
[3] SRC = SRC Tyrosine Kinase; INSR = Insulin Receptor Tyrosine Kinase

[4] Type VI cytoplasmic domain is derived from ssWC1-8, which was not placed in the current genomic assembly. Therefore, exon/intron structure cannot be determined.

Figure 2.1
A


Signal Sequence
5RA_2_d1
5RA_8_d1
5RA_22_d1
5RA_26_d1
5RA_30_d1
5RA_1_1.2
5RA_3_1.2
5RA_35_1.2
5RA_12_1.1

B


btWC1-4 b2
btWC1-3 b7
btWC1-3 b2
btWC1-4 b7
Domain b
btWC1-3 c3
btWC1-4 c3
btWC1-3 c8
btWC1-4 c8
Domain c
btWC1-3 d11
btWC1-4 d11
Domain d'
btWC1-4 e10
btWC1-3 e10
btWC1-3 e5
btWC1-4 e5
Domain e
btWC1-4 d4
btWC1-4 d9
btWC1-3 d9
btWC1-3 d4
btWC1-3 d6
btWC1-4 d6
Domain d
ssWC1-3
ssWC1-1
ssWC1-2
ssWC1-4
btWC1-3 a1
ssWC1-8
ssWC1-9
btWC1-4 a1
ssWC1-6
ssWC1-5
ssWC1-7
Domain a
0.50

C

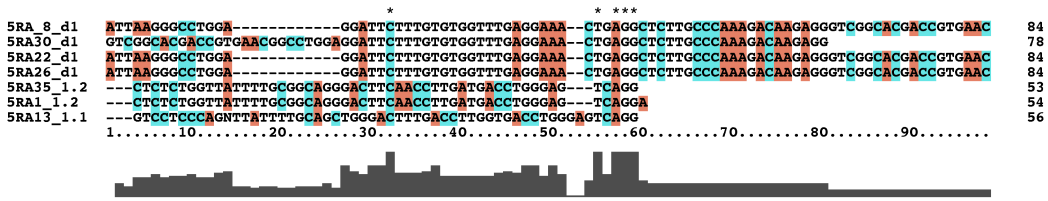
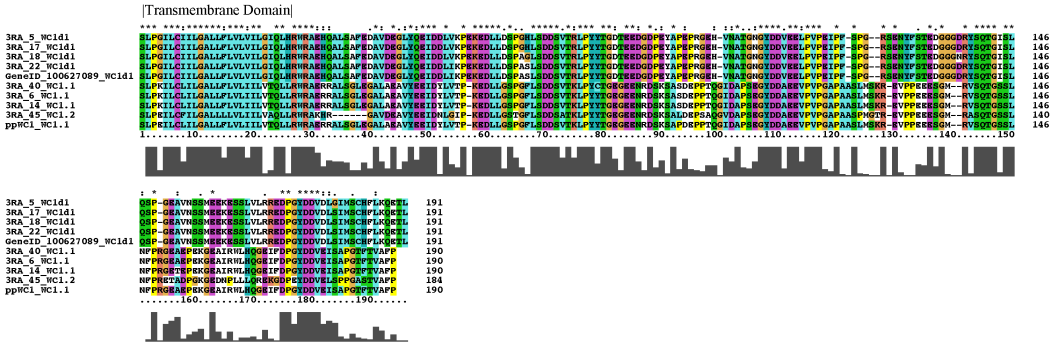


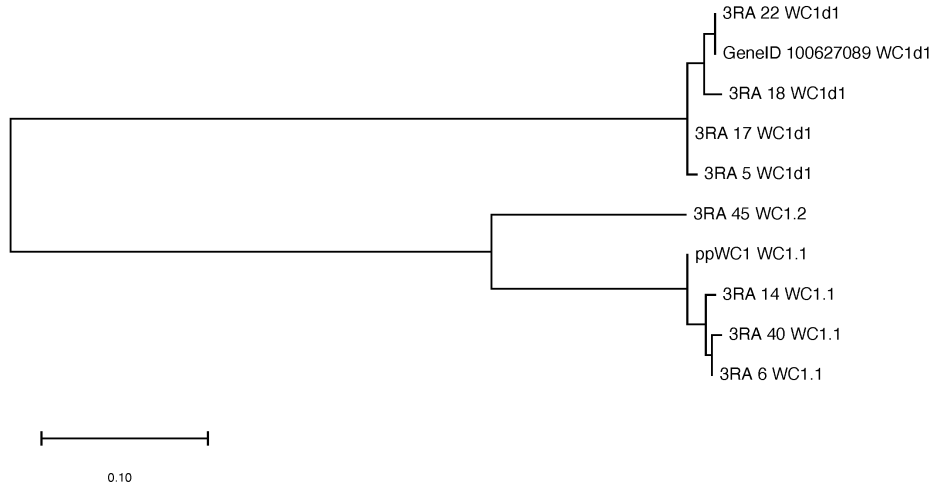

Figure 2.1 Analysis of WC1 sequences derived from 5'RACE PCR.
(A) WC1 signal sequence and SRCR domain 1 deduced amino acid sequences were aligned with Bioedit and visualized using ClustalX. Analysis includes all non-redundant cDNA clones derived from 5'RACE PCR. The signal sequence is bracketed (|) above the alignment and start of SRCR domain 1 is marked with a roman numeral (I). Gaps resulting from the alignment are indicated by dashes (-). (B) WC1 domain 1 deduced amino acid sequences obtained from the 5'RACE, along with cDNA clone ppWC1 were aligned in bioedit and pairwise analysis was performed using MEGAX with the complete deletion option. (C) SRCR domain 1 sequences derived from 5'RACE cDNA clones, along with cDNA clone ppWC1, were aligned with individual SRCR domain sequences representing domains 1 though 11 of bovine WC1-3 (btWC-3) and WC1-4 (btWC1-4) and a phylogenetic tree was constructed. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [261]. The tree with the highest log likelihood (-3105.16) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 31 amino acid sequences. There was a total of 109 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [262,263]. (D) 5'UTR sequences derived from unique 5'RACE cDNA clones were aligned with Bioedit and visualized using ClustalX. Gaps resulting from the alignment are indicated by dashes (-).

Figure 2.2

A




B




C

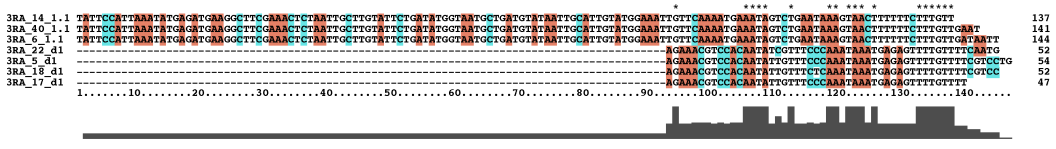
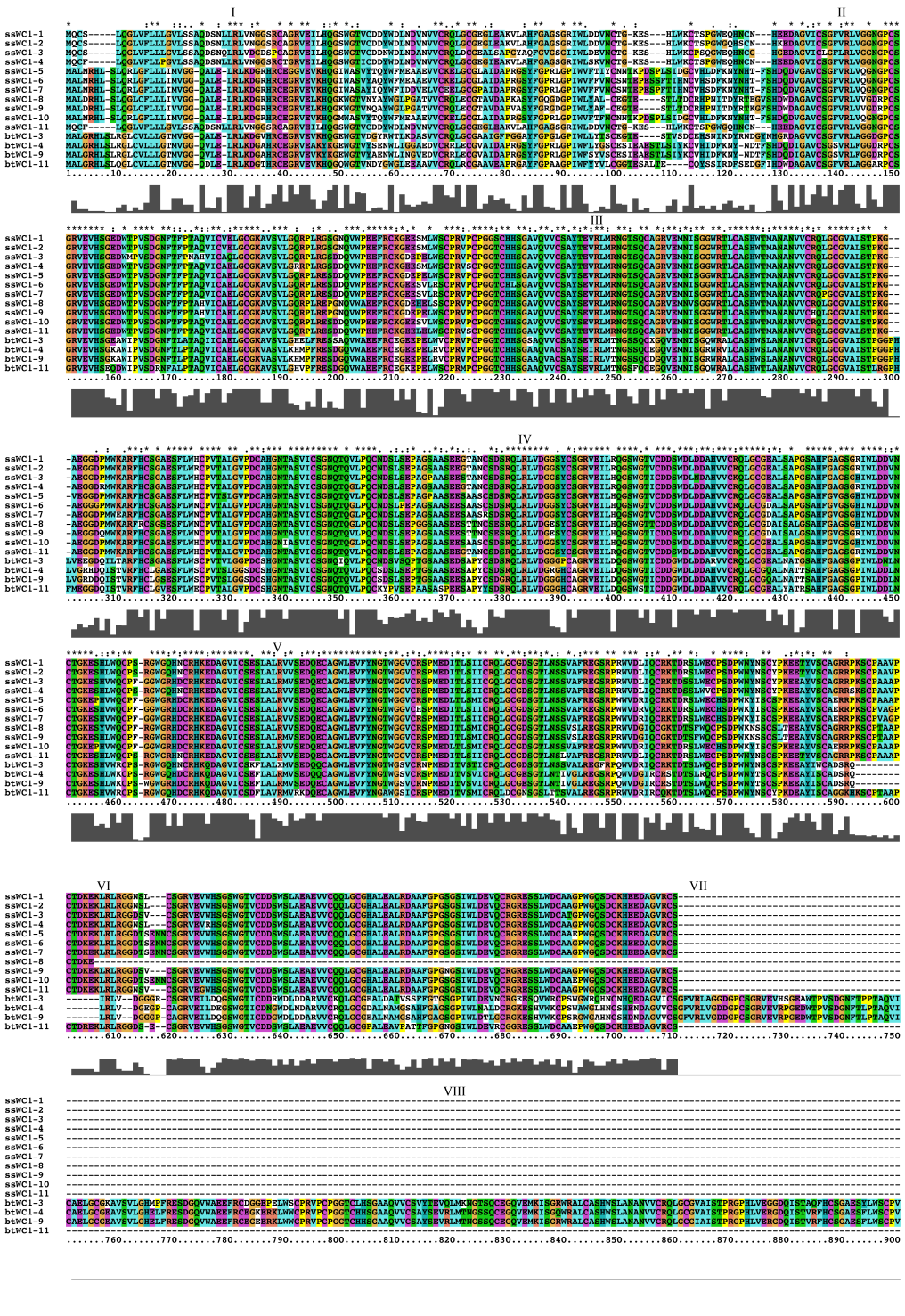
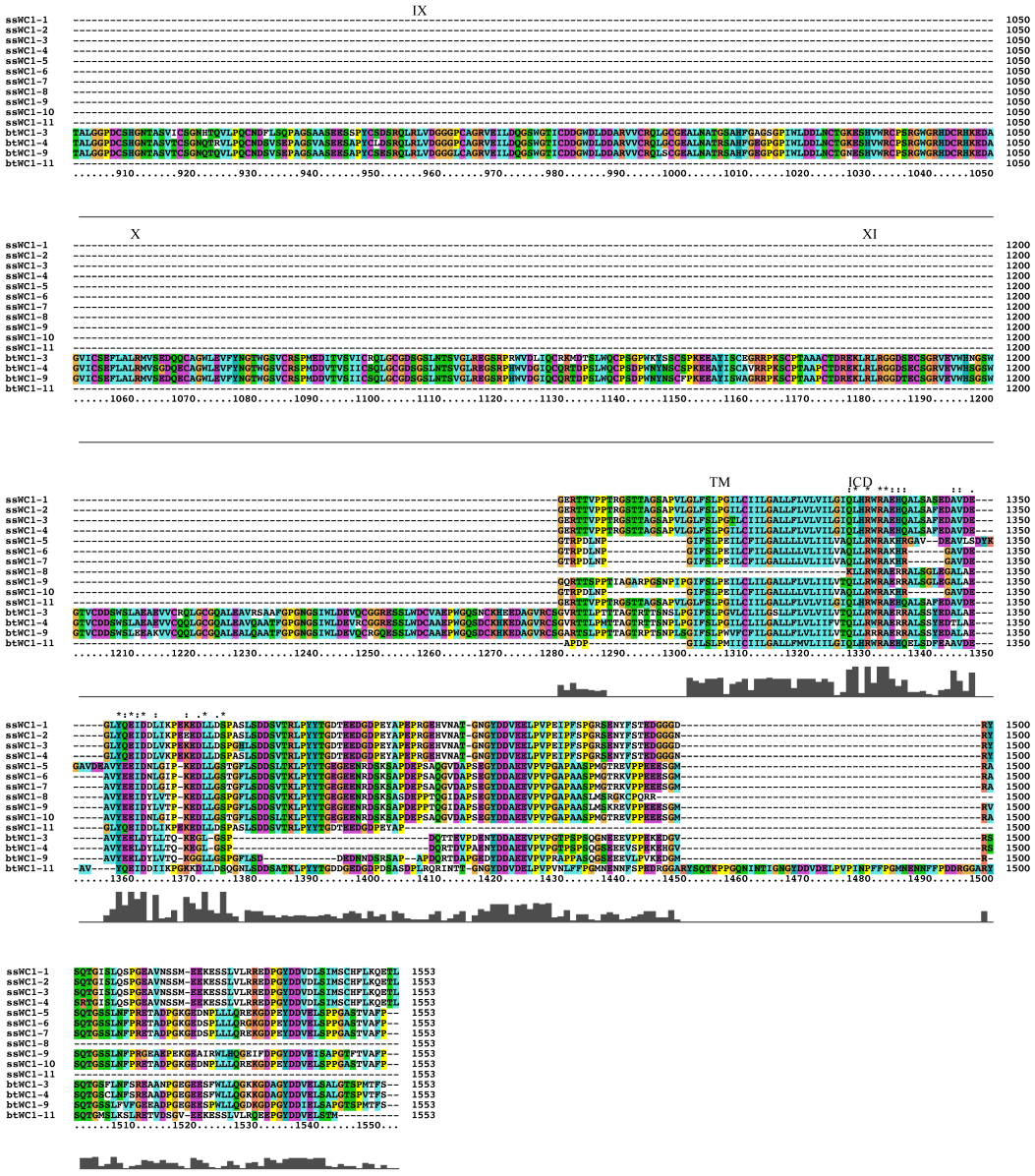


Figure 2.2 WC1 sequences derived from 3'RACE PCR.
(A) WC1 transmembrane and cytoplasmic domain deduced amino acid sequences were aligned with Bioedit and visualized using BoxShade. Analysis includes all non-redundant cDNA clones derived from 3'RACE PCR (3RA_) and cDNA clone ppWC1. The transmembrane domain is bracketed (|) above the alignment. Gaps resulting from the alignment are indicated by dashes (-). (B) Cytoplasmic domain sequences derived from 3'RACE cDNA clones, along with cDNA clone ppWC1, and the predicted d1-WC1 gene (GeneID 100627089) were aligned in bioedit and visualized in JalView. A phylogenetic tree was constructed using the alignment generated in part (B). The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [261]. The tree with the highest log likelihood (-1174.73) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 10 amino acid sequences. There was a total of 196 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [262,263]. (C) 3'UTR sequences derived from unique 3'RACE cDNA clones were aligned with Bioedit and visualized using BoxShade. Gaps resulting from the alignment are indicated by dashes (-).

Figure 2.3
A



A (continued from previous page)



B

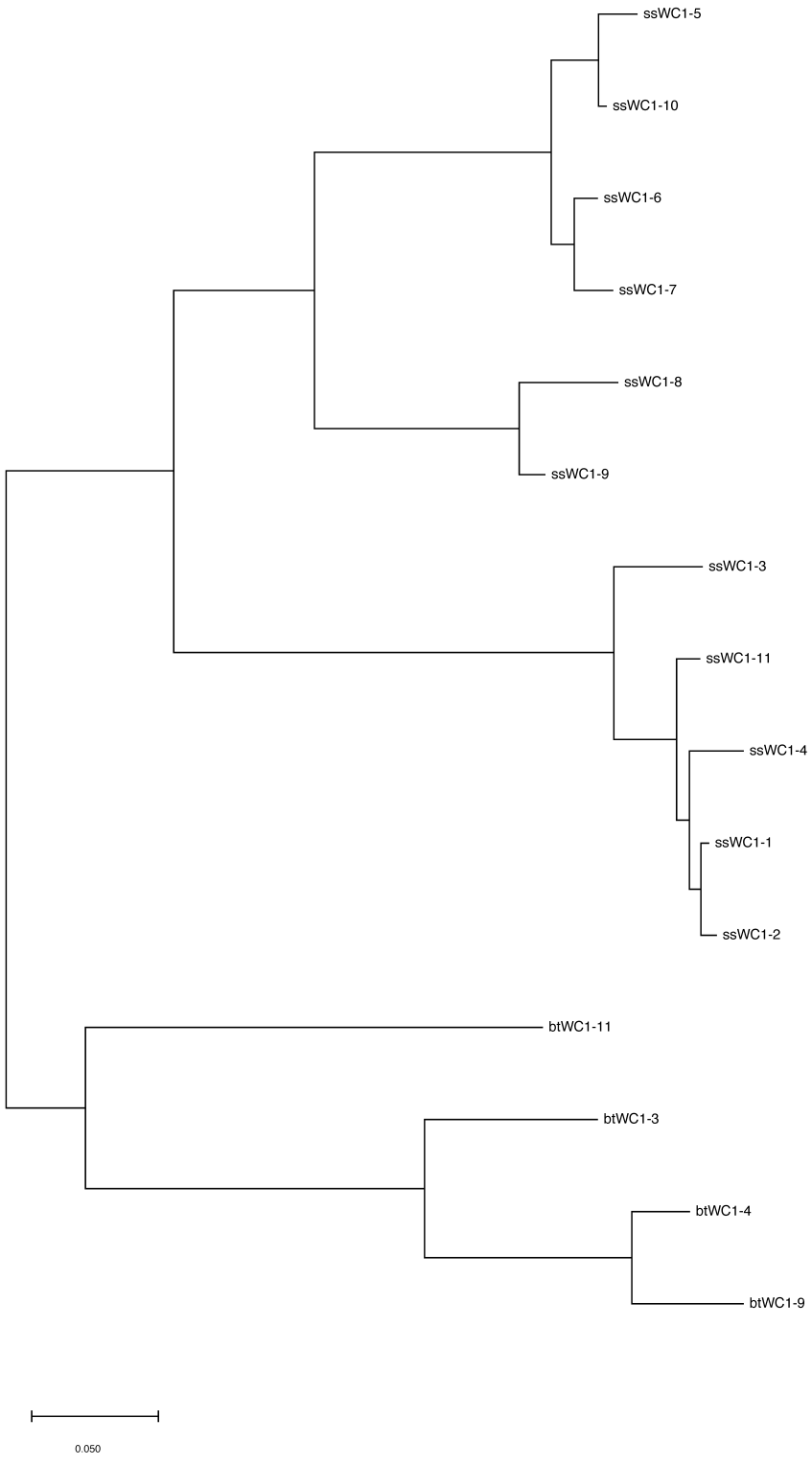

ssWC1-5
ssWC1-10
ssWC1-6
ssWC1-7
ssWC1-8
ssWC1-9
ssWC1-3
ssWC1-11
ssWC1-4
ssWC1-1
ssWC1-2
btWC1-11
btWC1-3
btWC1-4
btWC1-9
0.050

Figure 2.3 Comparison of full length porcine WC1 with bovine WC1.
(A) Deduced amino acid sequences representing full length porcine (ss) and bovine (bt) WC1 genes were aligned using ClustalX with default parameters. Manual adjustments to the alignment were performed in bioedit. SRCR domains are indicated above the alignment in roman numerals. The transmembrane domain is marked (TM) above the alignment. Gaps resulting from the alignment are indicated by dashes (-). (B) A phylogenetic tree was constructed from the alignment in part (A). The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [261]. The tree with the highest log likelihood (-10968.16) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 15 amino acid sequences. There was a total of 1553 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [262,263].

Figure 2.4

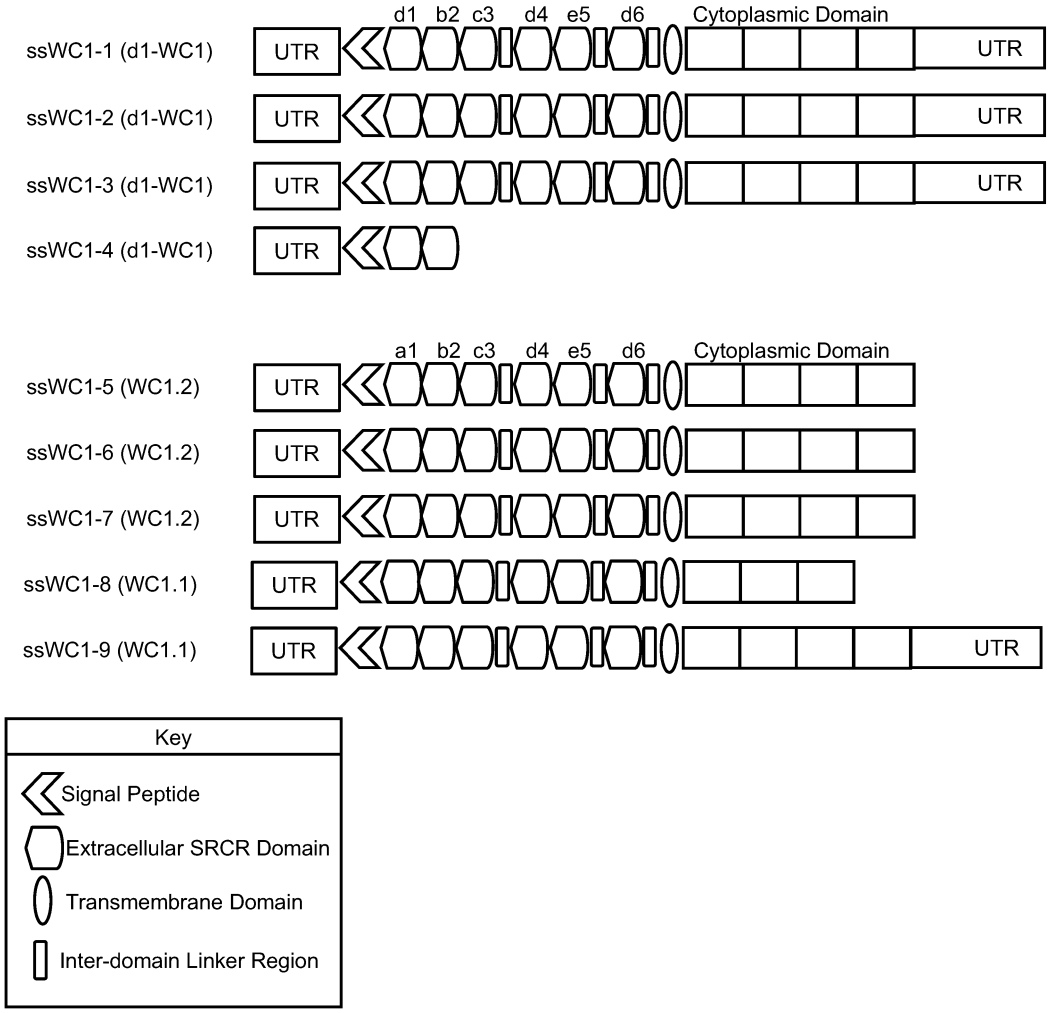



Figure 2.4 cDNA evidence for 9 unique porcine WC1 genes.
Schematic representation of cDNA sequences derived from standard PCR amplification of full length WC1 genes. Gene number is indicated to the left of the schematic with gene type indicated in parenthesis. SRCR and cytoplasmic domains are indicated above the models.

