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Characterization of porcine tripartite motif genes as host restriction factors against PRRSV and PEDV infection

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ABSTRACT

Members of the tripartite motif (TRIM) family are the important effectors of the innate immune response against viral infections. However, it is still unknown whether porcine TRIM (pTRIM) genes may restrict the infection of porcine reproductive and respiratory syndrome virus (PRRSV) and porcine epidemic diarrhea virus (PEDV). In this study, we firstly defined the entire pTRIM family. Fifty-seven pTRIMs were classified into 12 sub-families (C-I to C-XII) based on variable C-terminus, and 17 out of them were identified as positively selected genes. Nine pTRIMs were identified as the IFN-stimulated genes in IFN-β treated porcine alveolar macrophages (PAMs). Twelve pTRIMs were regulated in PRRSV or PEDV-infected PAMs, respectively. The mRNA expression of the implicated restriction factors (pTRIM5, 14, 21, 25 and 38) was detectable in all swine tissues studied, with the high expression in the spleen and lung tissues. These results firstly present the comprehensive characterization of pTRIM genes, and suggest the pTRIM5, 14, 21, 25, and 38 genes as the implicated host restriction factors against PRRSV and PEDV infection, which provide a basis to further study the functions of pTRIMs and the mechanism by which pTRIMs may act during viral infection.

1. Introduction

Tripartite motif (TRIM) protein family is a conservative, rapidly evolving polygenic family, which is widely found in insects, teleosts and higher vertebrates (Sardiello et al., 2008; McNab et al., 2011). However, the number of TRIM genes varies greatly among different species. It has been found that there are over 70 TRIM genes in human and mouse, 208 in zebrafish and 66 in puffer fish (van der Aa et al., 2009; Boudinot et al., 2011; Reymond et al., 2001). TRIM proteins are characterized by a tripartite motif comprising of a RING zinc finger domain, one or two B-box domains and a Coiled-coil domain, also known as RBCC (RING-Bbox-Coiled coil) proteins (Nisole et al., 2