Figure 2.5

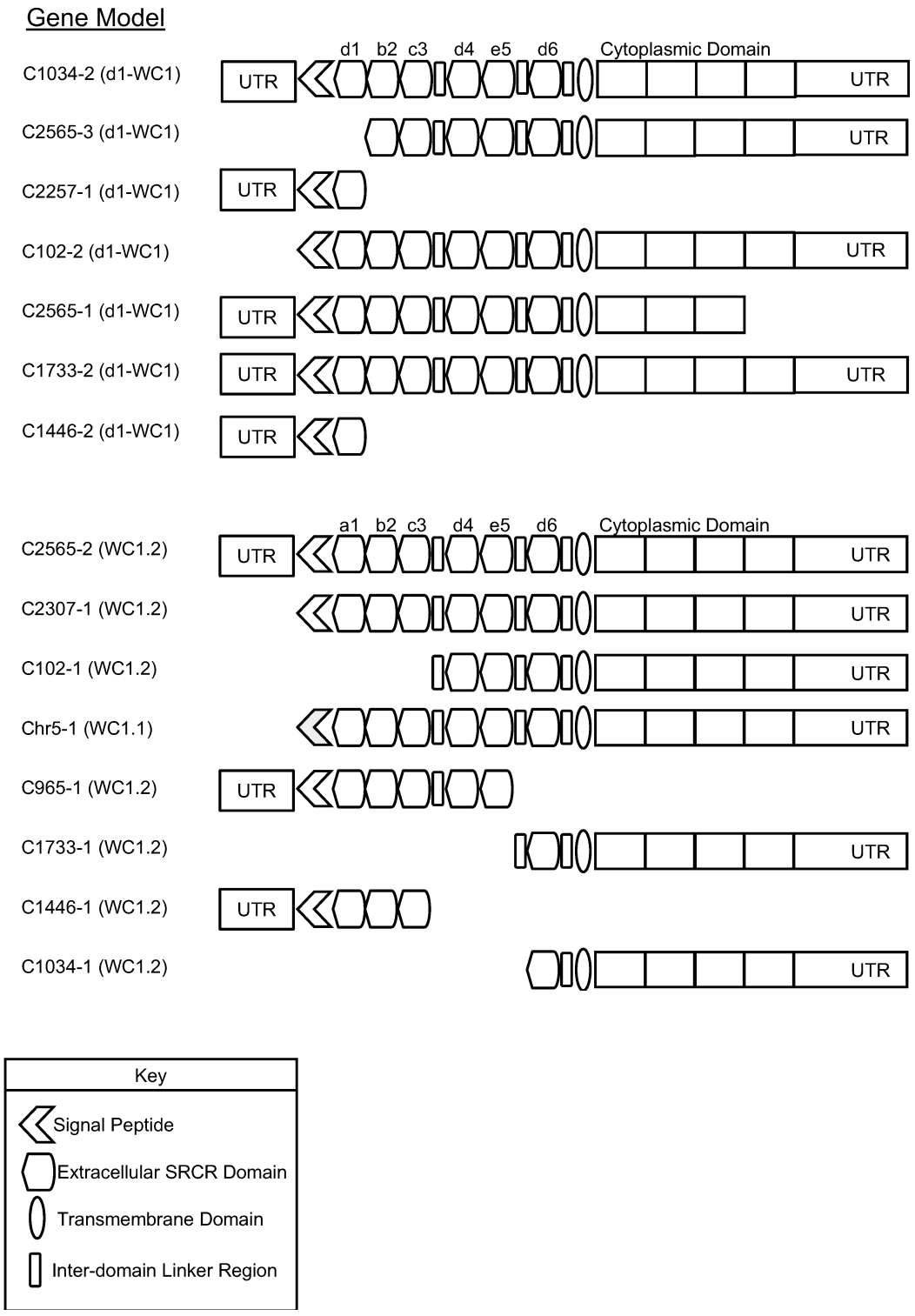

Gene Model
d1 b2 c3 d4 e5 d6 Cytoplasmic Domain
C1034-2 (d1-WC1)
C2565-3 (d1-WC1)
C2257-1 (d1-WC1)
C102-2 (d1-WC1)
C2565-1 (d1-WC1)
C1733-2 (d1-WC1)
C1446-2 (d1-WC1)
UTR
a1 b2 c3 d4 e5 d6 Cytoplasmic Domain
C2565-2 (WC1.2)
C2307-1 (WC1.2)
C102-1 (WC1.2)
Chr5-1 (WC1.1)
C965-1 (WC1.2)
C1733-1 (WC1.2)
C1446-1 (WC1.2)
C1034-1 (WC1.2)
Key
Signal Peptide
Extracellular SRCR Domain
Transmembrane Domain
Inter-domain Linker Region

Figure 2.5 Schematic representation of gene models generated by MAKER with manual curation.
Deduced amino acid sequences derived from the gene models generated by MAKER were aligned with amino acid sequence representing full-length ssWC1-1 derived from cDNA. This alignment was used to determine the structures of each gene model produced by MAKER. The name of each gene model and they type of gene is indicated on the left.

Figure 2.6

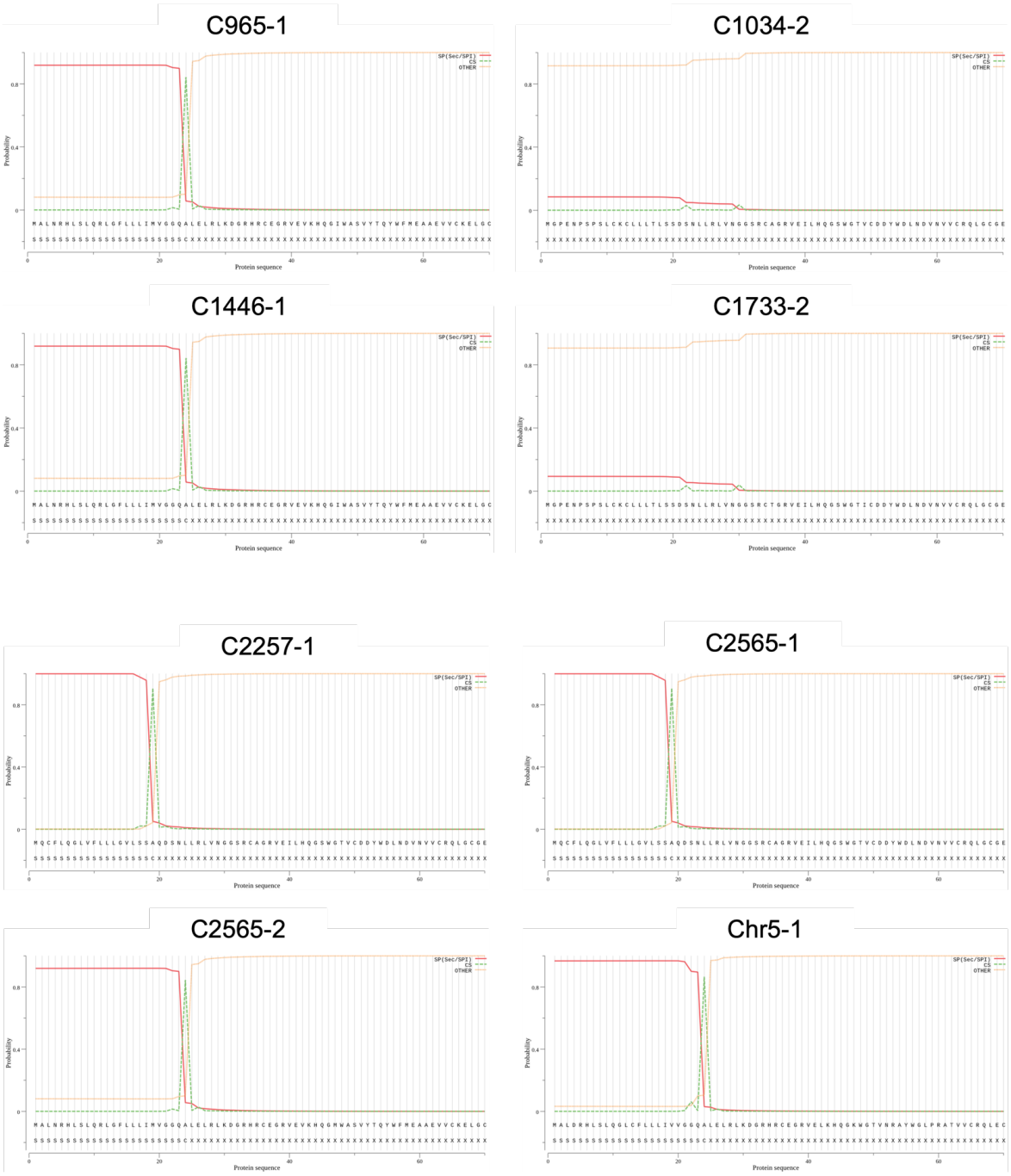
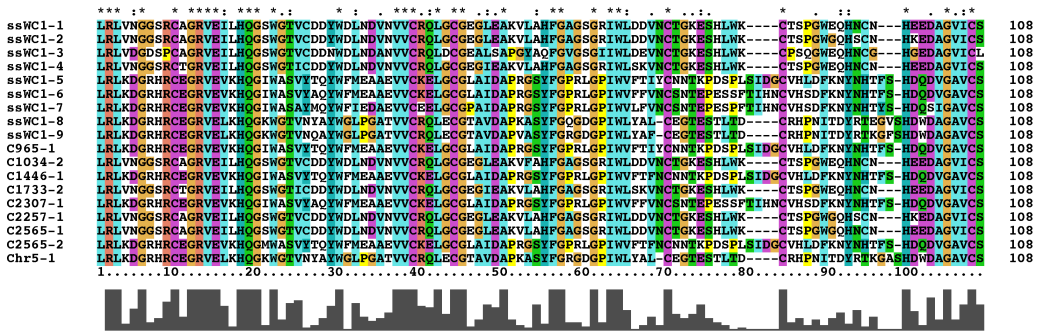



Figure 2.6 Confirmation of signal peptide in WC1 gene models.
Deduced amino acid sequences derived from gene models generated with MAKER which appeared to have signal peptide (C965-1, C1034-2, C1446-1, C1733-2, C2257-1, C2565-1, C2565-2 and Chr5-1) were analyzed using SignalP 5.0 (http://www.cbs.dtu.dk/services/SignalP/).

Figure 2.7
A


ssWC1-1 LRLVNGGSRCAGRVEILHQGSWGTVCDDYWDLNDVNVVCRQLGCGEGLEAKVLAHFGAGSGRIWLDDVNCTGKESHLWK----CTSPGWEQHNCN---HEEDAGVICS 108
ssWC1-2 LRLVNGGSRCAGRVEILHQGSWGTVCDDYWDLNDVNVVCRQLGCGEGLEAKVLAHFGAGSGRIWLDDVNCTGKESHLWK----CTSPGWGQHSCN---HKEDAGVICS 108
ssWC1-3 LRLVDGDSPCAGRVEILHQGSWGTVCDDNWDLNDANVVCRQLDCGEALSAPGYAQFGVGSGIIWLDEVNCTGKESHLWK----CPSQGWEQHNCG---HGEDAGVICL 108
ssWC1-4 LRLVNGGSRCTGRVEILHQGSWGTICDDYWDLNDVNVVCRQLGCGEGIEAKVLAHFGAGSGRIWLSKVNCTGKESHLWK----CTSPGWEQHNCN---HEEDAGVICS 108
ssWC1-5 LRLKDGRHRCEGRVEVKHQGIWASVTTQYWFMEAAEVVCKELGCGLAIDAPRGSYFGPRLGPIWVFTIYCNNTKPDSPLSIDGCVHLDFKNYNHTFS-HDQDVGAVCS 108
ssWC1-6 LRLKDGRHRCEGRVEVKHQGIWASVTAQYWFMEAAEVVCKELGCGLAIDAPRGSYFGPRLGPIWVFPVNCSNTEPESSFTIHNCVHSDFKNYNHTFS-HDQDVGAVCS 108
ssWC1-7 LRLKDGRHRCEGRVEVKHQGIWASAIMQYWFIEDAEVVCEELGCGPAIDAPRGSYFGPRLGPIWVLFVNCSNTEPESPFTIHNCVHSDFKNYNHTYS-HDQSIGAVCS 108
ssWC1-8 LRLKDGRHRCEGRVELKHQGKWGTVNYAYWGLPGATVVCRQLECGTAVDAPKASYFGQGDGPIWLYAL-CEGTESTLTD----CRHPNITDYRIEGVSHDWDAGAVCS 108
ssWC1-9 LRLKDGRHRCEGRVELKHQGKWGTVNQAYWGLPGATVVCRQLECGTAVDAPVASYFGRGDGPIWLYAF-CEGTESTLTD----CRHPNITDYRIKGFSHDWDAGAVCS 108
C965-1 LRLKDGRHRCEGRVEVKHQGIWASVTTQYWFMEAAEVVCKELGCGLAIDAPRGSYFGPRLGPIWVFTIYCNNTKPDSPLSIDGCVHLDFKNYNHTFS-HDQDVGAVCS 108
C1034-2 LRLVNGGSRCAGRVEILHQGSWGTVCDDYWDLNDVNVVCRQLGCGEGLEAKVFAHFGAGSGRIWLDDVNCTGKESHLWK----CTSPGWEQHNCN---HEEDAGVICS 108
C1446-1 LRLKDGRHRCEGRVEVKHQGIWASVTTQYWFMEAAEVVCKELGCGLAIDAPRGSYFGPRLGPIWVFTFNCNNTKPDSPLSIDGCVHLDFKNYNHTFS-HDQDVGAVCS 108
C1733-2 LRLVNGGSRCTGRVEILHQGSWGTICDDYWDLNDVNVVCRQLGCGEGIEAKVLAHFGAGSGRIWLSKVNCTGKESHLWK----CTSPGWEQHNCN---HEEDAGVICS 108
C2307-1 LRLKDGRHRCEGRVEVKHQGIWASVTAQYWFMEAAEVVCKELGCGLAIDAPRGSYFGPRLGPIWVFPVNCSNTEPESSFTIHNCVHSDFKNYNHTFS-HDQDVGAVCS 108
C2257-1 LRLVNGGSRCAGRVEILHQGSWGTVCDDYWDLNDVNVVCRQLGCGEGLEAKVLAHFGAGSGRIWLDDVNCTGKESHLWK----CTSPGWGQHSCN---HKEDAGVICS 108
C2565-1 LRLVNGGSRCAGRVEILHQGSWGTVCDDYWDLNDVNVVCRQLGCGEGLEAKVLAHFGAGSGRIWLDDVNCTGKESHLWK----CTSPGWGQHNCN---HEEDAGVICS 108
C2565-2 LRLKDGRHRCEGRVEVKHQGMWASVTTQYWFMEAAEVVCKELGCGLAIDAPRGSYFGPRLGPIWVFTFNCNNTKPDSPLSIDGCVHLDFKNYNHTFS-HDQDVGAVCS 108
Chr5-1 LRLKDGRHRCEGRVELKHQGKWGTVNYAYWGLPGATVVCRQLECGTAVDAPKASYFGRGDGPIWLYAL-CEGTESTLTD----CRHPNITDYRIKGASHDWDAGAVCS 108
1.....10.....20.....30.....40.....50.....60.....70.....80.....90.....100.....

B

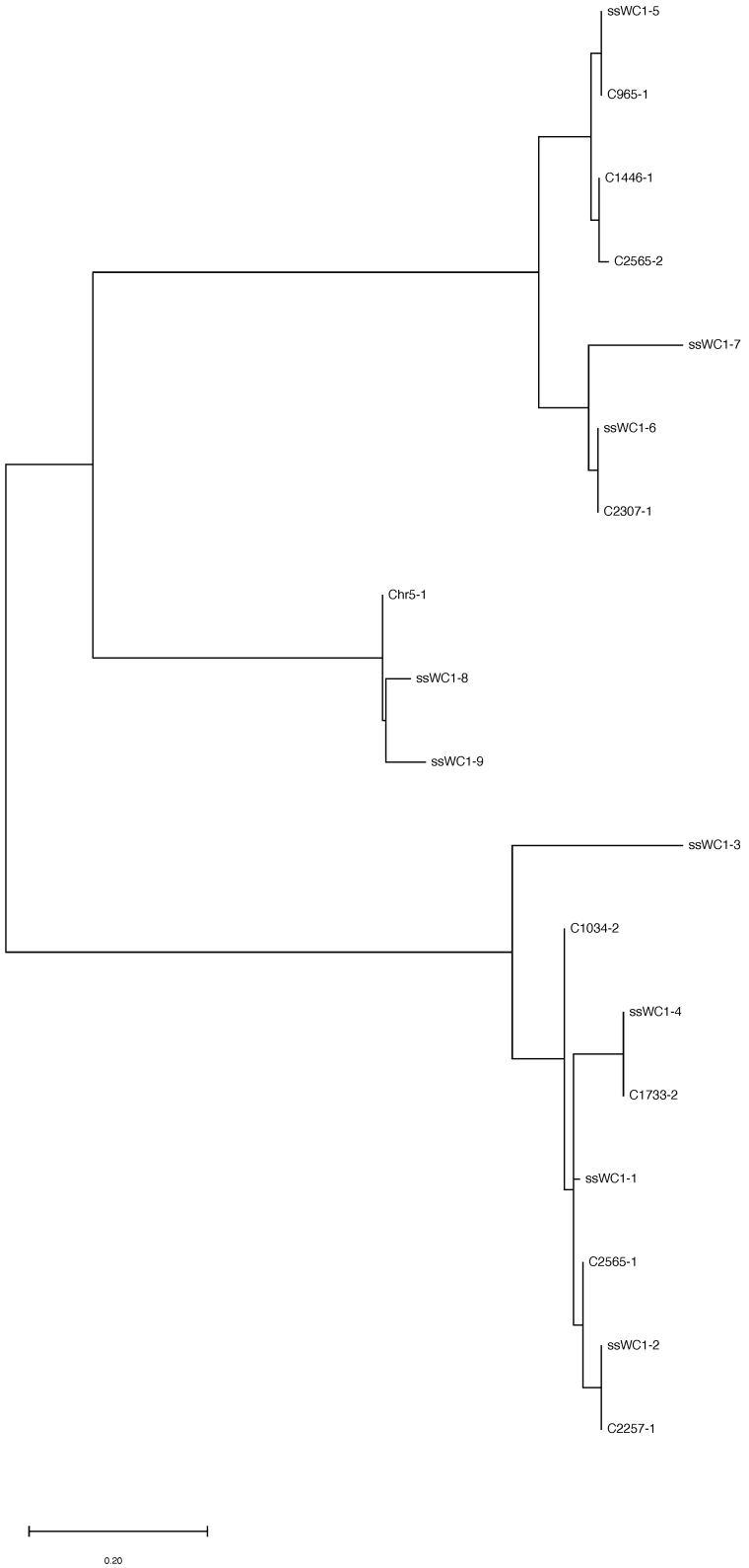
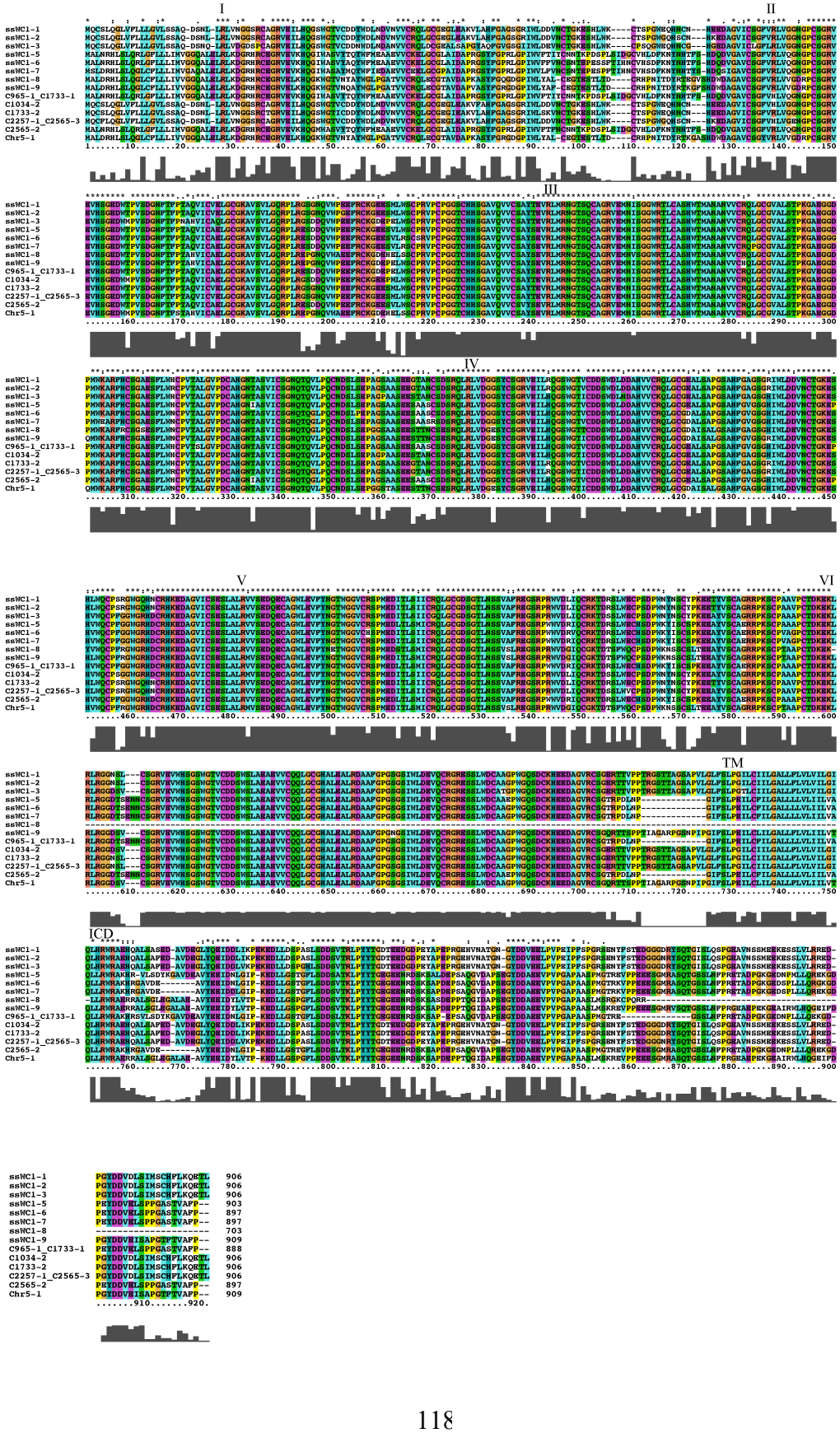

ssWC1-5
C965-1
C1446-1
C2565-2
ssWC1-7
ssWC1-6
C2307-1
Chr5-1
ssWC1-8
ssWC1-9
ssWC1-3
C1034-2
ssWC1-4
C1733-2
ssWC1-1
C2565-1
ssWC1-2
C2257-1
0.20

Figure 2.7 Analysis of SRCR domain 1 derived from genomic sequences.
(A) Deduced amino acid sequences representing SRCR domain 1 derived from gene models generated with MAKER (C2257-1, C965-1, C1446-1, C2307-1, C2565-1, C2565-2, and Chr5-1) were aligned SRCR domain 1 sequences derived from cDNA (ssWC1-1, ssWC1-2, ssWC1-3, ssWC1-4, ssWC1-5, ssWC1-6, ssWC1-7, ssWC1-8 and ssWC1-9) using ClustalX with default parameters. (B) A phylogenetic tree was constructed from the alignment in part (A). The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [261]. The tree with the highest log likelihood (-1077.54) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 16 amino acid sequences. There was a total of 108 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [262,263].

Figure 2.8
A



B

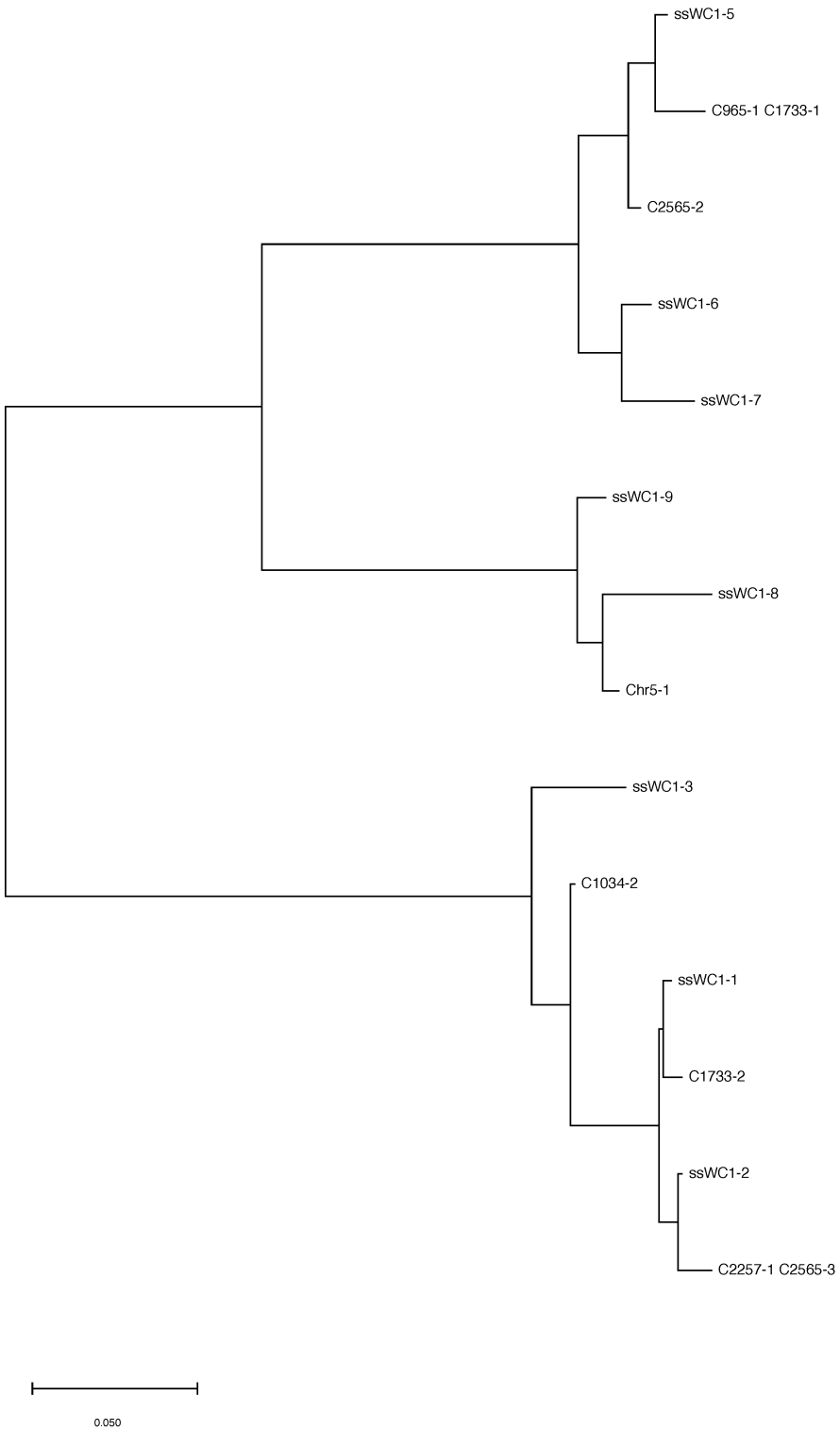
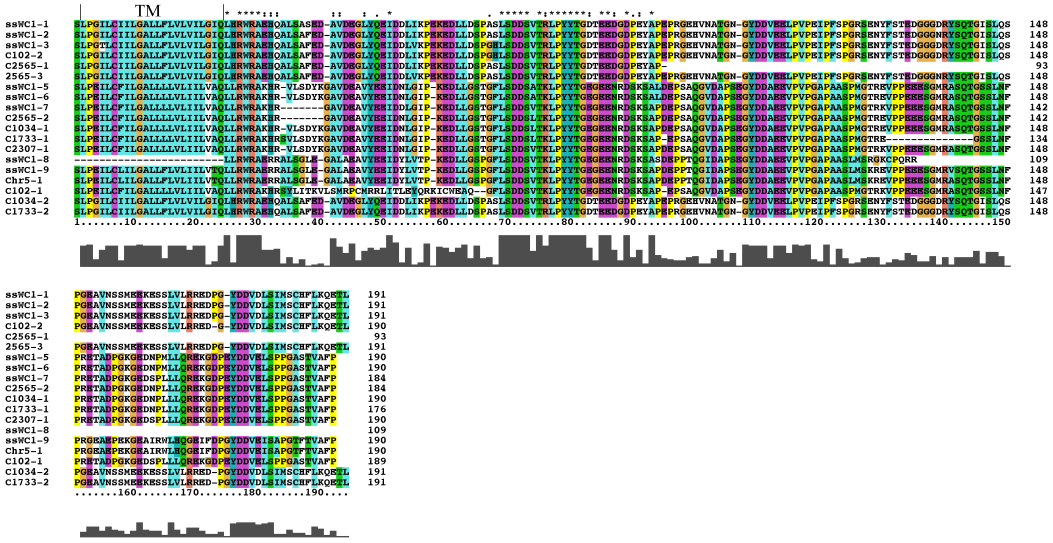

ssWC1-5
C965-1 C1733-1
C2565-2
ssWC1-6
ssWC1-7
ssWC1-9
ssWC1-8
Chr5-1
ssWC1-3
C1034-2
ssWC1-1
C1733-2
ssWC1-2
C2257-1 C2565-3
0.050

Figure 2.8 Full length gene models derived from genomic evidence**.**
**(**A) Gene models that appeared to split across two scaffolds were contigged in bioedit (C2257-1_C2565-3 and C965-1_C1733-1) and aligned in ClustalX with the four full length models generated by MAKER (C1034-2, C1733-2, C2565-2 and Chr5-1) and full-length cDNA sequences (ssWC1-1, ssWC1-2, ssWC1-3, ssWC1-5, ssWC1-6, ssWC1-7, ssWC1-8 and ssWC1-9) using ClustalX with default parameters. (B) A phylogenetic tree was constructed from the alignment in part (A). The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [261]. The tree with the highest log likelihood (-5603.91) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 14 amino acid sequences. There was a total of 921 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [262,263].

Figure 2.9
A


TM
ssWC1-1
ssWC1-2
ssWC1-3
C102-2
C2565-1
2565-3
ssWC1-5
ssWC1-6
ssWC1-7
C2565-2
C1034-1
C1733-1
C2307-1
ssWC1-8
ssWC1-9
Chr5-1
C102-1
C1034-2
C1733-2

B

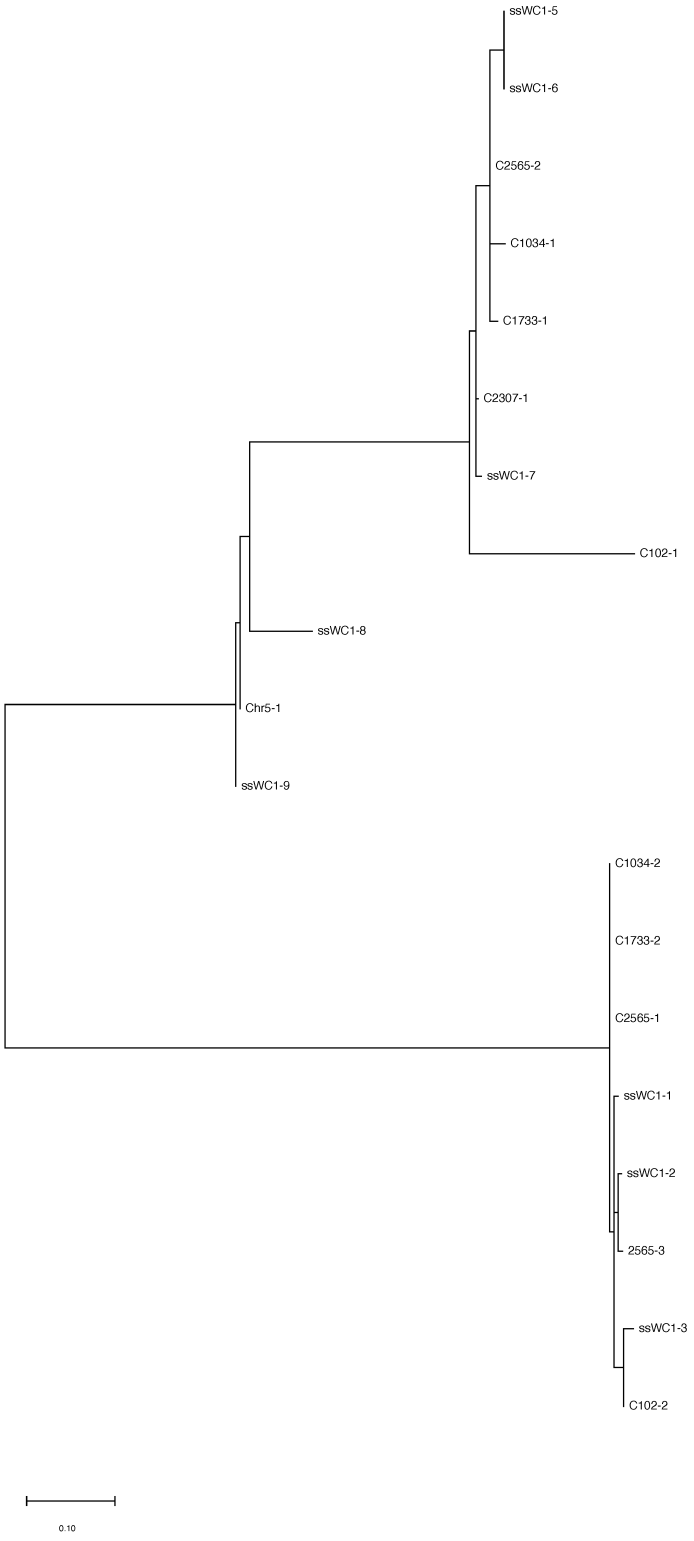
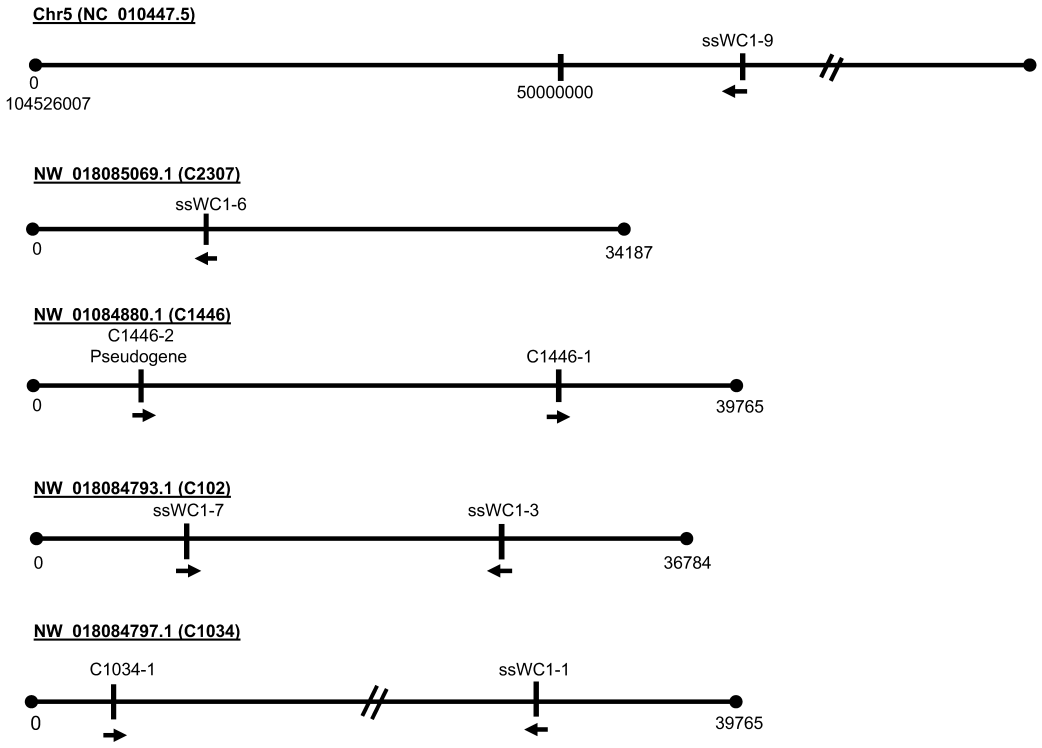

ssWC1-5
ssWC1-6
C2565-2
C1034-1
C1733-1
C2307-1
ssWC1-7
C102-1
ssWC1-8
Chr5-1
ssWC1-9
C1034-2
C1733-2
C2565-1
ssWC1-1
ssWC1-2
2565-3
ssWC1-3
C102-2
0.10

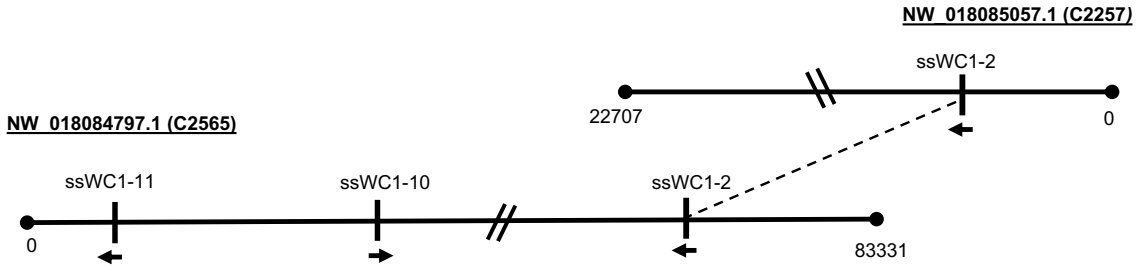
Figure 2.9 Intracytoplasmic domain sequences derived from genomic evidence. **(**A) Deduced amino acid sequences representing WC1 cytoplasmic domain sequences derived from gene models generated with MAKER (C2565-1, C2565-2, C2565-3, C1034-1, C2307-1, C102-1, C102-2 and Chr5-1) were aligned with cytoplasmic domain sequences derived from cDNA (ssWC1-1, ssWC1-2, ssWC1-3, ssWC1-5, ssWC1-6, ssWC1-7, ssWC1-8 and ssWC1-9) using ClustalX with default parameters. (B) A phylogenetic tree was constructed from the alignment in part (A). The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [261]. The tree with the highest log likelihood (-1521.84) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 17 amino acid sequences. There was a total of 194 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [262,263].

**Figure 2.10**

A




B




C

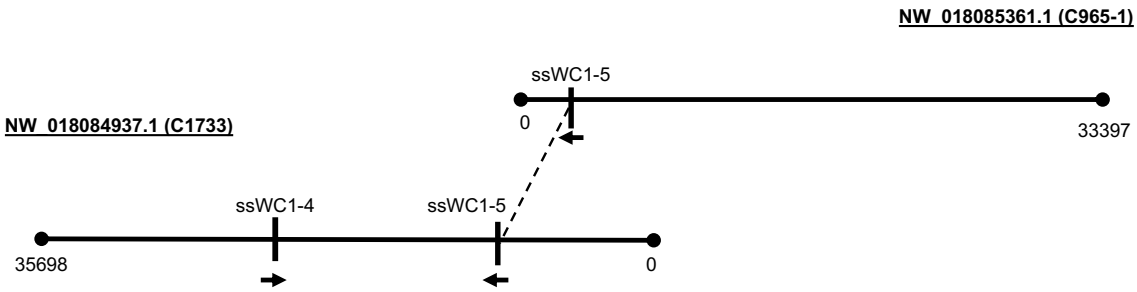


Figure 2.10 Organization of WC1 genes in *Sscrofa11.1*
Eight WC1 genes with supporting cDNA evidence were found in *Sscrofa11.1*. WC1 gene designations, orientations and Porcine Genome Scaffold identifications are as indicated. Four pseudogenes were identified and are indicated. Diagram is not to scale. (B) ssWC1-2 was split between two contigs (C2565 and C2257) (C) ssWC1-5 was split between two contigs (C965-1 and C1733).

Figure 2.11

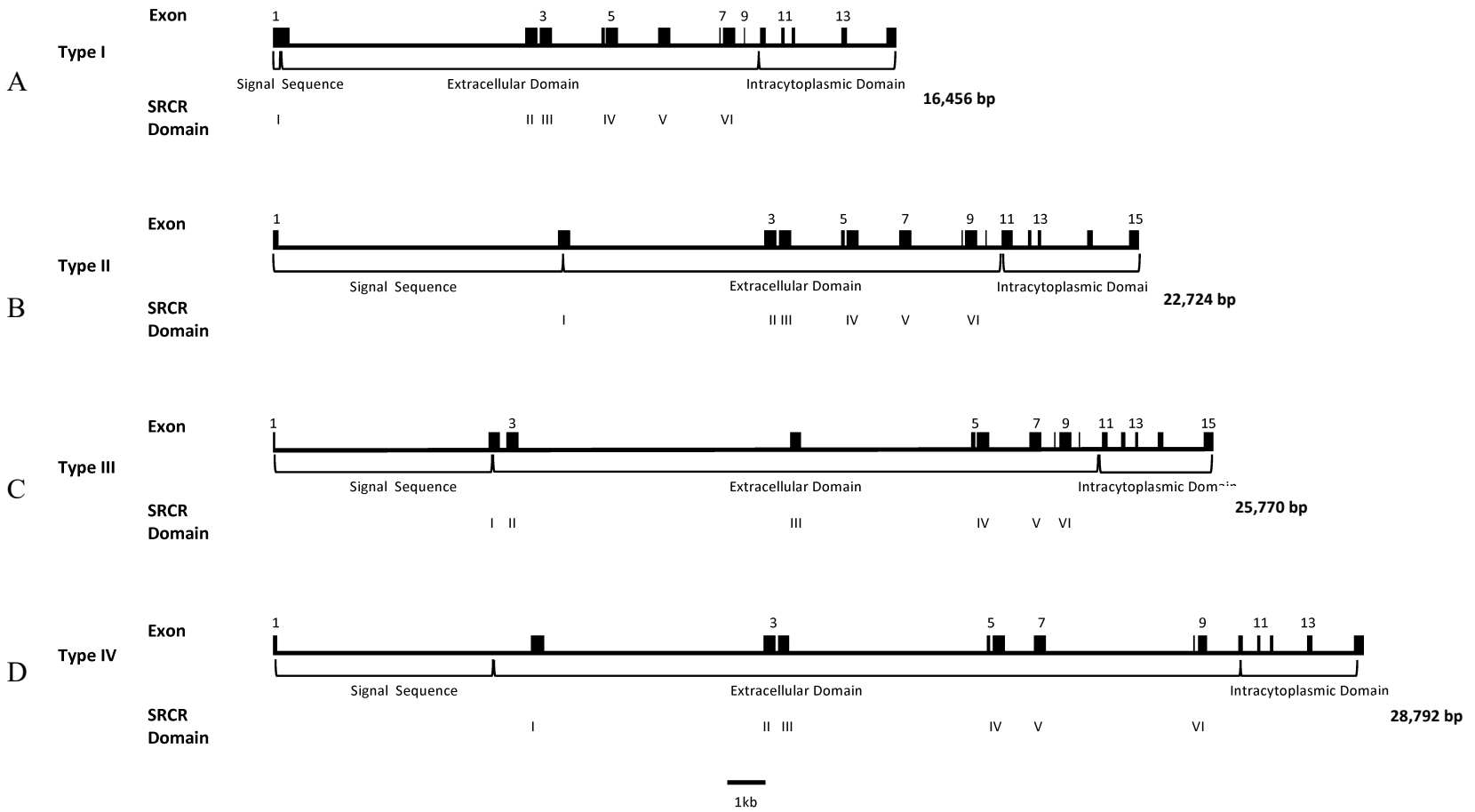
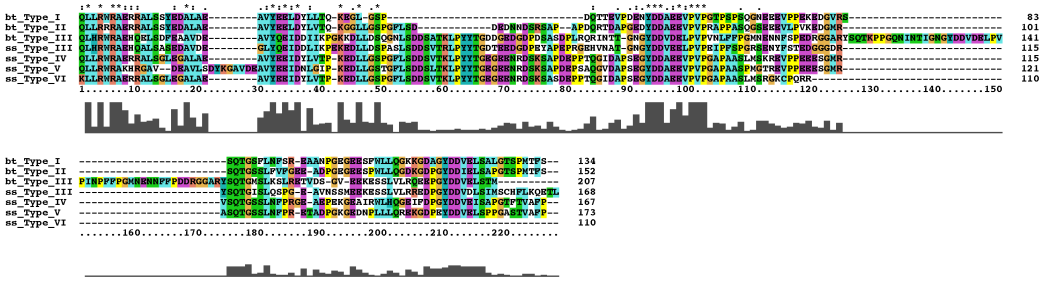

A
Type I
Exon
1 3 5 7 9 11 13
Signal Sequence
Extracellular Domain
Intracytoplasmic Domain
16,456 bp
SRCR Domain
I II III IV V VI
B
Type II
Exon
1 3 5 7 9 11 13 15
Signal Sequence
Extracellular Domain
Intracytoplasmic Domai
22,724 bp
SRCR Domain
I II III IV V VI
C
Type III
Exon
1 3 5 7 9 11 13 15
Signal Sequence
Extracellular Domain
25,770 bp
SRCR Domain
I II III IV V VI
D
Type IV
Exon
1 3 5 7 9 11 13
Signal Sequence
Extracellular Domain
Intracytoplasmic Domain
28,792 bp
SRCR Domain
I II III IV V VI
1kb

Figure 2.11 Schematic representation of porcine WC1 exon-intron structure.
Gene structures are drawn to scale. Scale bar (bottom) represents 1 kb. Exon numbers are labeled above each model and SRCR domains are indicated below each model. (A) *ssWC1-1* and *ssWC1-4* gene structure, 14 exons, 16,456 bp. (Type I; based on genomic sequence derived from gene models C1034-2 and C1733-2 constructed with MAKER). (B) *ssWC1-2* and *ssWC1-11* gene structure, 15 exons, 22,724 bp (Type II; based on genomic sequence derived from gene models C2257-1, C2565-3, and C2565-1 constructed with MAKER) (C) *ssWC1-9* gene structure, 15 exons, 25,770 bp. (Type III; based on genomic sequence derived from gene model Chr5-1 constructed with MAKER). (D) *ssWC1-5, ssWC1-6, ssWC1-7,* and *ssWC-10* gene structure, 14 exons, 28,792 bp. (Type IV; based on genomic sequence derived from gene models C965-1, C1733-1, C2307-1, C102-1, and C2565-2 constructed with MAKER)

Figure 2.12

A




B

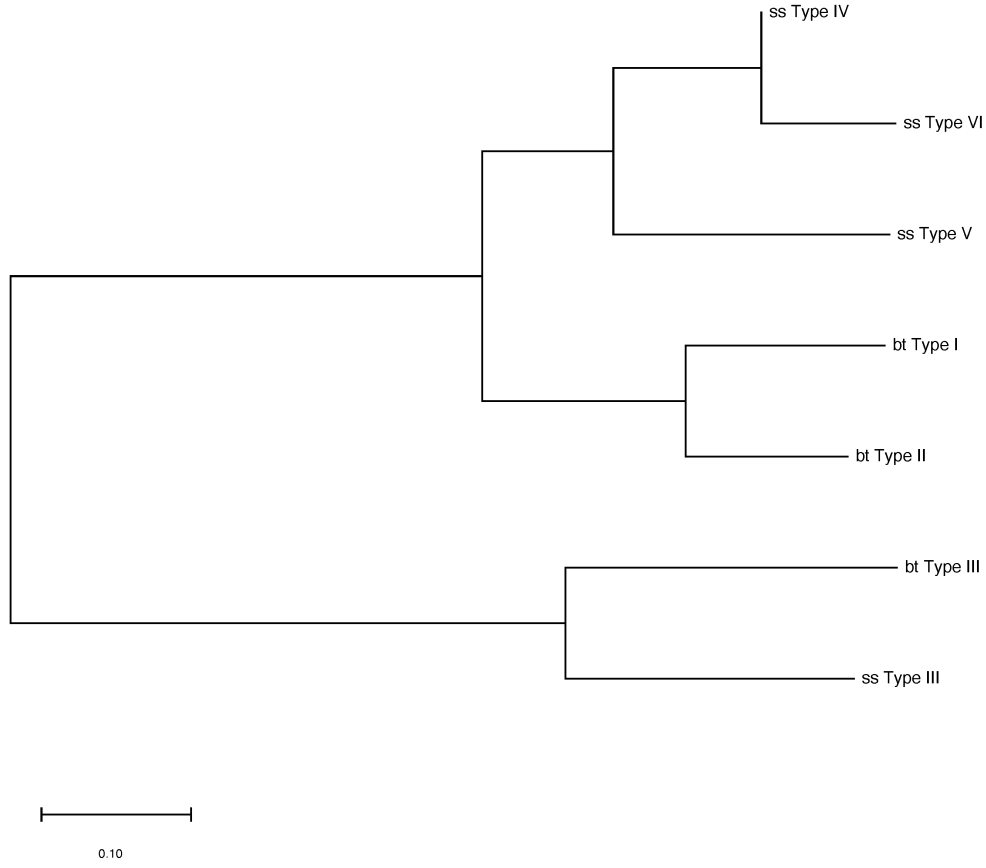
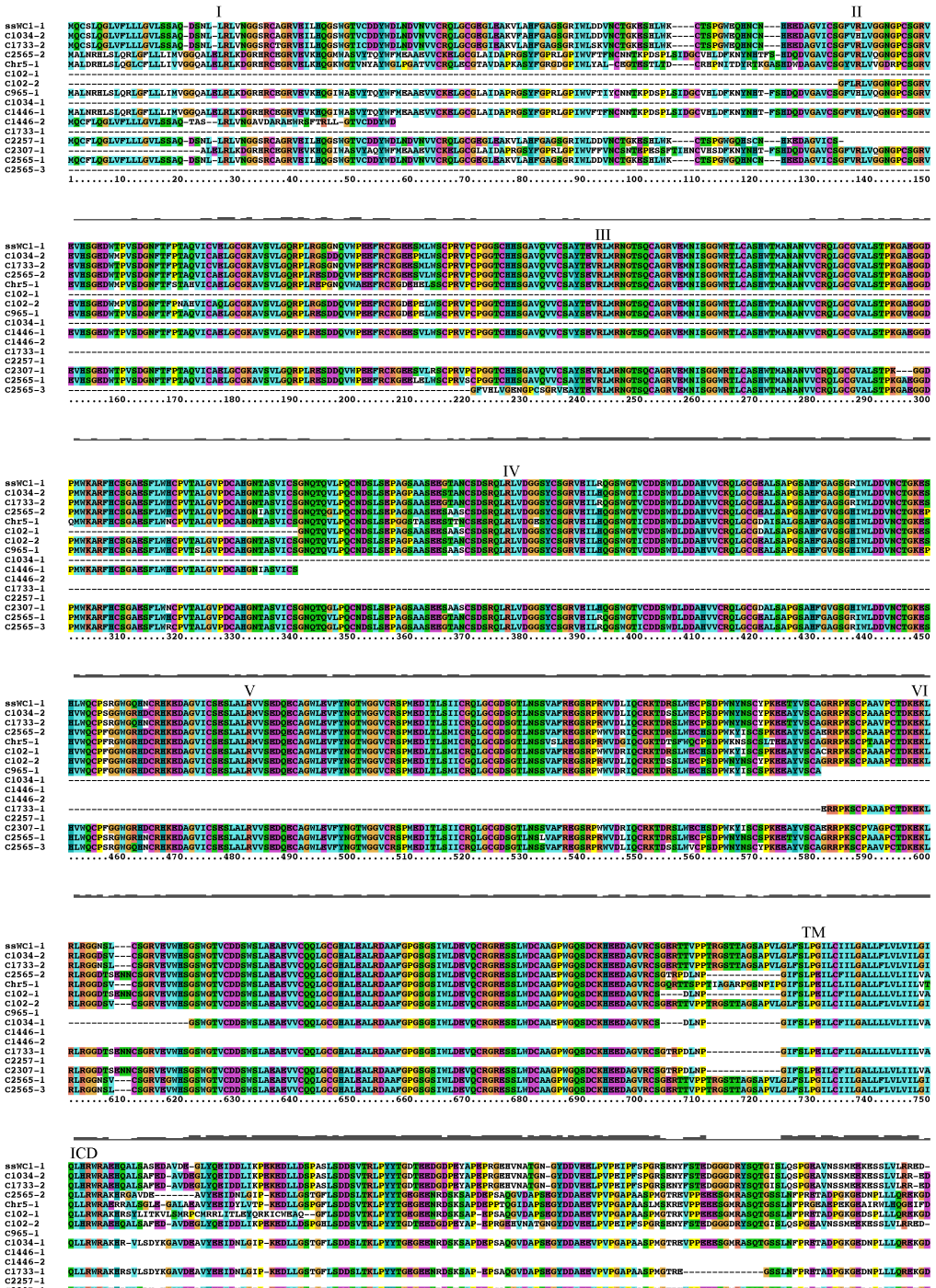
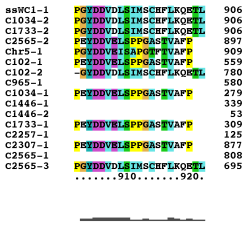


Figure 2.12 Porcine WC1 Cytoplasmic Domains
(A) Deduced amino acid sequences representing the three bovine cytoplasmic domains btWC1-3 (Type I), btWC1-4 (Type I), btWC-9 (Type II) and btWC1-11 (Type III) were aligned with cytoplasmic domain sequences derived from porcine WC1 genes (ssWC1-1, ssWC1-2, ssWC1-3, ssWC1-5, ssWC1-6, ssWC1-7, ssWC1-8 and ssWC1-9) in ClustalX with default parameters. Tyrosine residues found in porcine WC1 cytoplasmic domains are indicated by arrows above the alignment. (B) Deduced amino acid sequences representing the three bovine cytoplasmic domains (bt_Type_I, bt_Type_II and bt_Type_III) were aligned with cDNA sequences representing the four types of porcine WC1 cytoplasmic domains (ss_Type_III, ss_Type_IV, ss_Type_V and ss_Type_VI) in ClustalX with default parameters and a phylogenetic tree was constructed. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [261]. The tree with the highest log likelihood (-1777.45) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 7 amino acid sequences. There was a total of 228 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [262,263].

Supplemental Figure 2.1



Supplemental Figure 2.1 (continued from previous page)




Supplemental Figure 2.1 Alignment of MAKER gene models with ssWC1-1**.**
(A) Deduced amino acid sequences derived from gene models generated with MAKER (C102-1, C102-2, C2257-1, C965-1, C1034-1, C1034-2, C1446-1, C1446-2, C1733-1, C1733-2, C2307-1, C2565-1, C2565-2, C2565-3 and Chr5-1) were aligned with sequence derived from cDNA representing porcine WC1-1 (ssWC1-1). The initial alignment was carried out in ClustalX with default parameters and manual adjustments were made in Bioedit. SRCR domains are indicated in roman numerals above the alignment.

# CHAPTER 3

# SUBPOPULATIONS OF PORCINE γδ T CELLS ARE DEFINED BY WC1 GENE EXPRESSION

A significant portion of this chapter is from the publication: Le Page L, Gillespie A, Schwartz JC, Prawits LM, Schlerka A, Farrell CP, Hammond JA, Baldwin CL, Telfer JC, Hammer SE. "Subpopulations of swine γδ T cells defined by TCRγ and WC1 gene expression." Dev Comp Immunol. 2021 Jul 27;125:104214. doi: 10.1016/j.dci.2021.104214. Epub ahead of print. PMID: 34329647.

## 3.1 Introduction

γδ T cells represent a major portion of lymphocytes in the blood of ruminants and swine [12,13,15,18]. Livestock γδ T cells form a heterogeneous population of functionally diverse subsets which are involved in specific immune responses. Restricted TCR gene usage observed in γδ T cell populations that are localized to specific compartments of the body suggests that they are programmed for recognition of tissue specific antigens [52,145,146]. Expression of pattern recognition receptors such as WC1 further diversifies antigen recognition potential of γδ T cells in these species.

γδ T cell subpopulations in ruminants have been distinguished based on differential expression of WC1 [12,40–42]. The multigenic array of WC1 molecules is defined in cattle to encompass thirteen unique genes [107]. Differential expression of WC1 gene products is observed on distinct subpopulations of bovine γδ T cells, and it is known that WC1 plays an active role in determining what pathogens $WC1^+$ γδ T cells will respond to [13,49,120]. For example, it was found that $WC1.1^+$ γδ T cell respond to *Lepstopira* while $WC1.2^+$ γδ T

cell clones respond to *Anaplasma* [13,74]. Interestingly, both WC1.1$^+$ and WC1.2$^+$ γδ T cell populations share the same TCR restriction utilizing genes from only one TCRγ cassette (Cγ5) while WC1$^-$ γδ T cells are not restricted in TCR gene usage [74,121]. This suggests that expression of WC1 receptors may encode antigen specificity. Additionally, expression of WC1 genes is correlated with unique cytokine expression profiles of γδ T cells in cattle as WC1$^+$ γδ T cells are a significant source of IFN-γ and TNFα.

Prior to our work of defining the multigenic array of WC1 in the porcine model, the genetic diversity of WC1 molecules expressed on porcine γδ T cells had not been explored. It is now known that the WC1 gene family in swine is polygenic, as cDNA and genomic evidence suggests the existence of up to ten unique genes in this species [126]. The influence and correlation of differential WC1 gene usage on porcine γδ T cells regarding cytokine production and pathogen response has not been explored. The ability to identify specific swine γδ T cell populations is essential for understanding functional differences as well as their role in inflammatory pathology and immune protection. Identification of specific lymphocyte subpopulations has improved investigation of the immune response to porcine infections including African swine fever virus, classical swine fever virus and porcine reproductive and respiratory syndrome virus [273–275].

The process of defining porcine γδ T cell subsets relies upon interrogation with monoclonal antibodies which recognize molecules found on the surface of porcine γδ T cells. The success of this effort hinges upon defining the cellular targets of such monoclonal antibodies. The majority of CD2$^-$ γδ T cells in porcine blood co-express the

WC1 orthologue SWC5, and are identified by monoclonal antibodies CC101, PG92a and B37C10. These monoclonal antibodies differentially stain populations of porcine peripheral blood lymphocytes (PBL), however, the exact cellular target(s) of these antibodies remains to be defined [102,105]. Cell surface differentiation markers most used to distinguish subsets of porcine γδ T cells include CD2 and CD8α. These two markers differentiate subsets of γδ T cells found in the lymph nodes and spleen ($CD2^+CD8\alpha^+$ and $CD2^+CD8\alpha^-$) from those found in the blood ($CD2^-CD8\alpha^-$) [16,82,132].

## 3.2 Methods

### 3.2.1 Animals and cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from porcine blood collected at commercial slaughter from normal healthy pigs by centrifugation over Ficoll-Hypaque. Cells were suspended in complete-RPMI medium (RPMI-1640 supplemented with 10 % heat-inactivated fetal bovine serum (v/v; HyClone Laboratories, Logan, UT), 200 mM l-glutamine (Millipore Sigma, Burlington, MA), $5 \times 10^{-5}$ M 2-mercaptoethanol (Sigma) and 10 mg/ml gentamycin (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA)) and viable cell counts were determined by trypan blue exclusion. Lymphatic and non-lymphatic organs were derived from a four-month-old healthy domestic pig housed at the University Clinic for Swine of the University of Veterinary Medicine Vienna. Experimental procedures were performed in accordance with the terms of the institutional ethics committee, the Advisory Committee for Animal Experiments (§12 of Law for Animal Experiments, Tierversuchsgesetz – TVG) and the Federal Ministry for Science and Research. Isolation of PBMC, splenocytes, lung-resident T cells, IEL were as

described previously [150,151,276–278] and intrahepatic lymphocytes (IHL) were isolated by modification of the protocol described by Crispe (2001) [279].

### 3.2.1 Cloning and sequencing of WC1 transcripts

Primers used for PCR in these studies are found in (Table 3.1) cDNA for subsequent PCR targeting *TRGC* and *WC1* genes was generated with the AMV Reverse Transcription kit (Promega Corporation, Madison, WI) with oligo(dT) primers. PCR was performed with the following conditions: 1) 95 °C for 2 min; 2) 95 °C for 30 sec; 3) 55–63 °C for 45 sec; 4) 72 °C for 30 sec; and then steps 2–4 repeated for 30 cycles. Amplicons were viewed on a SYBR™ safe 1 % low melting agarose gel and amplicons of the correct size were extracted and purified with the NEB gel extraction kit (New England Biolabs Inc., Ipswich, MA). Some products were ligated into PCR2.1 using the TOPO TA Cloning Kit (Invitrogen), transformed into *Escherichia coli* DH5α competent cells, plasmids isolated by miniprep (Qiagen, Beverly, MA) and then Sanger sequenced by commercial vendors using M13R and T7 sequencing primers. For some gels, the intensity of the bands was determined by ImageJ (imagej.net) and presented as integrated density.

### 3.2.2 Full length WC1 expression

Full-length porcine WC1-1 and WC1-3 were cloned into vector pBK-CMV at EcoRI and XhoI restriction sites (See Table 2 for primers). Sequences were confirmed using sangar sequencing (Genewiz) prior to transfection. Expi293 cells (ThermoFisher) underwent a minimum of three passages and displayed a viability of at least 95% at the time of transfection. Cells were seeded at 1 x $10^6$ cells/ml in 80ml 24 hours prior to transfection.

The following day, 20 x $10^6$ were transfected with 1.25µg of purified plasmid DNA per 1 x $10^6$ cells using polyethylenimine HCl MAX, linear (40K PEI max 24765–1, Polysciences Inc) and 0.1% Pluronic F-68 (A1288.0100, VWR)[280]. The cells were incubated under standard conditions for three hours and diluted to a final concentration of 1 x $10^6$ cells/mL using pre-warmed Expi293 media (ThermoFisher) supplemented with 3.5 mM valproic acid (P4543, Sigma Aldrich).

### 3.2.3 Immunofluorescence staining

1x$10^6$ - 1x$10^7$ cells were washed three times in FACs buffer (1X PBS, 5% FBS, 0.02% sodium azide). Cells were stained with mAbs and fluorochrome-conjugated secondary antibodies indicated using standard techniques (see Table 3.2 and Table 3.3). Data was acquired using FACS DIVA (BD Biosciences) and analyzed in FlowJo (www.flowjo.com). Cells transfected with empty vector were used as a negative control, as well as unstained cells. Suspension 293s were gated for live cells based on forward (FCS) and side scatter (SSC) profiles. Additional gaiting was performed using unstained cells (Figure 3.1)

### 3.2.4 Recombinant WC1 SRCR proteins

Individual SRCR domains of porcine WC1 genes were PCR amplified using GoTaq Mastermix (Invitrogen) and cloned into pSeqTag2A (Invitrogen) (See table 3 for primers). Sequences were confirmed using sangar sequencing (Genewiz) prior to transfection. 7.5 x $10^8$ cells were transfected with 30µg of purified plasmid DNA using the ExpiFectamine 293 Transfection kit (ThermoFisher). Supernatant was collected for 6 to 8 days, supplemented with 1mM PMSF, 3mM Nickel II Sulfate, 5-10% glycerol and

incubated (end over end rotation) with 200µl Ni-NTA agarose beads (QIAGEN) per 10ml of supernatant overnight at 4°C. The following day, Ni-NTA agarose beads were washed 3 times with a minimum of 30ml of wash buffer (50mM $NH_2PO_4$, 300mM NaCl, 20mM imidazole and 0.05% Tween 20 [pH 8.0]), and incubated with elution buffer (50mM $NH_2PO_4$, 300mM imidazole and 300mM NaCl) for 2 hr at 4°C. Purified protein was concentrated, and elution buffer exchanged for PBS, using Amicon Ultra-15 Centrifugal Filter Units (Millipore, Billerica, MA). Protein was stored at -80°C in a 1:1 ratio with 100% glycerol. Purified protein was quantified by immunoblotting with comparison to a standard using ImageJ.

### 3.2.5 Dot blots

25ng of recombinant protein of individual SRCR domains isolated from ssWC1-1, ssWC1-5, and ssWC1-9 was pipetted directly onto pre-wetted PVDF membrane using a DotBlot manifold. Blots were allowed to stand for twenty minutes at room temperature followed by a rinse in distilled water. The blots were then blocked for 2 hours at room temperature in 5% non-fat milk dissolved in tricine buffered saline supplemented with 0.1% tween-20 (TBS-T) before staining overnight at 4°C with primary mAb (Table 9). The next day, blots were washed in 100mls of 1X TBS-T with a minimum of six buffer changes. The blots were then stained with 1:10,000 goat α-mouse (H + L) conjugated with HRP at room temperature for two hours prior to developing with WesternPico Plus substrate (ThermoFisher).

## 3.3 Results

### 3.3.1 αSWC5 mAbs recognize porcine WC1

In swine, γδ T cells of the blood are subdivided based on the expression of CD2 [132,281]. The majority of $CD2^-$ γδ T cells co-express the molecule SWC5, and are identified by monoclonal antibodies CC101, PG92a and B37C10. Monoclonal antibody CC101 specifically recognizes bovine WC1 molecules and reacts γδ T cells of swine staining ~39.8% of PBL [102,105]. Additionally, it has been shown that CC101 recognizes a 180,000 MW molecule, consistent with the size of porcine WC1, on the majority of $CD2^-CD4^-$ CD8- cells within the blood [105]. PG92a, a monoclonal antibody which reacts with SWC5 molecules on porcine γδ T cells stains ~18.5% of porcine PBL [102]. The αSWC5 monoclonal antibody B37C10 stains ~17.9% of porcine PBL and precipitates a 180,000 MW molecule consistent with porcine WC1 [102,105,282]. While these mAbs recognize the SWC5 molecule, their staining patterns of porcine PBL vary. We hypothesize that this difference in staining is due to differential recognition of WC1 gene products on the surface of porcine γδ T cells.

To evaluate αSWC5 mAb recognition of different porcine WC1 gene products, we individually expressed full-length transcripts encoding two d1-WC1 genes (ssWC1-1 and ssWC1-3) on the surface of suspension 293 cells. Suspension 293s expressing either ssWC1-1 or ssWC1-3 were stained with mAb CC101, B37C10 and PG92A for assessment by flow cytometry. It was found that PG92A did not stain cells from either group (Figure), thus we concluded that this mAb does not recognize ssWC1-1 or ssWC1-

3. CC101 positively stained cells expressing full-length WC1-1 as well as those expressing full-length ssWC1-3 (Figure 13A), and so we concluded that it recognizes both genes. mAb B37C10 differentially stained full-length WC1 gene products as it was found to positively stain cells expressing ssWC1-1, but not those expressing ssWC1-3 (Figure 13B).

### 3.3.2 αSWC5 mAbs differentially recognize WC1 SRCR domains 1 & 3

Porcine WC1 genes are comprised of six extracellular SRCR domains which vary in amino acid composition [53,126]. The N-terminal SRCR domain (a1 or d1 in swine) of WC1 molecules is the most variable, lending each WC1 gene with a unique signature. While there is slight variation within the latter 5 SRCR domains (b2, c3, d4, e5 and d6), these domains are more conserved across genes. Given the results from our flow cytometry experiments, CC101 positively stained cells expressing either ssWC1-1 or ssWC1-3, we hypothesized that this mAb was reacting with a conserved epitope found in one of the latter SRCR domains (b2, c3, d4, e5 and d6) evidenced by its recognition of both gene products. In contrast, mAb B37C10 only recognized cells expressing ssWC1-1, thus we hypothesized it recognized an epitope found in the d1 SRCR domain of ssWC1-1. We sought to probe which SRCR domains were recognized by the mAbs evaluated. To test these hypotheses, a dotblot analysis was performed. Recombinant protein of individual SRCR domains derived from ssWC1-1 (d1-WC1), ssWC1-9 (WC1.1-type), and ssWC1-5 (WC1.2-type) was pipetted directly onto PVDF membrane and the membrane was stained with CC101, B37C10 or PG92A. The αCD163 (2A10/11) was also evaluated for cross reactivity with WC1 in this experiment.

As anticipated, mAb CC101 positively stained c3 SRCR domains derived from ssWC1-5 and ssWC1-9 (Figure 14). We did not possess recombinant protein encompassing the c3 SRCR domain derived from the d1-WC1 gene ssWC1-1, but we expect that this mAb recognizes the c3 domain of ssWC1-1 given our results from the flow cytometry experiments and the conserved nature of the c3 domain (Figure 15). In line with our previous results, mAb B37C10 positively stained SRCR d1 derived from ssWC1-1 and did not stain any of the other SRCR domains that were evaluated (Figure 14). In agreement with our results from the flow cytometry experiment, PG92A did not stain any of the SRCR domains evaluated (data not shown). Additionally, CD163A mAb 2A10/11 did not stain any of the SRCR domains evaluated (Figure 14). However, this is not to say that mAb 2A10/11 doesn't recognize WC1 molecules. While we included a variety of SRCR domains derived from multiple porcine WC1 genes, we did not include recombinant protein for every porcine WC1 SRCR domain in existence. It is possible that this mAb, along with PG92A may recognize SRCR domains derived from other porcine WC1 genes.

### 3.3.3 CD2 and WC1 expression define three major subpopulations of porcine γδ T cells

In ruminants, $CD2^+$ and $CD2^-$ γδ T cells are largely defined as being $WC1^-$ and $WC1^+$ respectively, therefore we hypothesized that this was the case for porcine $CD2^+$ and $CD2^-$ γδ T cells as well [127]. To test this hypothesis, we utilized mAb CC101 and B37C10 which we had shown differentially recognize SRCR domains derived from porcine WC1 genes. PGLB22a, a mAb directed to the γδ TCR, was used to define porcine γδ T cells of the blood. γδ T cells were first evaluated for CD2 expression, and as anticipated there were

both $CD2^+$ and $CD2^-$ cells within the γδ $TCR^{+\ population}$ (Figure 3.6 A). We next looked at WC1 on γδ $TCR^+$ cells using the αSWC5 mAb B37C10, which we had demonstrated reacts with porcine WC1-1. Within the γδ $TCR^+$ population, there were $SWC5^+$ and $SWC5^-$ cells (Figure 3.6 B). In agreement with studies performed on bovine γδ T cells using an αWC1 mAb, WC1 expression was restricted to the $CD2^-$ γδ T cell subset and (Figure 3.6 C). The relative proportion of $CD2^+$ and $CD2^-$ γδ T cells in porcine blood resembled that which is found in ruminants [127]. $WC1^+$ cells accounted for roughly half of the $CD2^-$ population (Figure 3.6 C). We previously determined that mAb CC101 recognizes porcine WC1, thus we sought to evaluate this mAb on porcine PBMC. While CC101 reacted with porcine PBMC, we found that it recognized lymphocytes other than γδ T cells (Figure 3.7), thus it was not used further in these studies. From our prior experiments, we concluded that mAb CC101 recognizes the highly conserved SRCR domain c3 in both WC1.1 and WC1.2-type genes. It's possible that this mAb is reacting with the c3 domain of another group B scavenger receptor CD163A, which possesses a c6 SRCR domain that shares amino acid identity with WC1 c3 SRCR domains (Figure 3.5 A and B). Based on these results, swine peripheral blood γδ T cells were divided into three major subpopulations defined by CD2 and WC1 expression (Figure 3.6 D).

### 3.3.4 TCR and WC1 gene usage by three subpopulations of porcine γδ T cells

Three subpopulations of porcine γδ T cells were defined by CD2 and WC1 expression. To further characterize these subpopulations, we evaluated their WC1 and TCRγ gene usage. We utilized flow cytometric sorting to enrich for the three subpopulations: γδ$TCR^+$/$CD2^-$/$SWC5^-$, γδ$TCR^+$/$CD2^+$/$SWC5^-$, and γδ $TCR^+$/$CD2^-$/$SWC5^+$ (Figure 3.8 A).

Primers were designed to amplify TRGC genes and groups of WC1 gene sequences. The current porcine assembly describes two WC1 genes, however, as described above we have cDNA evidence to suggest nine unique porcine WC1 genes, and genomic evidence to suggest a tenth gene [53,126]. Porcine WC1 genes are defined by the N-terminal SRCR domain, and these N-terminal SRCR domains are subdivided based on amino acid identity into WC1.1-like, WC1.2-like and d1-WC1. Six of the ten putative WC1 genes (ssWC1-5, ssWC1-6, ssWC1-7, ssWC1-8, ssWC1-9 and ssWC1-10) begin with the classic a1 N-terminal SRCR domain previously defined in cattle [119]. Genes possessing the a1 N-terminal SRCR domain can be further subdivided into WC1.1-like or WC1.2-like based on characteristics defined in ruminant WC1 [119,126]. Swine possess two WC1.1-like genes (ssWC1-9 and ssWC1-10) and four WC1.2-like genes (ssWC1-5, ssWC1-6, ssWC1-7 and ssWC1-8) fig. The remaining porcine WC1 genes (ssWC1-1, ssWC1-2, ssWC1-3 and ssWC1-4) begin with an N-terminal d1 SRCR domain as defined by clustering patterns of these domains following phylogenetic analysis [51,126]. Specifically, WC1 primer sets were designed to amplify the porcine WC1 gene subsets d1-WC1, WC1.1-type and WC1.2-type [126]. It's been shown that bovine $WC1^{+}$ γδ T cells can co-express multiple WC1 genes potentially diversifying their ligand recognition potential [163]. Therefore, it was of interest to investigate if this phenomenon was observed in porcine γδ T cells. Primers were tested against porcine PBMC (Figure 3.8 B) and these amplicons were cloned and sequenced to confirm primer specificity (data not shown). We then evaluated the sorted γδ T cell subpopulations for WC1 gene expression. Both $CD2^{-}$ /$SWC5^{-}$ and $CD2^{-}$/$SWC5^{+}$ subpopulations expressed transcripts for WC1 genes (Figure 3.8 C). We previously showed that SWC5 mAb B37C10 positively stained cells

expressing ssWC1-1, but not those expressing ssWC1-3 (Figure 3.3 B.) We also showed that mAb B37C10 recognizes the most unique N-terminal SRCR domain, d1, derived from ssWC1-1 (Figure 3.4). mAb B37C10 was not found to recognize other WC1 SRCR domains that were evaluated. Additionally, the fact that this mAb recognizes the d1-SRCR domain of ssWC1-1, but not the d1-SRCR domain of ssWC1-3 suggests that the epitope is highly specific. The primers used to evaluate the subpopulations of γδ T cells were designed to be pan-reactive based on WC1 gene subset. Therefore, the fact that the $CD2^-/SWC5^-$ population was found to express transcripts for all three WC1 gene subsets despite its lack of recognition by mAb B37C10 is not surprising. The $CD2^+$ γδ T cells were WC1 negative, which is in agreement with studies done in ruminants regarding CD2 expression (Figure 3.8 C) [127]. The two $CD2^-$ subpopulations ($SWC5^+$ and $SWC5^-$) were also distinguishable by WC1 receptor expression. The $SWC5^+$ population possessed a strong band of amplicons when WC1.1a1 and d1-WC1 primers were used while the $SWC5^-$ cells only had a strong amplicon band when pan-reactive a1 primers were used. Based on our previous results in studies regarding B37C10 recognition of WC1 genes, we can confidently say that the $CD2^-SWC5^-$ population does not express ssWC1-1. While it is tempting to assume that the $CD2^-SWC5^+$ population expressed ssWC1-1, we cannot definitively say this is the case. While B37C10 did not recognize the d1-SRCR domain of ssWC1-3, it is possible that B37C10 may recognize the d1 SRCR domain derived from ssWC1-2, as it shares more amino acid identities with ssWC1-1 than ssWC1-3 (Figure 3.9 A and B).

Regarding TCRγ gene usage, the two $CD2^-$ populations ($SWC5^+$ and $SWC5^-$) possessed strong bands of amplicons representing transcription of genes within the TRGC1 cassette, but also had amplicons representing transcripts for other TRGC genes (Figure 3.8 C). $CD2^+$ γδ T cells had stronger amplicon bands representing transcription of TRGC2/3 genes in addition to transcripts for TRGC1. No transcripts were found for TRGC4. Counting this with the WC1 gene expression results and the phenotypic differences regarding CD2 and SWC5 expression, these results further suggest that these populations represent distinct subpopulations of porcine γδ T cells.

### 3.4 Discussion

γδ T cells constitute a major portion of lymphocytes in the blood of swine. Defining porcine γδ T cell subsets requires interrogation with monoclonal antibodies that recognize molecules on the surface of porcine γδ T cells. To date, subpopulations of porcine γδ T cells are primarily distinguished based on CD2 and CD8α expression. A large portion of $CD2^-$ γδ T cells in porcine blood co-express the WC1 orthologue SWC5, and are identified by monoclonal antibodies CC101, PG92a and B37C10. Prior work has shown that CC101 recognizes a 180,000 MW molecule consistent with the size of porcine WC1 on the majority of $CD2^-CD4^-CD8^-$ cells in porcine PBMC [105]. The αSWC5 monoclonal antibody B37C10 also precipitates a 180,000 MW molecule that is consistent with the size porcine WC1 [102,105,282]. Despite this knowledge, the molecular targets of these mAbs had not been confirmed. We hypothesized that the differential staining patterns of porcine PBL presented by these three mAb were due to their recognition of different WC1 genes on the surface of porcine γδ T cells.

We showed that two mAbs against the SWC5 molecule expressed on porcine γδ T cells, B37C10 and CC101, differentially recognize porcine WC1 gene products. In our hands, mAb PG92A did not positively stain any of the WC1 genes that were evaluated. While mAb CC101 positively stained cells expressing full length ssWC1-1 and ssWC1-3 gene products, B37C10 stained cells expressing ssWC1-1 only. The differential recognition of swine WC1 genes by these mAb was further interrogated by evaluating recognition of individual SRCR domains. It was found that mAb CC101 recognizes the highly conserved SRCR domain c3 in both WC1.1 and WC1.2-type genes. While th c3 SRCR domains of ssWC1-1 and ssWC1-3 were not tested in this analysis, given our results from the flow analysis of full-length WC1 gene recognition, it is likely that CC101 recognizes the SRCR c3 domain of these two genes as well. It was also found that mAb CC101 reacts with molecules expressed on non-γδ T cells. It's possible that this mAb is reacting with the c3 domain of another group B scavenger receptor CD163A, which possesses a c6 SRCR domain that shares amino acid identity with WC1 c3 SRCR domains [51]. In contrast, mAb B37C10 recognized the more diverse N-terminal SRCR domain d1 derived from ssWC1-1.

In cattle, γδ T cell subpopulations are distinguished by differential expression of the hybrid coreceptor and pattern recognition receptor WC1. WC1 is expressed as a multigenic array that is comprised of 13 unique genes in this species. Within the $WC1^{+}$ population of bovine γδ T cells, subpopulations are distinguished by expression of specific WC1 genes and this correlates with their ability to respond to pathogens. We had

previously determined that WC1 in swine is expressed as a multigenic array, and we estimate that there are 10 unique genes within this family. Expression of WC1 genes has not been evaluated on swine γδ T cells. We sought to determine if swine γδ T cells could be divided into subpopulations based on WC1 gene expression. In ruminant $WC1^+$ γδ T cell populations, TRGC gene expression is restricted to those within the *TRGC5*-containing cassette [121,283]. Thus, we asked if restricted use of TRG genes occurs in swine $WC1^+$ γδ T cells.

Using the SWC5 mAb B37C10, which we had confirmed recognizes the d1 SRCR domain derived from ssWC1-1, in conjunction with CD2 we defined three populations of peripheral blood γδ T cells. The three subpopulations include $CD2^+/SWC5^-$, $CD2^-/SWC5^+$ and $CD2^-/SWC5^-$ γδ T cells. In line with prior results obtained from ruminant species, SWC5 expression was only observed on the $CD2^-$ population of γδ T cells. To further characterize the three subpopulations, we evaluated their WC1 and TCRγ gene usage through RT-PCR analysis. Within the $CD2^-$ populations of peripheral blood γδ T cells ($SWC5^+$ and $SWC5^-$) we found varying patterns of WC1 gene expression. Expression of WC1 genes by $SWC5^-$ γδ T cells can be explained by the fact that mAb B37C10 recognizes the most diverse SRCR domain, SRCR domain 1 of the d1-WC1 gene ssWC1-1. Thus, the epitope recognized by B37C10 is highly specific. While B37C10 was confirmed to recognize ssWC1-1, it did not recognize another WC1-d1 gene ssWC1-3. cDNA and genomic evidence support the existence of up to ten WC1 genes in swine, therefore we can definitively say that $CD2^-SWC5^-$ cells express WC1 genes which are not recognized by mAb B37C10. Regarding TCRγ usage, both $CD2^-$ populations of γδ

T cells displayed evidence of transcription of genes in the *TRGC1* cassette. Of note, these cells also displayed evidence of amplicons representing transcripts for genes within casette *TRGC2/3* albeit at lower levels. In contrast, $CD2^{+}$ cells possessed stronger amplicon bands representing transcription of the *TRGC2/3* gene locus in addition to transcripts for *TRGC1*.

Table 3. 1 RT-PCR Primers for amplifying swine TRGC and WC1

| Set | Primer # | Gene | Orientation | Sequence (5'→3') | Size (bp) |
|---|---|---|---|---|---|
| A | 1 | TRGC2 | fwd | TATTGGAAAGAAAAGAATG(G/A)C | - |
| | 2 | TRGC1 | rev | CACCACTGTCCCTCAGTGTC | 760 |
| | 3 | TRGC2 | rev | TTTCTGGGTTTGGCTTC(G/A)TTCAGAG | 601 |
| B | 4 | WC1.1 a1 | fwd | TGAAGGGACAGAGTCAACTCTCACTG | 411 |
| | 5 | Pan WC1 d1 | fwd | CTCCGCCTGGTGAATGGGGGCAGT | 611 |
| | 6 | WC1.2 a1 | fwd | CTGCAACAATACCAAGCCAGATT | 423 |
| | 7 | Pan WC1 a1 | fwd | CTCGAGCTGAGGCTGAAGGATGGA | 617/629 |
| | 8 | Pan WC1 b2 | rev | GAACAGACAACTTGAACAGCTCCACTGTGG | - |
| C | 9 | Pan TRGC | fwd | CATGAAATTCAGCTGGGTGACCTGAA | - |
| | 10 | TRGC1 | rev | GGCGCTACAAGACTGTTGTTTCTCGAAA | 290 |
| | 11 | TRGC2+3 | rev | CCCACTGCTGCAGACTGTTCTCCTAAA | 404 |
| | 12 | TRGC4 | rev | CTTTTTTGGAATCAGTAACAGTGACTTCA | 260 |

Table 3. 2 Primary mAbs used for immunofluorescence staining and dot blots

| mAb Name | Target | Species[1] | Isotype |
|---|---|---|---|
| PGLB22A | γδ TCR | Swine | IgG1 |
| PPT27 | γδ T cell subset | Swine | IgG1 |
| GB21a | TCRδ | Bovine | IgG2b |
| MSA4 | CD2 | Swine | IgG2a |
| CC101 | WC1 | Bovine | IgG2a |
| PG92a | SWC5 | Swine | IgM |
| B37C10-AF647 | SWC5 | Swine | IgG1 |
| B37C10 | SWC5 | Swine | IgG1 |
| 2A10/11 | CD163 | Swine | IgG1 |
| LND68A | CD163 | Bovine | IgG1 |
| α-myc 9e10 | myc epitope | n/a | IgG1 |

Table 3. 3 Secondary mAbs for immunofluorescence staining and dot blots

| Isotype Target | Conjugate | Used With |
|---|---|---|
| murine IgG1 HC[2] | RPE | B37C10, 2A10/11 |
| murine IgG2a | AF488 | CC101 |
| murine IgG2a | FITC | CC101 |
| murine IgG2a | PE | CC101 |
| murine IgM | PE | PG92a |
| murine IgG HC + LC[3] | HRP | α-myc 9e10 |

[1] Refers to the target species
[2] HC = Heavy Chain
[3] LC = Light Chain

Figure 3.1

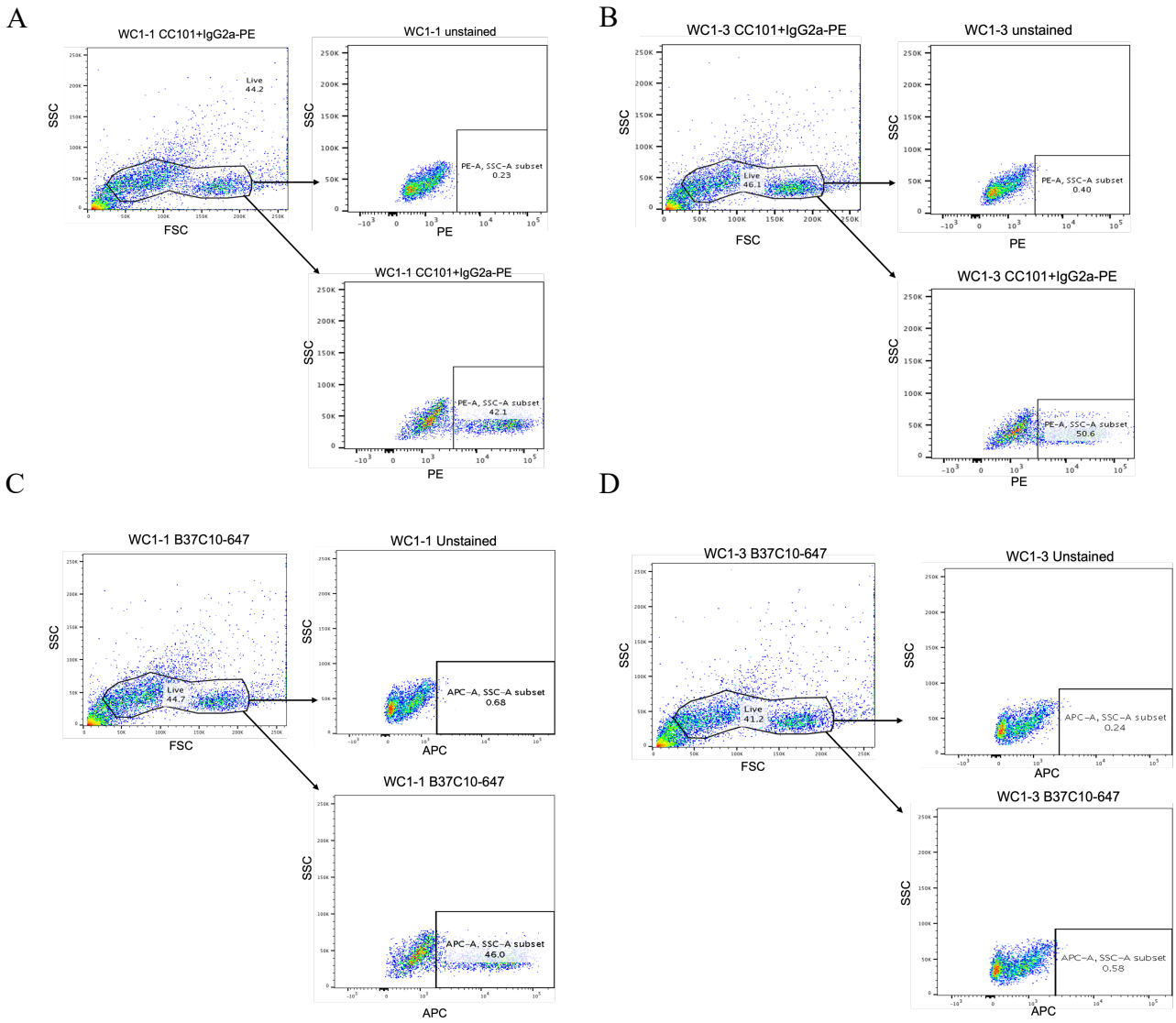

A
WC1-1 CC101+IgG2a-PE
Live
44.2
SSC
FSC
WC1-1 unstained
PE-A, SSC-A subset
0.23
PE
WC1-1 CC101+IgG2a-PE
PE-A, SSC-A subset
42.1
PE
B
WC1-3 CC101+IgG2a-PE
Live
46.1
SSC
FSC
WC1-3 unstained
PE-A, SSC-A subset
0.40
PE
WC1-3 CC101+IgG2a-PE
PE-A, SSC-A subset
50.6
PE
C
WC1-1 B37C10-647
Live
44.7
SSC
FSC
WC1-1 Unstained
APC-A, SSC-A subset
0.68
APC
WC1-1 B37C10-647
APC-A, SSC-A subset
46.0
APC
D
WC1-3 B37C10-647
Live
41.2
SSC
FSC
WC1-3 Unstained
APC-A, SSC-A subset
0.24
APC
WC1-3 B37C10-647
APC-A, SSC-A subset
0.58
APC

Figure 3. 1 Expression of full length WC1 on Expi293s

Suspension 293s were gated for live cells based on forward (FCS) and side scatter (SSC) profiles. Additional gating was performed using unstained cells. (A) Cells expressing full length ssWC1-1 were stained with mAb CC101 and secondary ab αIgG2a-PE. (B) Cells expressing full length ssWC1-3 were stained with mAb CC101 (αSWC5) and secondary ab αIgG2a-PE. (C) Cells expressing full length WC1-1 were stained with mAb B37C10 (αSWC5) directly conjugated with APC. (D) Cells expressing full length WC1-3 were stained with mAb B37C10 directly conjugated with APC.

Figure 3.2

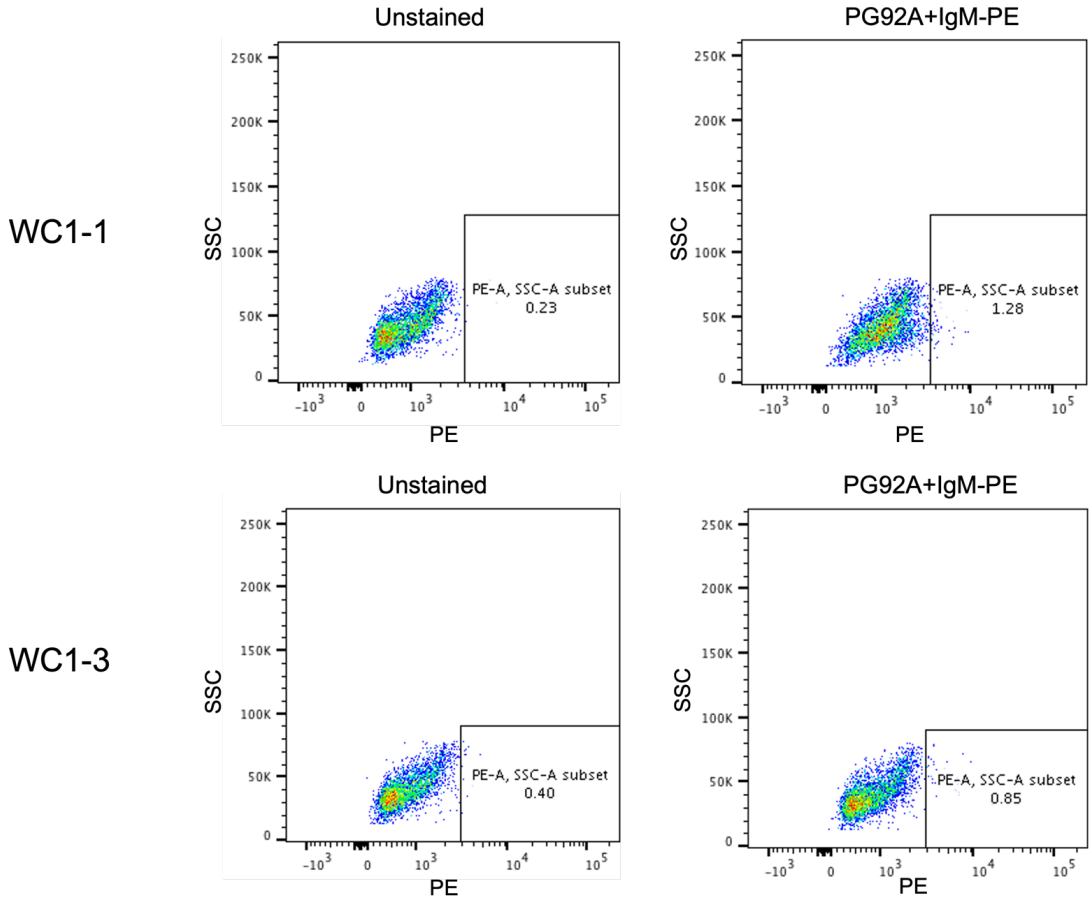
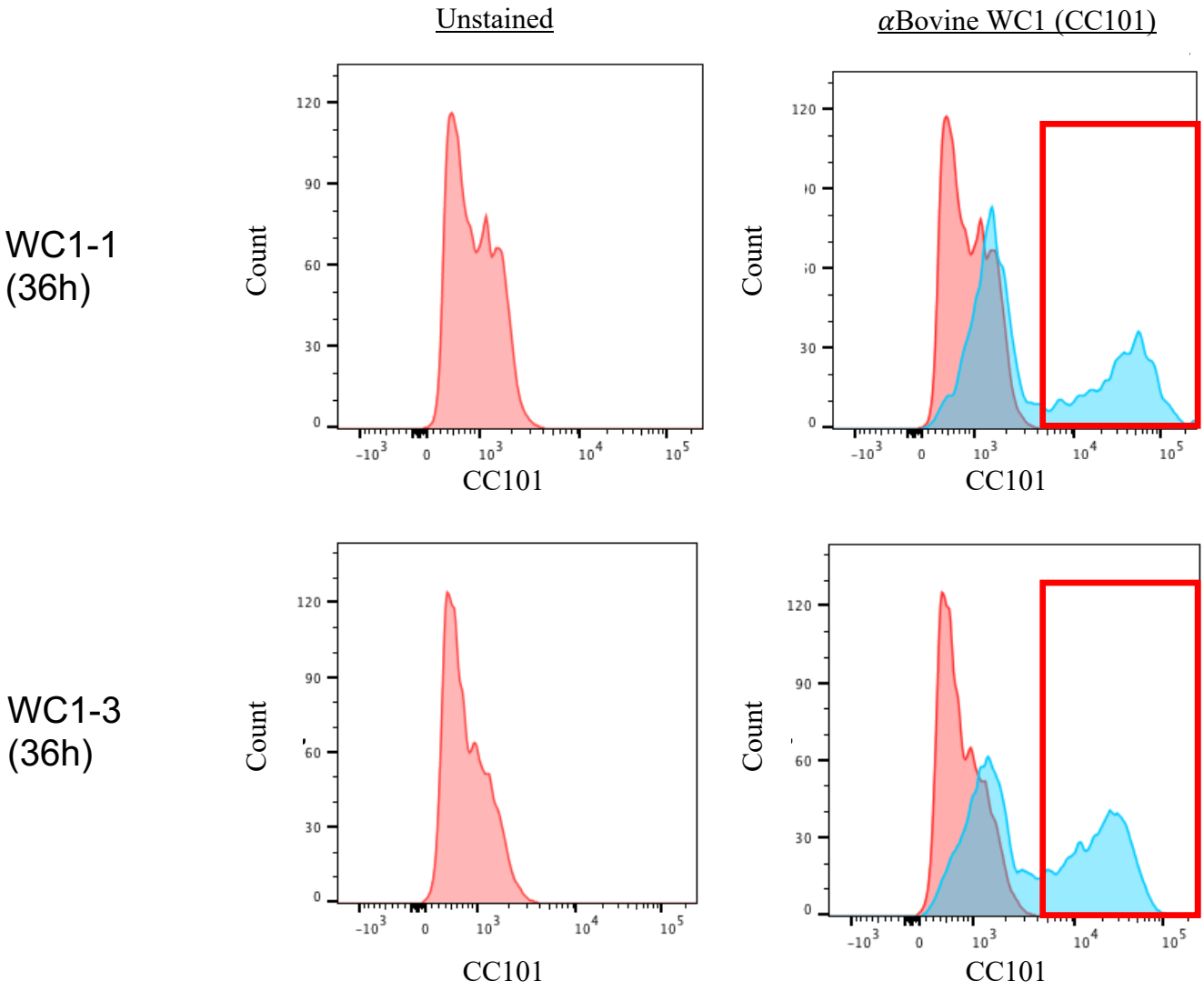



Figure 3. 2 mAb PG92A does not recognize ssWC1-1 or ssWC1-3
See Figure 3.1 for gates. Cells expressing full length ssWC1-1 or full length ssWC1-3 were stained with mAb PG92A (αSWC5) and secondary ab αIgM-PE.

Figure 3. 3

A



B

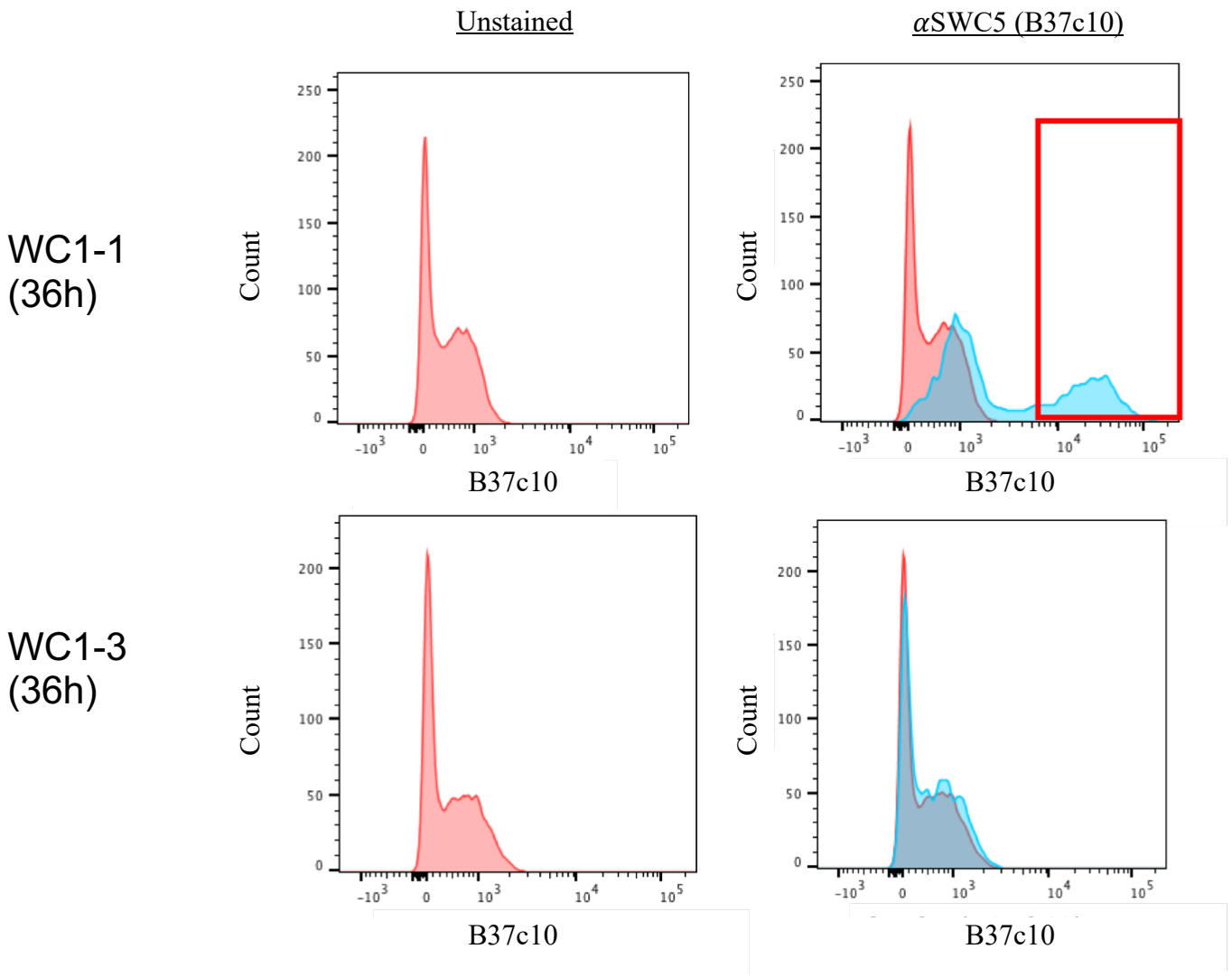



Figure 3. 3 mAb CC101 and B37C10 recognize full length WC1 expressed on the surface of 293 cells

See Figure 3.1 for gates. (A) Cells expressing full length ssWC1-1 or full length ssWC1-3 were stained with mAb CC101 (αSWC5) and secondary ab αIgG2a-PE. (B) Cells expressing full length ssWC1-1 or full length ssWC1-3 were stained with mAb B37C10 (αSWC5) directly conjugated with APC.

Figure 3.4

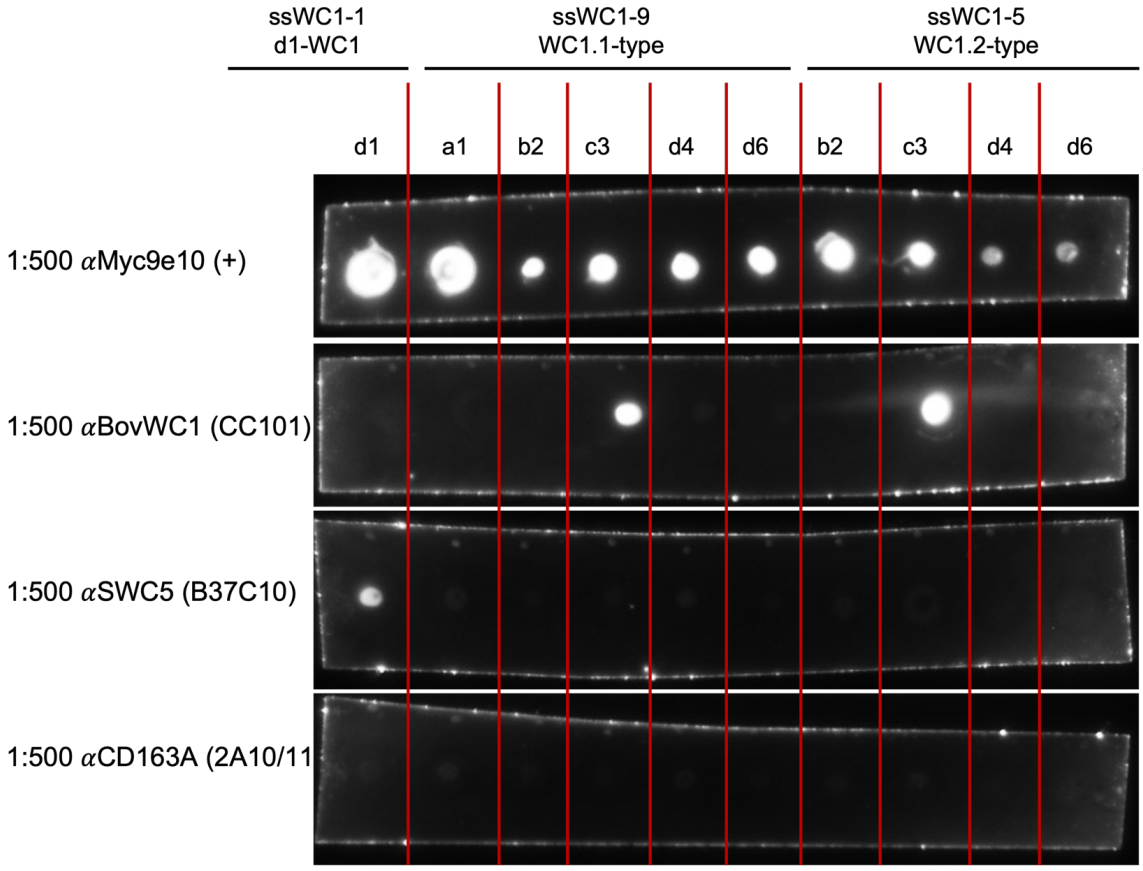
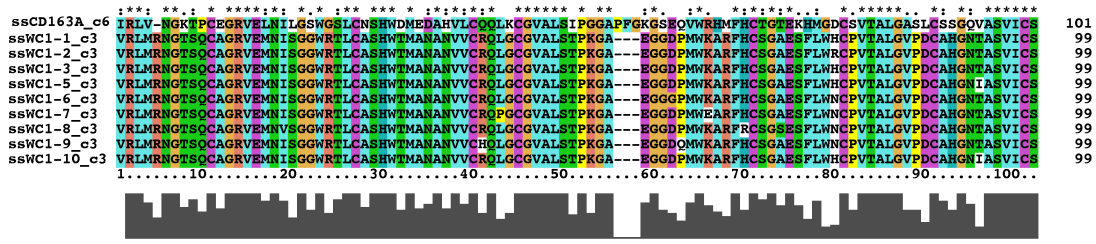



Figure 3. 4 mAb B37C10 and CC101 differentially recognize SRCR domains 1 and 3 of porcine WC1

5ng of recombinant protein encompassing individual SRCR domains derived from swine WC1 genes (indicated above the photograph) were pipetted directly onto pre-wetted PVDF membrane and incubated at room temperature for 20 minutes. The blots were blocked for 2 hours at room temperature in 5% non-fat milk. The blots were then incubated with respective primary mAbs (indicated on to the left of the photograph) overnight in 5% non-fat milk at 4ºC. Secondary staining with goat αmouse IgG(H+L)-HRP conjugated mAb was carried out for 2 hours at room temperature. Blots were developed using West Pico PLUS chemiluminescent substrate (ThermoFisher).

Figure 3.5

A


ssCD163A_c6 IRLV-NGKTPCEGRVELNILGSWGSLCNSHWDMEDAHVLCQQLKCGVALSIPGGAPFGKGSEQVWRHMFHCTGTEKHMGDCSVTALGASLCSSGQVASVICS 101
ssWC1-1_c3 VRLMRNGTSQCAGRVEMNISGGWRTLCASHWTMANANVVCRQLGCGVALSTPKGA---EGGDPMWKARFHCSGAESFLWHCPVTALGVPDCAHGNTASVICS 99
ssWC1-2_c3 VRLMRNGTSQCAGRVEMNISGGWRTLCASHWTMANANVVCRQLGCGVALSTPKGA---EGGDPMWKARFHCSGAESFLWRCPVTALGVPDCAHGNTASVICS 99
ssWC1-3_c3 VRLMRNGTSQCAGRVEMNISGGWRTLCASHWTMANANVVCRQLGCGVALSTPKGA---EGGDPMWKARFHCSGAESFLWHCPVTALGVPDCAHGNTASVICS 99
ssWC1-5_c3 VRLMRNGTSQCAGRVEMNISGGWRTLCASHWTMANANVVCRQLGCGVALSTPKGA---EGGDPMWKARFHCSGAESFLWHCPVTALGVPDCAHGNIASVICS 99
ssWC1-6_c3 VRLMRNGTSQCAGRVEMNISGGWRTLCASHWTMANANVVCRQLGCGVALSTPKGA---EGGGPMWKARFHCSGAESFLWNCPVTALGVPDCAHGNTASVICS 99
ssWC1-7_c3 VRLMRNGTSQCAGRVEMNISGGWRTLCASHWTMANANVVCRQPGCGVALSTPKGA---EGGDPMWEARFHCSGAESFLWNCPVTALGVPDCAHGNTASVICS 99
ssWC1-8_c3 VRLMRNGTSQCAGRVEMNVSGGWRTLCASHWTMANANVVCRQLGCGVALSTPKGA---EGGDPMWKARFRCSGSESFLWNCPVTALGVPDCAHGNTASVICS 99
ssWC1-9_c3 VRLMRNGTSQCAGRVEMNISGGWRTLCASHWTMANANVVCHQLGCGVALSTPKGA---EGGDQMWKARFHCSGAESFLWNCPVTALGVPDCAHGNTASVICS 99
ssWC1-10_c3 VRLMRNGTSQCAGRVEMNISGGWRTLCASHWTMANANVVCRQLGCGVALSTPKGA---EGGDPMWKARFHCSGAESFLWHCPVTALGVPDCAHGNIASVICS 99
1.....10.....20.....30.....40.....50.....60.....70.....80.....90.....100..

B

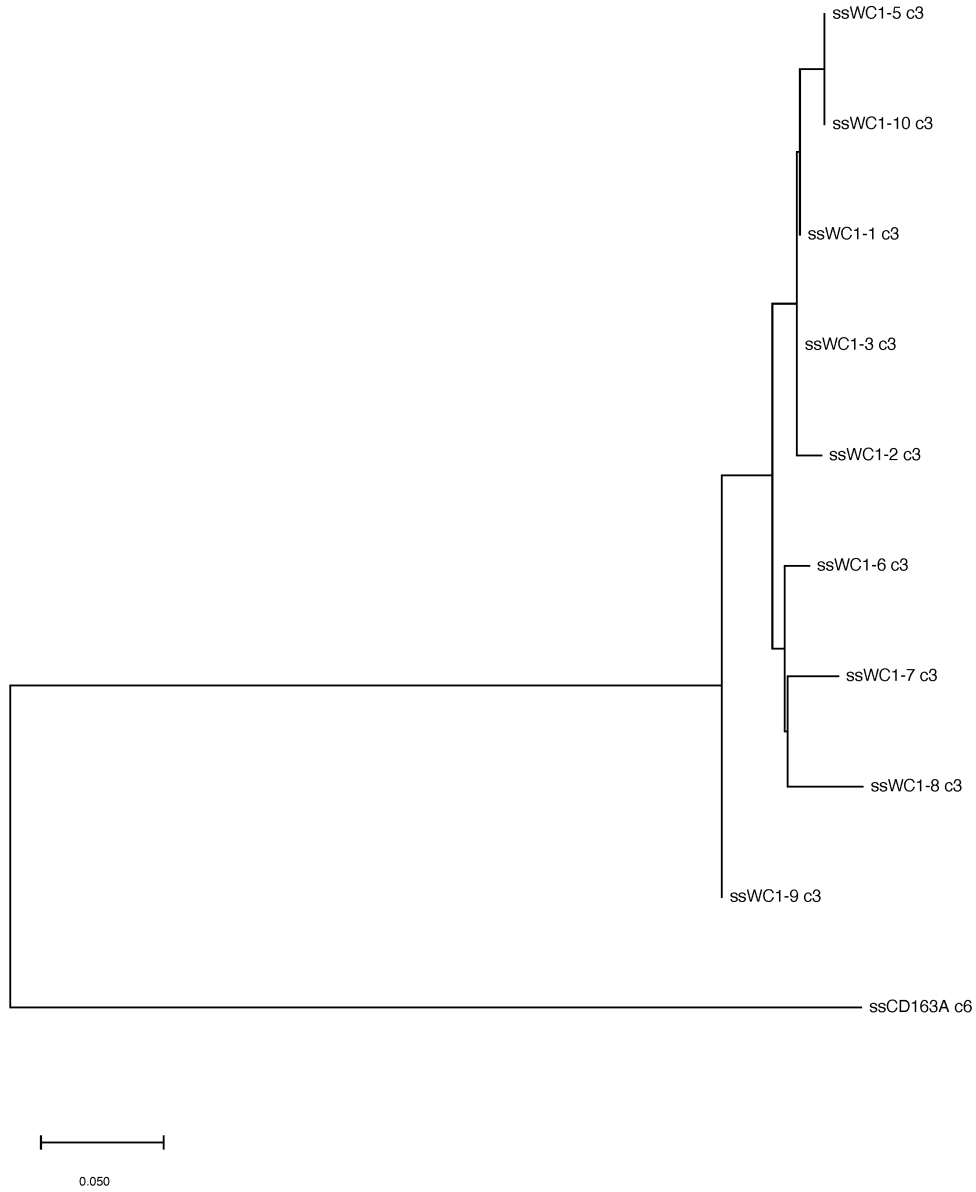

ssWC1-5 c3
ssWC1-10 c3
ssWC1-1 c3
ssWC1-3 c3
ssWC1-2 c3
ssWC1-6 c3
ssWC1-7 c3
ssWC1-8 c3
ssWC1-9 c3
ssCD163A c6
0.050

Figure 3. 5 SRCR domain c3 is highly conserved.
(A) Deduced amino acid sequences representing SRCR c3 domain derived from porcine WC1 genes (ssWC1-1, ssWC1-2, ssWC1-3, ssWC-5, ssWC1-6, ssWC1-7, ssWC1-8, ssWC1-9 and ssWC1-10) were aligned with SRCR domain c6 from porcine CD163A (ssCD163A_c6) using ClustalX with default parameters. (B) A phylogenetic tree was constructed from the alignment in part (A). The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [261]. The tree with the highest log likelihood (-551.04) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 10 amino acid sequences. There was a total of 102 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [262,263].

Figure 3.6

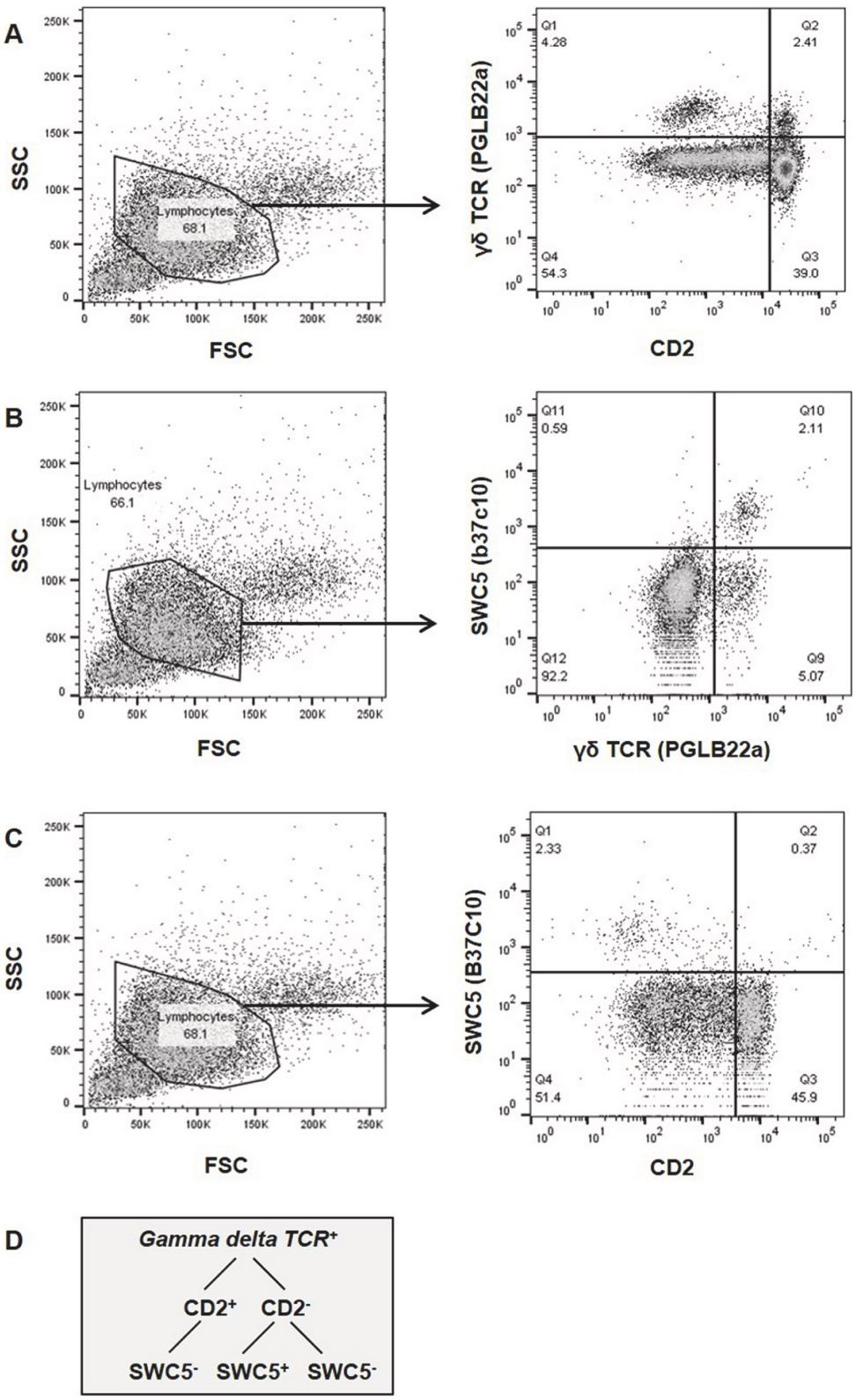

A
SSC
FSC
Lymphocytes
68.1
γδ TCR (PGLB22a)
CD2
Q1
4.28
Q2
2.41
Q4
54.3
Q3
39.0
B
SSC
FSC
Lymphocytes
66.1
SWC5 (b37c10)
γδ TCR (PGLB22a)
Q11
0.59
Q10
2.11
Q12
92.2
Q9
5.07
C
SSC
FSC
Lymphocytes
68.1
SWC5 (B37C10)
CD2
Q1
2.33
Q2
0.37
Q4
51.4
Q3
45.9
D
Gamma delta TCR+
CD2+
CD2-
SWC5-
SWC5+
SWC5-

Figure 3. 6  Flow cytometry of swine γδ T cell populations.
Flow cytometry of swine γδ T cell subpopulations. Swine PBMC were gated on lymphocytes according to forward (FSC) and side scatter (SSC) profiles and stained by immunofluorescence with: (A) mAb PGLB22A (α-γδTCR) and secondary Ab αIgG1-FITC and mAb MSA4 (αCD2) with secondary Ab αIgG2a-PE; (B) mAb b37b10-AF647 (αSWC5) and mAb PGLB22a (α-γδTCR) with secondary Ab αIgG1-FITC; or (C) mAb b37c10-AF647 (αSWC5) and mAb MSA4 (αCD2) with secondary Ab αIgG2a-FITC. (D) Diagram of the γδ T cell subpopulations defined by mAb staining. See also Fig. 3.6A for precise definition of the 3 populations.

From: Le Page L, Gillespie A, Schwartz JC, Prawits LM, Schlerka A, Farrell CP, Hammond JA, Baldwin CL, Telfer JC, Hammer SE.  "Subpopulations of swine γδ T cells defined by TCRγ and WC1 gene expression." Dev Comp Immunol. 2021 Jul 27;125:104214. doi: 10.1016/j.dci.2021.104214. Epub ahead of print. PMID: 34329647.

Figure 3.7

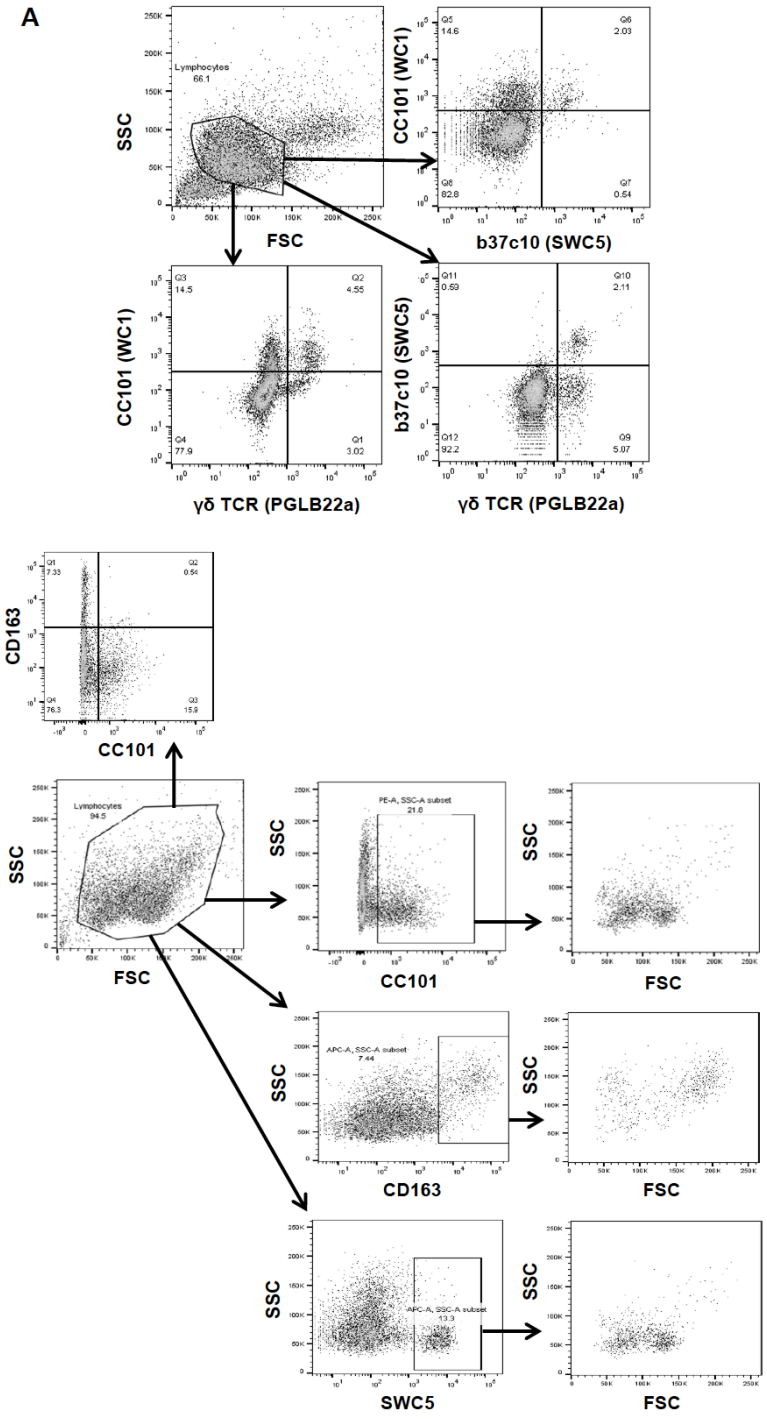
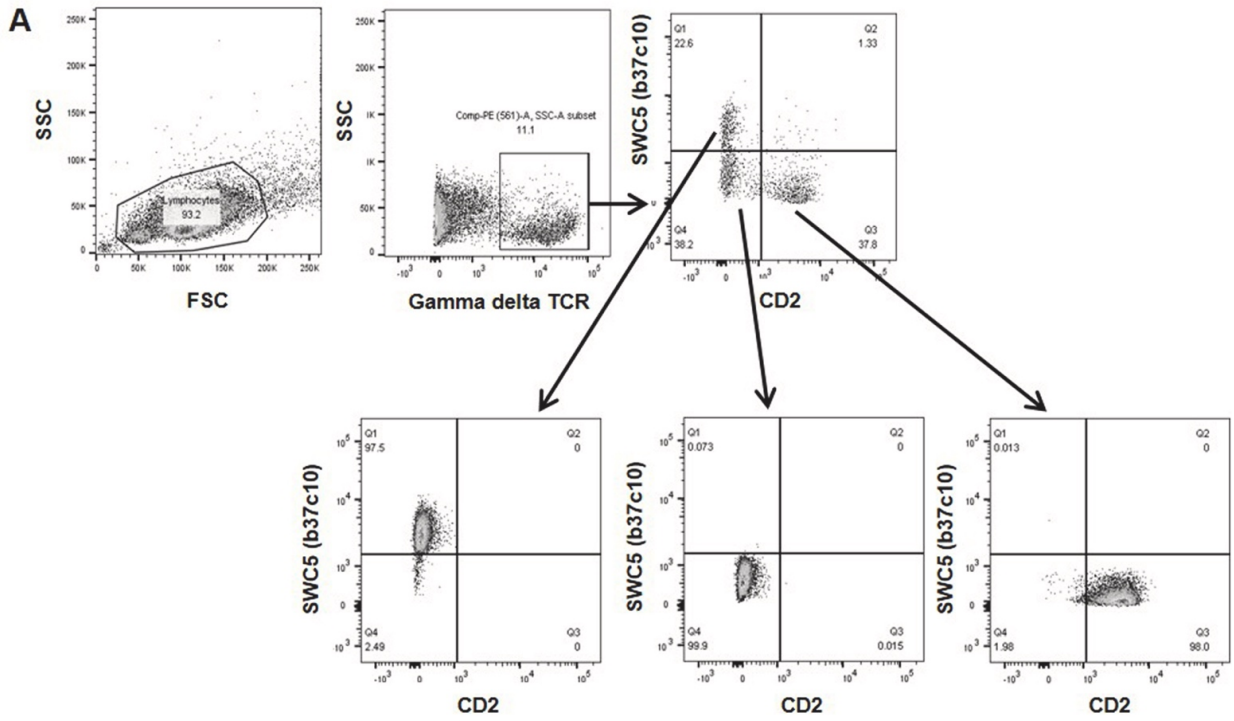
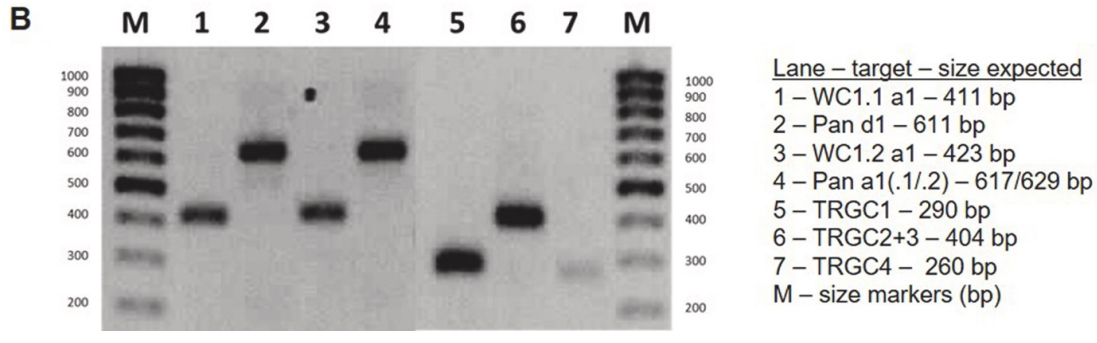

A
SSC
FSC
Lymphocytes
66.1
CC101 (WC1)
b37c10 (SWC5)
γδ TCR (PGLB22a)
CD163
CC101
Lymphocytes
94.5
PE-A, SSC-A subset
21.6
APC-A, SSC-A subset
7.44
APC-A, SSC-A subset
13.3
SWC5

Figure 3. 7 Ligand of mAb CC101 investigated with swine PBMC.
Flow cytometry of porcine PBMC gated on lymphocytes based on forward (FCS) and side scatter (SSC) and stained with mAb B37C10-AF647 (αSWC5), mAb PGLB22A (α-γδ TCR) with secondary αIgG1-FITC antibody and mAb CC101 with secondary αIgG2a-PE antibody. For CD163A staining, αCD163A mAb 2A10/11 was used.

From: Le Page L, Gillespie A, Schwartz JC, Prawits LM, Schlerka A, Farrell CP, Hammond JA, Baldwin CL, Telfer JC, Hammer SE. "Subpopulations of swine γδ T cells defined by TCRγ and WC1 gene expression." Dev Comp Immunol. 2021 Jul 27;125:104214. doi: 10.1016/j.dci.2021.104214. Epub ahead of print. PMID: 34329647.

Figure 3.8




Lane – target – size expected
1 – WC1.1 a1 – 411 bp
2 – Pan d1 – 611 bp
3 – WC1.2 a1 – 423 bp
4 – Pan a1(.1/.2) – 617/629 bp
5 – TRGC1 – 290 bp
6 – TRGC2+3 – 404 bp
7 – TRGC4 – 260 bp
M – size markers (bp)

Figure 3.8

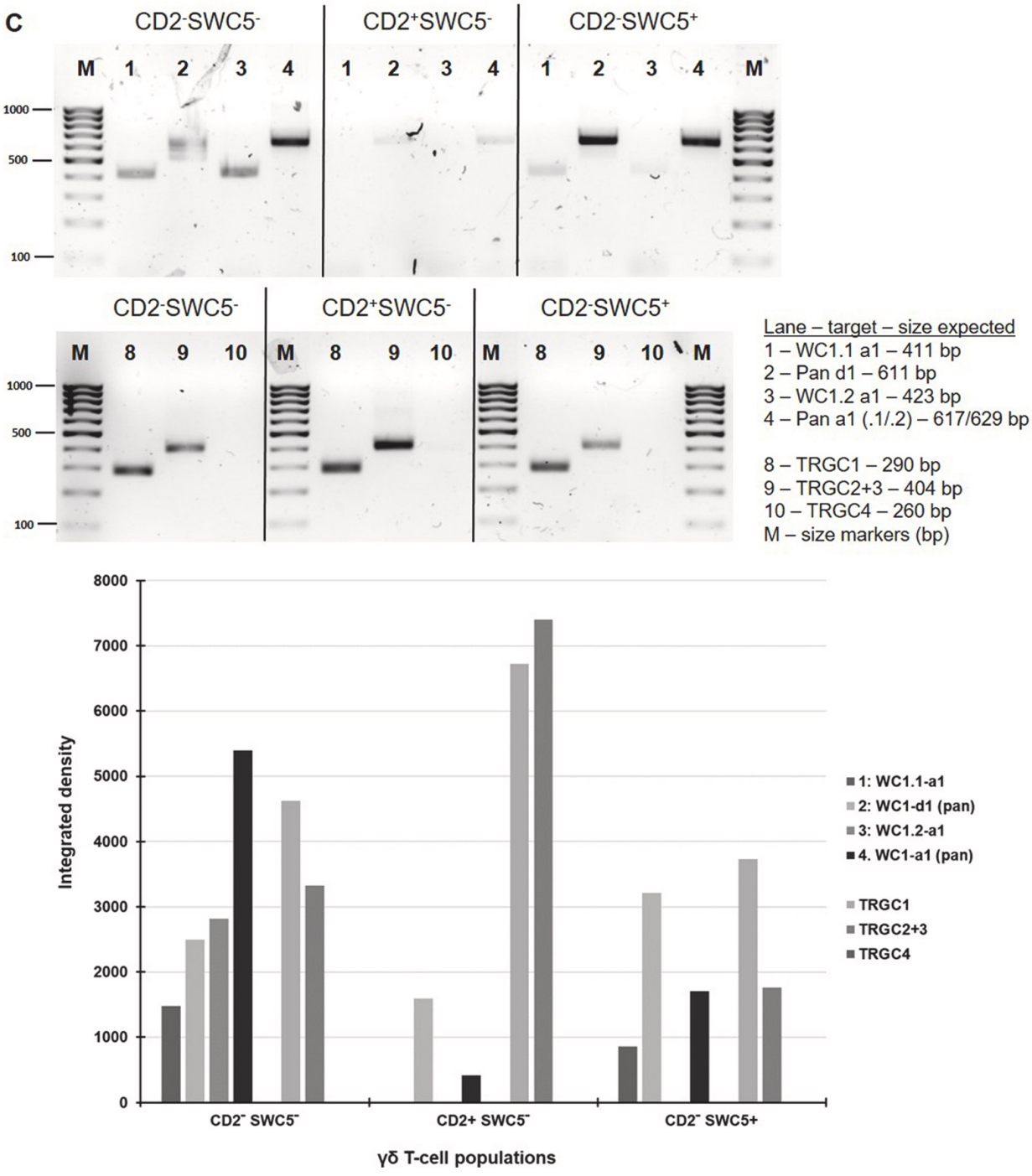
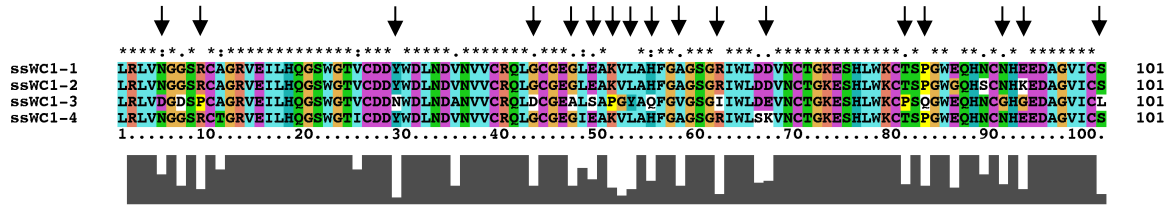

C
CD2-SWC5-
CD2+SWC5-
CD2-SWC5+
M 1 2 3 4 1 2 3 4 1 2 3 4 M
1000
500
100
CD2-SWC5-
CD2+SWC5-
CD2-SWC5+
M 8 9 10 M 8 9 10 M 8 9 10 M
1000
500
100
Lane – target – size expected
1 – WC1.1 a1 – 411 bp
2 – Pan d1 – 611 bp
3 – WC1.2 a1 – 423 bp
4 – Pan a1 (.1/.2) – 617/629 bp
8 – TRGC1 – 290 bp
9 – TRGC2+3 – 404 bp
10 – TRGC4 – 260 bp
M – size markers (bp)
Integrated density
8000
7000
6000
5000
4000
3000
2000
1000
0
CD2- SWC5-
CD2+ SWC5-
CD2- SWC5+
γδ T-cell populations
1: WC1.1-a1
2: WC1-d1 (pan)
3: WC1.2-a1
4. WC1-a1 (pan)
TRGC1
TRGC2+3
TRGC4

Figure 3. 8 TCRγ and WC1 gene expression by subpopulations of porcine γδ T cells in the blood.
A) PBMCwere gated by forward (FSC) and side scatter (SSC) and then by expression of γδ TCR followed by CD2and/or SWC5 expression. They were sorted by flow cytometry, as shown, into three populations. These were used in experiments to evaluate TCRγ and WC1 gene transcription below. (B) Qualitative analysis to test primers and expression of WC1 and TRGC genes in porcine PBMC using the primer combinations shown in Table 3.1 (C) RT-PCR of WC1 and TRGC genes in porcine SWC5 and CD2-defined γδ T cell subpopulations. Summary table of the results of the semi-quantitative expression of WC1 and TRGC genes in porcine SWC5/CD2-defined γδ T cells is shown below with lane number corresponding to the sample order in B and C above along with the integrated density readings of the gel.

From: Le Page L, Gillespie A, Schwartz JC, Prawits LM, Schlerka A, Farrell CP, Hammond JA, Baldwin CL, Telfer JC, Hammer SE. "Subpopulations of swine γδ T cells defined by TCRγ and WC1 gene expression." Dev Comp Immunol. 2021 Jul 27;125:104214. doi: 10.1016/j.dci.2021.104214. Epub ahead of print. PMID: 34329647.

Figure 3.9

A




B

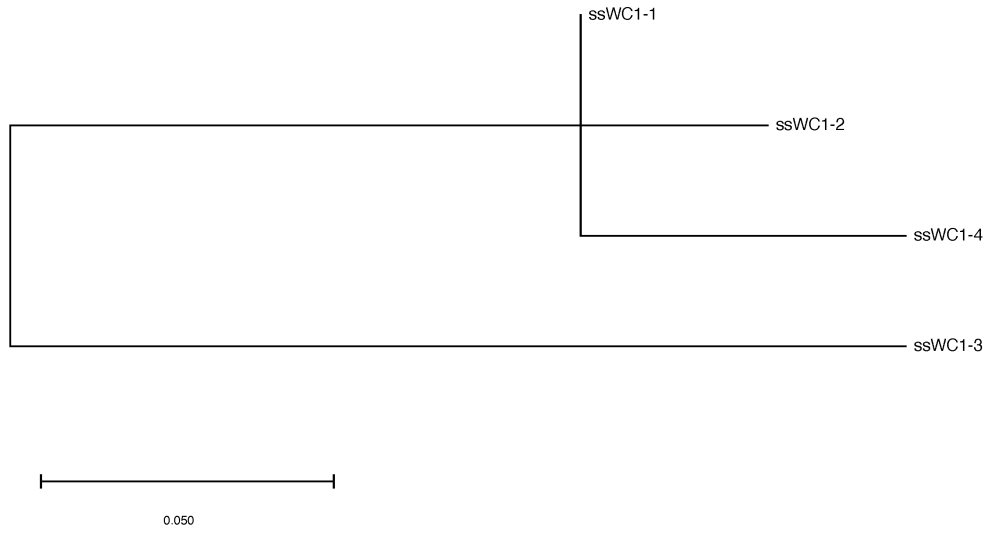



Figure 3. 9 Comparison of d1 SRCR domains
(A) Deduced amino acid sequences representing SRCR domain d1 derived from porcine WC1 genes (ssWC1-1, ssWC1-2, ssWC1-3 and ssWC1-4) were aligned using ClustalX with default parameters. Areas of divergence between ssWC1-1 and ssWC1-3 are indicated with arrows above the alignment. (B) A phylogenetic tree was constructed from the alignment in part (A). The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [261]. The tree with the highest log likelihood (-455.32) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 4 amino acid sequences. There was a total of 101 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [262,263]

# CHAPTER 4

# PATHOGEN BINDING POTENTIAL OF PORCINE WC1 SRCR DOMAINS

## 4.1 Introduction

γδ T cells participate in the immune response to infection with Gram-negative [284,285] and Gram-positive bacteria [286] through the production of effector molecules such as IFN-γ and TNFα [287,288]. Moreover, γδ T cells demonstrate memory responses to *Leptospira* and *Mycobacteria* [195,289]. Despite their established role in cellular immunity, the mechanisms by which γδ T cell activation occurs are not well understood. Unlike the αβ TCR, which interacts with peptides presented in the context of MHC molecules, the γδ TCR interacts directly with ligand [61,62,66]. However, the γδ TCR signals through the CD3 complex, thus establishing a requirement for its ligand to be restrained. In some cases, this requirement is achieved through the γδ TCR interacting with unconventional antigens such as MHC-related T22, MHC class I polypeptide-related sequence (MIC), and the lipid-presenting MHC CD1d molecules [59,62,290,291]. In another example, the γδ TCR interacts with a transmembrane protein butyrophilin 3A (BTN3A), which undergoes conformational changes in its extracellular domains following intracellular binding of phosphoantigens (pAgs) such as endogenous isopentenyl pyrophosphate (IPP) and the (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) [292–294]. IPP is a non-peptide intermediate of the mevalonate pathway, and its accumulation within cells indicates malignant transformation [295]. HMBPP is an intermediate of the non-mevalonate pathway employed by prokaryotes, including mycobacteria [296]. Conformational changes in the extracellular

portion of BTN3A occur in response to elevated intracellular levels of pAgs and allow for its direct interaction with the non-complementary-determining region (CDR3) portion of the γδ TCR [297].

Coreceptors, such as CD8, which is expressed on αβ T cells, are known to potentiate activation of T cells through direct binding with MHC class I [298]. However, classic examples of coreceptors, such a CD8 and CD4, do not confer specificity for ligands but increase TCR affinity for peptide plus MHC. In contrast, pattern recognition receptors (PRR), often expressed on the surface of innate immune cells, are proteins capable of directly binding to molecules derived from pathogens called Pathogen Associated Molecular Patterns (PAMPs) [299]. Previously, we demonstrated that WC1 functions as a hybrid coreceptor and PRR for the γδ TCR in cattle [49]. Bovine $WC1^+$ γδ T cell populations are serologically defined in $WC1.1^+$ or $WC1.2^+$ γδ T cells based on the specific WC1 genes that they express, and we've also shown that WC1 gene expression plays a determining role in the pathogen responsiveness of $WC1^+$ γδ T cells. [44,119]. That is, $WC1.1^+$ bovine γδ T cells, specifically those expressing btWC1-3, respond to *Leptospira spp.* In contrast, $WC1.2^+$ γδ T cells expressing btWC1-4 do respond [43]. This was later correlated with btWC1-3 directly binding to *Leptospira spp* via its SRCR domains [49]. Mutational analysis of the *Leptospira spp.* binding SRCR domains derived from btWC1-3 revealed that single amino acid residues within the SRCR domain convey differential pathogen binding ability [49]. We have recently determined that SRCR domains of the bovine WC1.2-type gene, btWC1-4, directly bind to *Mycobacterium bovis* BCG Danish and Pasteur strains (unpublished data). Because $WC1^+$ γδ T cells share a restricted

set of TCR genes, yet respond to different pathogens, we hypothesize that WC1 gene expression plays the determining role in which pathogens $WC1^+$ γδ T cells will respond to.

Like cattle, swine belong to the order Artiodactyl and maintain a large subset of $WC1^+$ γδ T cells. Additionally, swine are also susceptible to infection with Leptospira and Mycobacterium. Porcine γδ T cells mount a Th1-like immune response characterized by lymphoproliferation and IFN-γ production in response to vaccination with BCG [25]. In young pigs vaccinated with BCG, stimulation with mycobacterial antigens induces γδ T cell proliferation and IFN-γ production [25]. Given this, we were interested in applying the bovine model of WC1 hybrid coreceptor and PRR activity to porcine WC1.

## 4.2 Methods

### 4.2.1 Bacterial culture

*Escher coli* (DH10B; Invitrogen) was cultured in Luria-Bertani (LB) broth at 37°C, 250rpm overnight. Cell count was determined by diluting samples 1:10 in LB, measuring the OD 600, and converting via the algorithm 1.0 OD 600 = $8x10^{8}$ CFU/ml prior to fixing. *M. Bovis* BCG Danish and Pasteur strains were cultured in Middlebrook 7H9 Liquid Medium (Difco catalog no. 0.713-01-7) supplemented with 10% oleic acid-dextrose-catalase enrichment (Middlebrook OADC Enrichment, cat no 212351) and 0.05% Tween-80. For all experiments, cells were fixed in neutral buffered formalin (10% formaldehyde) and stored at 4ºC. Fixed *M. Bovis* samples were diluted at 1:10 in TBS-Ca Buffer and quantified using the algorithm 1.0 OD 600 = 3.13 x $10^{7}$ CFU/ml [300]. *Leptospira*

*borgpetersenii* serovar Hardjo-bovis from Spirovac (Zoetis, Florham Park, NJ) was stored at 4ºC and used in all experiments involving Leptospira.

### 4.2.2 Expression, purification, and quantification of recombinant WC1 SRCR proteins

Individual SRCR domains from each WC1 gene found in *Sus scrofa* were PCR amplified using GoTaq Mastermix (Invitrogen) and cloned into pSeqTag2A (Invitrogen). Sequences were confirmed (GeneWiz) before transfection. 7.5 x $10^8$ cells were transfected with 30µg of purified plasmid DNA using the ExpiFectamine 293 Transfection kit (ThermoFisher). The supernatant was collected for 6 to 8 days, supplemented with 1mM PMSF, 3mM Nickel II Sulfate, 5-10% glycerol, and incubated with 200µl Ni-NTA agarose beads (QIAGEN) per 10ml of supernatant overnight at 4°C. The following day, Ni-NTA agarose beads were washed three times with wash buffer (50mM $NH_2PO_4$, 300mM NaCl, 20mM imidazole, and 0.05% Tween 20 [pH 8.0]) and incubated with elution buffer (50mM $NH_2PO_4$, 300mM imidazole, and 300mM NaCl) for 2 hr at 4°C. Purified protein was concentrated, and elution buffer was exchanged for PBS, using Amicon Ultra-15 Centrifugal Filter Units (Millipore, Billerica, MA). Protein was stored at -80°C in a 1:1 ratio with 100% glycerol. Purified protein was quantified by immunoblotting and comparison to standard using ImageJ.

### 4.2.3 Bacterial binding assay and immunoblotting

*E. coli* and *M. Bovis* BCG strains Danish, and Pasteur were fixed in neutral buffered formalin (10% Formaldehyde) at room temperature for 2 hr, or 4°C overnight. Prior to the binding assays, fixed bacteria were washed twice with TBS-Ca Buffer (20mM Tris,

150mM NaCl, and 5mM $CaCl_2$). Bacterial pull-down assays were carried out as described previously [49]. Briefly, individual SRCR domains (5-10ng) were incubated with 1 x $10^7$ bacteria in 400µl of TBS-Ca Buffer plus 1% BSA, rotating end over end for 1 hour at 4°C. Quantification of bacteria is described above. The bacterial pellet was washed twice with TBS-Ca Buffer plus 1% BSA and once with TBS-Ca Buffer alone. The bacterial pellet was resuspended in 20µl of 2X SDS-PAGE sample buffer containing 2-Mercaptoethanol and boiled at 95°C for 5 min. Bacteria were pelleted by centrifugation at 16,000 x g for two minutes, and the supernatant containing the eluted protein was resolved on a 12% SDS-PAGE gel at 200V. Western blots were performed with a constant amperage of 350mA for one hour or 20mA overnight at 4°C. WC1 SRCR domains were detected by hybridization with a 1:20 dilution of anti-Myc antibody (clone 9e10) in 5% non-fat milk, incubated overnight at 4°C with constant agitation, and followed by secondary staining with 1:10,000 dilution of HRP-conjugated goat anti-mouse mAb (Biorad) for 1 hour at room temperature with constant rocking. Blots were developed using either Clarity Western ECL blotting substrate (Biorad) or ECL Western blotting detection reagents (GE Healthcare Life Sciences, Pittsburgh, PA). E. coli was used as a negative control as no WC1 SRCR domains (in cattle or swine) are known to bind to E. coli.

### 4.2.4 Far Western Blot analysis

Bacterial sonicates were treated at 1 x $10^7$ /ml with 50µg/ml of Proteinase K at 37°C for 1 hour, followed by a wash and treatment with 5mM PMSF at 37°C for 0.5 hours. Whole cell sonicates (WCS), and Proteinase-K treated sonicates were loaded onto a 12% SDS-page gel. The SDS page gel was run at 200 constant voltage for sixty minutes. The

sonicates were then transferred to PVDF membrane at 350mA for one hour. The resulting blots were blocked with 5% non-fat milk dissolved in 1X Tris-buffered saline with 0.1% Tween-20 (TBS-T) for two hours at room temperature. Blots were subsequently probed with 10ng of myc-tagged porcine or bovine recombinant WC1 SRCR proteins in 5% non-fat milk dissolved in 1X TBST overnight at 4$^{o}$C. Each blot was probed with recombinant protein encompassing a single SRCR domain. Blots were then washed in 100mLs of 1X TBS-T with a total of six buffer changes. After washing, blots were stained with 1:500 murine-derived α-myc 9e10 mAb (Cousin Lab, Umass Amherst) in 5% non-fat milk dissolved in 1X TBS-T overnight at 4$^{o}$C. The following day, blots were washed in 100mLs of 1X TBS-T with a total of six buffer changes. The blots were then stained with 1:10,000 goat α-mouse HRP conjugated antibody (Biorad) for two hours at room temperature, and wash steps were repeated. After washing, blots were developed using Clarity Western ECL blotting substrate (Biorad).

## 4.3 Results

### 4.3.1 Porcine WC1 SRCR domains differentially bind bacterial pathogens

We have previously shown that bovine WC1 functions as a hybrid coreceptor and PRR for the γδ TCR via direct interaction with bacteria [49]. The ability of WC1 to function as a coreceptor is dependent upon phosphorylation of specific tyrosine residues in its cytoplasmic domain [48,125]. As discussed in Chapter 1 of this dissertation, porcine WC1 genes possess multiple tyrosine residues in the cytoplasmic domain, which may be phosphorylated to potentiate activation through the γδ TCR [126]. Therefore, we wanted to

investigate the pathogen binding potential of SRCR domains derived from porcine WC1 to evaluate if this molecule can function as a PRR for the γδ TCR. To assess the pathogen binding potential of porcine WC1 SRCR domains, we utilized the bacterial binding pull-down assay (Figure 4.1 A) [49]. In this assay, individual recombinant WC1 SRCR proteins labeled with a Myc epitope are incubated with *L. borgpetersenii* serovar Hardjo-bovis from Spirovac (Zoetis, Florham Park, NJ) or *M. bovis* BCG Danish or Pasteur strain. Following incubation, the bacterial pellet is precipitated through centrifugation and washed several times to remove non-specifically bound recombinant protein. Recombinant SRCR proteins which bind to the bacteria of interest will remain bound to the bacterial pellet through subsequent wash steps. After washing, the bacterial pellet is reduced and boiled, and the supernatant is run on an SDS page gel, transferred to PVDF, and blotted with anti-Myc antibody.

A total of eight porcine WC1 SRCR domains were evaluated for binding to *E. coli* and *L. borgpetersenii* serovar Hardjo-bovis. The selected SRCR domains were chosen so that both WC1.1-type and WC1.2-type molecules were represented in the analysis. Ideally, the analysis would have included a1 SRCR domains derived from both WC1.1 and WC1.2-type molecules. However, an a1 SRCR domain derived from a WC1.2-type gene was not available. As anticipated, none of the SRCR domains tested were found to bind to *E. coli; however*, all eight bound to *L. borgpetersenii* serovar Hardjo-bovis (Figure 4.1 B). Four porcine WC1 SRCR domains were evaluated for binding to BCG Danish and Pasteur strains (Figure 4.1C). Of the four SRCR domains assessed, two were found to

bind BCG danish strain only (ssWC1-1 d1 and ssWC1-9 c3) while the remaining two domains bound both BCG Danish and Pasteur strains (ssWC-9 d4 and ssWC1-5 d6).

### 4.3.2 WC1 SRCR domains bind Proteinase-K treated bacterial sonicates

We have illustrated that SRCR domains of porcine and bovine WC1 molecules can directly bind to bacteria, including BCG Danish and Pasteur strains. However, we are unsure if the ligand recognized by WC1 molecules is a protein or non-protein antigen. To interrogate potential WC1 ligands, we performed a Far Western Blot analysis with WCS and Proteinase-K treated sonicates of *M. avium paratuberculosis* strains K10 and 3988 and *M. bovis* strains BCG and 1315. Proteinase-K is a broad-spectrum serine protease with the ability to digest native proteins [301]. We hypothesized that binding activity would be reduced by Proteinase-K treatment if the WC1 ligand was indeed proteinaceous. Interestingly, our results were quite different than we had anticipated. In the case of btWC1-4a1, btC1-12b7, and ssWC1-9 b2, we observed binding activity with the WCS for *M. bovis* BCG strain only, which emerged as a band between 20 and 25 kDa (Figure 4.2). All three SRCR domains demonstrated binding activity with Proteinase-K treated *M. avium paratuberculosis* strains K10 and 3988, as well as *M. bovis* strain 1315, but not with WCS (Figure 4.2). The binding activity of the Proteinase-K treated cell sonicates presented as a band between 25 and 37 kDa for all three SRCR domains evaluated. An additional band between 15 and 20 kDa was observed on the blot probed with btWC1-12b7 (Figure 4.2). These results suggest that the ligand is not proteinaceous, and in this specific context, treatment with Proteinase-K may have revealed an inaccessible epitope in the WCS.

## 4.4 Discussion

As discussed in Chapter 1 of this dissertation, γδ T cells have a demonstrated role in relevant infectious diseases which impact livestock production and human health. Engaging these cells through vaccination or immunomodulatory strategies may provide substantial benefits in the face of such diseases. Methods to prime γδ T cells with vaccine constructs might exploit features of γδ T cell activation, such as their utilization of PRR molecules. The WC1 hybrid coreceptor and PRR plays a multi-faceted role in the response of γδ T cells to bacterial pathogens. Thus we hypothesize that it may serve as a target to recruit specific subpopulations of γδ T cells with vaccine constructs and potentially increase γδ T cell participation following vaccination. Identifying specific ligands engaged by WC1 molecules may allow us to design vaccine constructs that specifically recruit $WC1^+$ γδ T cells. Here we've shown that individual SRCR domains derived from porcine and bovine WC1 molecules can directly bind *Leptospira spp*, *M. avium paratuberculosis,* and *M. bovis*. WC1 molecules are likely capable of binding to other pathogens that infect livestock, and the fact that WC1 is expressed as a multigenic array offers vast potential in this regard.

Porcine WC1 SRCR domains found only to bind BCG Danish likely interact with a ligand that is not found in BCG Pasteur. In contrast, porcine WC1 SRCR domains that bind to both BCG Danish and Pasteur strains probably recognize a ligand found in both strains. Our results demonstrate that porcine WC1 molecules interact with multiple ligands found on *M. bovis* BCG. In vitro, bovine γδ T cells proliferate and produce IFN-γ in recall responses to complex antigens like PPD-B and specific antigens like the protein

complex ESAT6:CFP10 and nonprotein antigen mAGP [68,69,302,303]. Bovine WC1$^+$ γδ T cell populations are serologically defined in WC1.1$^+$ or WC1.2$^+$ γδ T cells based on the specific WC1 genes that they express [44,119]. While WC1.1$^+$ γδ T cells produce IFN-γ in response to *L. borgpetersenii,* WC1.2$^+$ γδ T cell subsets produce IL-10 in response to mitogen stimulation [13,43,47,99]. However, WC1.2$^+$ γδ T cells produce IFN-γ in specific responses to *Anaplasma marginale* demonstrating some functional plasticity within this subset which may depend upon PRR stimulation [304]. While higher numbers of WC1.1$^+$ γδ T cells are recruited to the lungs and lymph nodes of animals inoculated with *M. bovis* BCG, both WC1.1$^+$ and WC1.2$^+$ γδ T cell subsets accumulate in vivo at sites of pulmonary *M. bovis* lesions [75,305]. Additionally, it was found that both WC1.1$^+$ and WC1.2$^+$ γδ T cell subsets produce cytokines in response to in vitro stimulation of mycobacterial antigens [75]. While porcine γδ T cells proliferate and produce IFN-γ following vaccination with BCG, the expression status of WC1 molecules on these cells is unknown [25]. Analysis of porcine γδ T cell populations in the context of WC1 gene expression has been hampered by a lack of defined mAbs that recognize WC1 molecules on the surface of porcine γδ T cells. While we have confirmed that two available mAbs (B37C10 and CC101) recognize porcine WC1 molecules, more work is needed to clarify which WC1 molecules are specifically identified. Here, we found that both porcine WC1.1-type SRCR domains and WC1.2-type SRCR domains were capable of binding to *M. bovis* BCG Danish and Pasteur strains. Given this, it is reasonable to hypothesize that both WC1.1$^+$ and WC1.2$^+$ porcine γδ T cells participate in the response to BCG, like what is observed in cattle. Future work may entail dividing porcine γδ T cells into subsets

based on WC1 gene expression and evaluating their participation in response to stimulation with BCG.

It was recently shown that WC1 receptors colocalize with the γδ TCR upon activation [248]. It was also found that *Leptospira* spirochetes bound specifically to WC1 on the surface of γδ T cells, supporting the concept that WC1, along with the TCR and ligand, form a signaling domain upon engagement [248]. These findings may indicate that both WC1 and the TCR bind the ligand together, or interaction between WC1 and the TCR occurs following WC1 ligand binding. The latter may represent a system similar to butyrophilin (BTN) molecules found on human γδ T cells, which bind to the antigen and then interact directly with germline-encoded portions of the TCR [249,250]. BTNs are expressed as a multigene family, and it has been found that multiple BTN molecules (encoding different genes) are involved in the activation of γδ T cells [249,251]. Upon antigen binding, BTN2A1 associates with BTN3A1, and together they initiate activation of γδ T cells. First, BTN2A1 binds to the Vγ9 TCRγ chain, followed by the binding of a second ligand, possibly BTN3A1, to a separate TCR domain within Vδ2 [249]. The mode of γδ T cell activation employed by BTNs is especially interesting when considering the fact that individual bovine γδ T cells express up to six variants of WC1 [163]. Moreover, it was shown that WC1 variants remain separated on resting γδ T cells, clustering together only with homologous WC1 molecules, but upon activation, islands containing different variants coalesce and merge with the γδ TCR islands [248]. It is also possible that WC1 functions more closely to coreceptors like CD4 and CD8 but through direct engagement

with the same ligand recognized by the γδ TCR instead of MHC molecules. To evaluate this possibility, more work is needed to identify the exact ligands of WC1 molecules.

While no specific ligands have been identified to date, experiments have been carried out to characterize the nature of the WC1 ligand in the *Leptospira* spp. model. Multiple SRCR domains derived from bovine WC1 bind to *Leptospira* spp., and pre-treatment of the bacteria with Proteinase-K does not diminish binding activity [49]. From this, we can conclude that the WC1 ligand found on *Leptospira* spp. is not a protein. Experiments using polymyxin B to block LPS signaling could also rule out LPS as a potential ligand [49]. Here, through bacterial binding assays using Proteinase-K treated WCS, we have demonstrated that at least one of the WC1 ligands found on *M. avium paratuberculosis* and *M. bovis* is non-proteinaceous. More work is needed to confirm the identity of such ligands.

Our bacterial binding assays comparing porcine WC1 SRCR domain binding to *M. bovis* BCG Danish and Pasteur strains may shed some light regarding this. Of the four SRCR domains evaluated, two bind BCG danish strain only (ssWC1-1 d1 and ssWC1-9 c3) while the remaining two domains bound both BCG Danish and Pasteur strains (ssWC-9 d4 and ssWC1-5 d6). Multiple daughter strains of BCG vaccines exist due to genetic changes incurred during repeated subculture in laboratories throughout the world before the introduction of lyophilization [306]. Studies focused on comparative genomics have uncovered coding regions present in *M. tuberculosis* complex that are absent from *M. bovis* BCG daughter strains [307]. Gene segments that vary in BCG daughter strains are

called regions of difference (RD), and the proteins encoded within these regions are referred to as RD proteins. It is hypothesized that the variable efficacy observed between vaccination with different BCG daughter strains is due to insertions, deletions, and single nucleotide polymorphisms (SNPs) found within RDs. One study compared the protective immune responses to bovine tuberculosis in cattle vaccinated with BCG Danish to those vaccinated with BCG Pasteur. This study found that vaccination with BCG Pasteur induced significantly higher and more sustained levels of bovine purified protein derivative (PPD)-specific IFN-γ production in whole-blood cultures when compared to vaccination with BCG Danish [308]. When comparing the genetic composition of BCG Danish and Pasteur strains, RD14 is missing from BCG strain Pasteur, but it is present in BCG Danish [56]. Thus, investigation of antigens contained in RD14 may be a good starting point for evaluating porcine WC1 ligands in this specific context. One antigen, RV1768 ($PE\_PGRS_{31}$), is encoded within RD14. RV1768 is a member of the PE_PGRS family [309]. PE_PGRS proteins are located in the cell wall and are implicated in mediating interactions between the bacteria and immune cells. Thus they may represent potential WC1 ligands that are worth investigating [310].

Figure 4.1

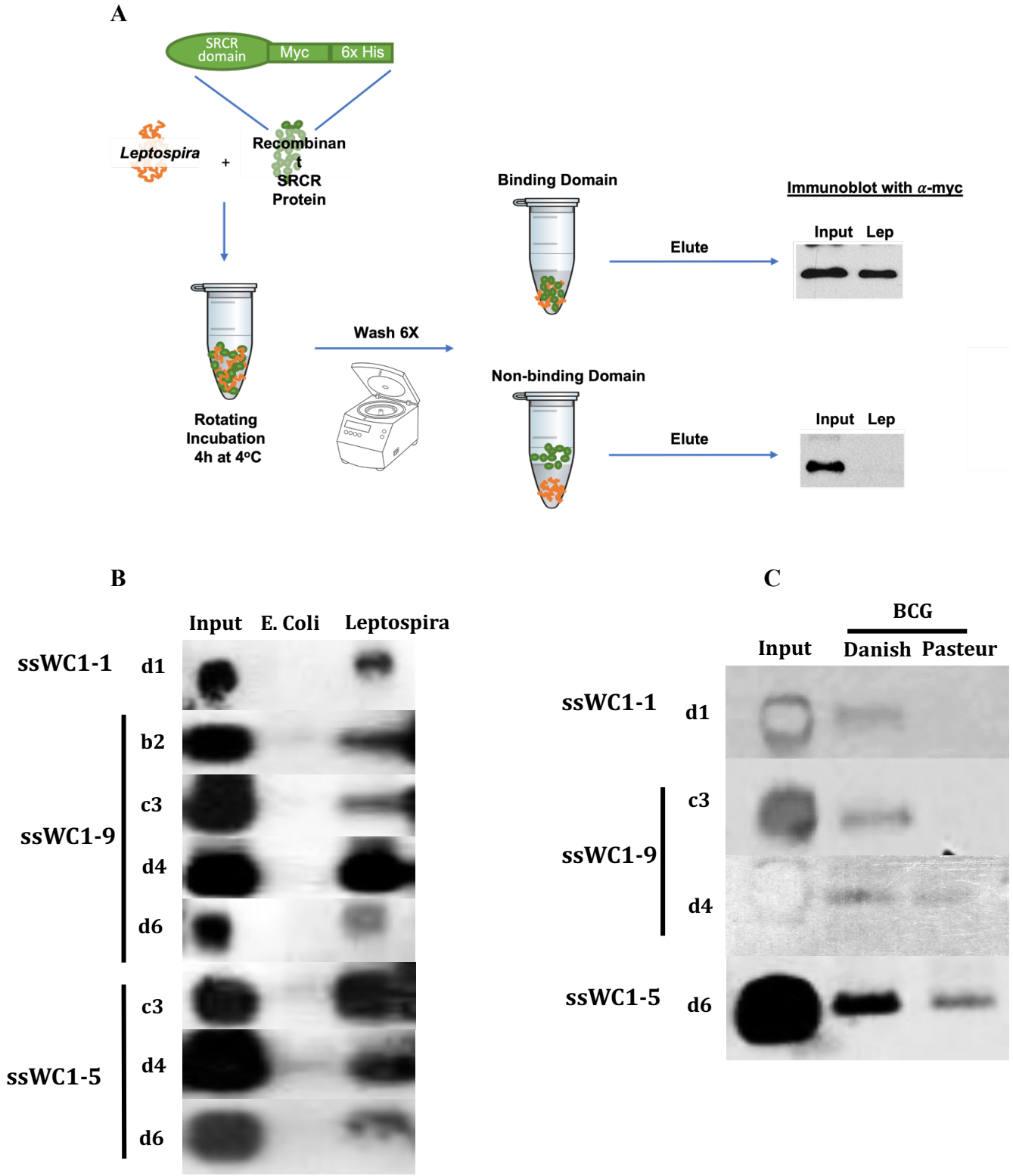



Figure 4. 1 Recombinant WC1 SRCR proteins bind *Leptospira spp.* and BCG

(A) Schematic representation of the bacterial binding assay. 5ng of Myc-tagged WC1 recombinant SRCR proteins are incubated with whole bacteria for 1 hour with end over end rotation. The bacterial pellet is precipitated through centrifugation and washed to remove unbound SRCR protein. The pellet is resuspended in 2X SDS-PAGE sample buffer containing 2-βme and boiled at 95°C for 5 min. The bacteria is then pelleted by centrifugation and the supernatant containing eluted SRCR protein is resolved on a 12% SDS page gel. Following transfer to PVDF, blots are stained with αmyc 9e10 mAb, followed by secondary staining with goat α mouse IgG (H+L)-HRP conjugated mAb. (B) Recombinant myc-tagged SRCR proteins (domain is indicated to the left of the image) were incubated with *E. coli* or *Leptospira spp.* as described in part (A). The input lane represents the total amount of recombinant WC1 protein that was used for the binding assay. (C) Recombinant myc-tagged SRCR proteins (domain is indicated to the left of the image) were incubated with BCG Danish and Pasteur strains as described in part (A). The input lane represents the total amount of recombinant WC1 protein that was used for the binding assay.

Figure 4.2

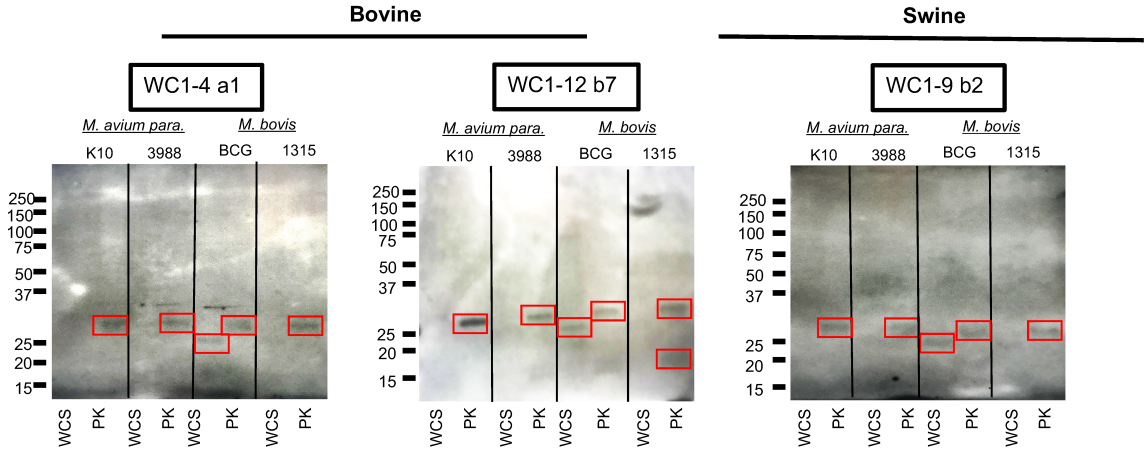



Figure 4. 2 Recombinant WC1 SRCR domains bind Mycobacterium spp. proteins in far western assay.
Whole cell sonicates (WCS) and Proteinase-K (PK) treated sonicates were loaded onto an SDS-Page gel and transferred to PVDF. The resulting blots were probed with 10ng of myc-tagged porcine or bovine recombinant WC1 SRCR proteins overnight, followed by staining with murine-derived α-myc mAb. The blots were then stained with goat α-mouse HRP-conjugated antibody and developed using ECL blotting substrate.

# CHAPTER 5

# DISCUSSION

The overarching goal of this research was to define the WC1 gene family in swine and to investigate its potential to function as a hybrid coreceptor and PRR in this species. We sought to characterize porcine WC1 in terms of 1) total number of genes, 2) gene structure, 3) propensity to signal and 4) pathogen binding potential. Through annotation of the genome *Sscrofa11.1* and phylogenetic comparison to bovine WC1, we determined that there is evidence suggesting eleven unique WC1 genes in swine. Additionally, it was revealed that some porcine WC1 molecules contain an N-terminal d1 SRCR domain which is not observed in bovine WC1. Of the eleven porcine genes five of them possess an N-terminal d1 (ssWC1-1, ssWC1-2, ssWC1-3 ssWC1-4, and ssWC1-11). Of the remaining six WC1 genes in swine, two are classified as WC1.1-type (ssWC1-8 and ssWC1-9) and four are classified as WC1.2-type genes (ssWC1-5, ssWC-6, ssWC-7 and ssWC1-10). Through annotation of the current genomic assembly, we found evidence for four WC1 gene structures. Gene structures varied in number of exons and size. We confirmed that individual SRCR domains are encoded within a single exon, as is the case with bovine WC1. Evidence for two additional WC1 genes that were not accounted for by cDNA (ssWC1-10 and ssWC1-11) was also uncovered in the genome annotation. Future experiments may entail attempting to PCR amplify these genes using the genomic sequences to design primers. While WC1 in cattle contains six to eleven SRCR domains, there is currently no evidence to support the existence of an eleven SRCR domain WC1 molecule in swine.

In the bovine model, phosphorylation of a specific tyrosine residue found in the intracytoplasmic domain is required to potentate WC1-mediated γδ T cell activation. Comparison of porcine intracytoplasmic domains with bovine intracytoplasmic domains revealed that there are four types of intracytoplasmic domain found within porcine WC1 genes (Type III, Type IV, Type V and Type VI). Analysis of genomic evidence for exon/intron structures of the cytoplasmic domains revealed that Type III, IV and V domains are encoded by five exons. The type VI porcine cytoplasmic domain was derived from ssWC1-8. This gene was unplaced in the current assembly; therefore, the exon/intron structure of its intracytoplasmic domain has not been determined. The porcine Type III intracytoplasmic domain has eight tyrosine residues, Type IV has five tyrosine residues, Type V has six tyrosine residues and Type VI has four tyrosine residues. Analysis of individual tyrosine residues and the surrounding amino acids in porcine WC1 cytoplasmic domains revealed that there are multiple tyrosine residues which could be phosphorylated by the src family of kinases. Moving forward, it would be interesting to mutate each tyrosine residue individually and evaluate phosphorylation events following WC1-mediated activation. We hypothesize that phosphorylation of one tyrosine residue is required for porcine WC1 to invoke coreceptor activity.

Once we had defined the multigenic array of WC1 genes in the porcine model, we sought to evaluate its expression on porcine γδ T cells using mAbs against the SWC5 antigen, CC101, PG92A and B37C10. Prior work had shown that these mAbs differentially stained porcine PBL, and we hypothesized that this was due to differential recognition of WC1 genes expressed on γδ T cells. Full length WC1 genes ssWC1-1 and ssWC1-3 were

expressed on the surface of suspension 293s and stained with CC101, PG92A and B37C10. While PG92A did not positively stain cells expressing either ssWC1-1 or ssWC1-3, mAb CC101 positively stained cells expressing ssWC1-1 and those expressing ssWC1-3. In contrast, mAb B37C10 positively stained cells expressing ssWC1-1 only. Porcine WC1 genes are comprised of six extracellular SRCR domains, and the N-terminal SRCR domain 1 (a1 or d1) is the most unique. The latter SRCR domains [b2-c3-d4-e5-d5] are highly conserved across different WC1 genes, and across species [51]. Thus, we hypothesized that mAb CC101 recognized an epitope found within one of the more conserved SRCR domains as opposed to SRCR 1. Both ssWC1-1 and ssWC1-3 are d1-WC1 molecules, meaning they begin with an N-terminal d1 SRCR domain. We postulated that mAb B37C10 was reacting with an epitope found in the d1 SRCR domain of ssWC1-1 which was not found in the d1 SRCR domain of ssWC1-3.

Using recombinant WC1 protein encompassing individual SRCR domains that were tagged with a myc epitope we performed a dot blot analysis to evaluate domain-specific reactivity of the mAbs. Through this analysis we confirmed that mAb CC101 recognized the c3 SRCR domain isolated from both a WC1.1 and WC1.2-type gene. We did not have recombinant protein encompassing the c3 SRCR domains of ssWC1-1 or ssWC1-3, however, based on our flow cytometry results, and a phylogenetic comparison of these domains, we concluded that CC101 is likely reacting with the c3 SRCR domain from these molecules as well. As anticipated, we found that mAb B37C10 reacted with the d1 SRCR domain of ssWC1-1. This was in line with our results derived from flow cytometry. One limitation of this study was that we did not have recombinant protein to

represent all the SRCR domains found in porcine WC1 genes. It would be useful to know which WC1 molecules are recognized by the tested mAbs. It is highly likely that CC101 reacts with other porcine WC1 genes outside of the ones evaluated here. An understanding of which WC1 genes are recognized by specific monoclonal antibodies would aid in our quest to characterize porcine $WC1^+$ γδ T cell populations based on WC1 expression. In cattle, it has been shown that subpopulations which differentially respond to pathogens can be distinguished with monoclonal antibodies. The ability to do this with porcine $WC1^+$ γδ T cells would be extremely useful for conducting research in a disease-specific context.

After confirming that mAbs CC101 and B37C10 react with WC1 molecules we sought to characterize porcine γδ T cell populations based on WC1 and CD2 expression. In ruminants, $CD2^+$ and $CD2^-$ γδ T cells are largely defined as being $WC1^-$ and $WC1^+$ respectively, therefore we hypothesized that this was the case for porcine $CD2^+$ and $CD2^-$ γδ T cells [127]. γδ T cells isolated from porcine peripheral blood were first evaluated for CD2 expression. As anticipated, both $CD2^+$ and $CD2^-$ cells existed within the γδ TCR+ population. Next, we looked at WC1 expression on γδ $TCR^+$ cells using αSWC5 mAb B37C10, which we had previously demonstrated reacts with porcine WC1. In agreement with studies performed on bovine γδ T cells, SWC5 expression was restricted to the $CD2^-$ γδ T cell subset. Additionally, both $SWC5^+$ and $SWC5^-$ cells existed within the $CD2^-$ population.

While CC101 reacted with porcine PBMC, we found that it recognized lymphocytes outside of the γδ $TCR^+$ population. In our previous studies we demonstrated that mAb CC101 reacted with a highly conserved SRCR domain, domain c3. We hypothesize that CC101 is reacting with another group B scavenger receptor CD163A on γδ $TCR^-$ cells. CD163A is a close relative of WC1, and it possesses a c6 SRCR domain which shares significant amino acid identity with WC1 c3 SRCR domains. Based on these results, swine peripheral blood γδ T cells were divided into three major subpopulations defined by CD2 and WC1 expression.

Using mAb B37C10, we established three subpopulations of porcine γδ T cells which were defined by CD2 and WC1 expression: γδ$TCR^+$/$CD2^-$/$SWC5^-$, γδ$TCR^+$/$CD2^+$/$SWC5^-$, and γδ $TCR^+$/$CD2^-$/$SWC5^+$. Using flow cytometric sorting, we enriched for these populations and evaluated WC1 and TCRγ gene usage. Using primers designed to amplify the three porcine WC1 gene subsets; d1-WC1, WC1.1 and WC1.2, we evaluated the sorted γδ T cell populations for WC1 gene expression. Both subpopulations of $CD2^-$ γδ T cells, $CD2^-$/$SWC5^-$ and $CD2^-$/$SWC5^+$, expressed WC1 transcripts. Of note, the $SWC5^-$ population expressed transcripts for all three WC1 gene subsets. This further confirmed our previous results, which demonstrated that mAb B37C10 recognized a unique epitope in the N-terminal d1 SRCR domain of ssWC1-1. $CD2^+$ γδ T cells were confirmed negative for WC1 expression. This was in agreement with studies conducted in ruminants regarding CD2 expression [127]. $CD2^-$ subpopulations ($SWC5^+$ and $SWC5^-$) varied in WC1 receptor expression. The $SWC5^+$ cells appeared to express higher levels of WC1.1a1 and d1-WC1 genes than the $SWC5^-$ cells.

Regarding TCR gene usage, the two $CD2^-$ subpopulations possessed strong bands of amplicons representing transcription of genes within the TRGC1 cassette, but also had amplicons representing transcripts for other TRGC genes. $CD2^+$ γδ T cells had stronger amplicon bands representing transcription of TRGC2/3 genes in addition to transcripts for TRGC1. Counting this with the WC1 gene expression results and the phenotypic differences regarding CD2 and SWC5 expression, these results further suggest that these populations represent distinct subpopulations of porcine γδ T cells.

Future work regarding these experiments might include evaluating αSWC5 mAbs against SRCR domains derived from porcine CD163A. mAb CC101 was found to stain lymphocytes other than γδ T cells. A small portion of the γδ $TCR^-$ $CC101^+$ lymphocytes were found to be positive for CD163A. Double staining of these cells with CC101 and αCD163A revealed that there was minimal co-expression of these two molecules. The $CD163A^+$ population did not account for the large proportion of $CC101^+$ cells that were observed. It would be interesting to evaluate the $CC101^+$γδ$TCR^-$ lymphocytes for WC1 gene expression. While WC1 gene expression is restricted γδ T cells in ruminants, it's possible that this is not the case in swine. Given that we know mAb CC101 is reactive with multiple swine WC1 genes, it may be worth exploring.

After confirming that WC1 gene expression defined three subpopulations of porcine γδ T cells, we sought to evaluate its propensity to directly bind to bacterial pathogens. In cattle, WC1 gene expression plays a major role in determining what pathogens $WC1^+$ γδ

T cells will respond to. γδ T cells expressing WC1.1-type genes respond to *Leptospira spp.* while those expressing WC1.2-type genes do not respond [43]. It was later found that btWC1-3, a WC1.1 molecule expressed on *Leptospira spp.* responsive γδ T cells, directly bound to *Leptospira spp.* via multiple SRCR domains. Moreover, populations of responsive and non-responsive $WC1^+$ γδ T cells can be distinguished by staining with mAbs [119]. Therefore, we sought to evaluate the pathogen binding potential of porcine WC1 SRCR domains.

Through bacterial pull-down assays, we confirmed that multiple porcine WC1 SRCR domains bound to *Leptospira spp.* It was also found that the SRCR domains differentially bound to BCG strains Danish and Pasteur. Two of the SRCR domains tested were found to bind both strains of BCG, while two SRCR domains bound to Pasteur only. While preliminary, this data has given us a starting point for investigation of potential WC1 ligands. When comparing the genetic composition of BCG Danish and Pasteur strains, RD14 is missing from BCG strain Pasteur, but it is present in BCG Danish [56]. SRCR domains which bind to BCG Danish are potentially binding to antigen found within RD14. Thus, investigation of antigens contained in RD14 may be a good starting point for evaluating WC1 ligands in this specific context. Additionally, far western blot analysis with cell sonicates of various mycobacterial origin revealed that the ligand in this specific context is likely not proteinaceous.

Through the work presented here we have uncovered that porcine WC1 is expressed as a multigenic array comprised of up to ten unique genes. Porcine WC1 is composed of six

extracellular SRCR domains, which are capable of directly binding to pathogens including *Leptospira spp.* and BCG Danish and Pasteur strains. We also confirmed that two mAbs, against the SWC5 molecule recognize WC1 molecules on porcine γδ T cells. We subsequently determined that porcine γδ T cells can be subdivided into three major populations based on CD2 and WC1 gene expression.

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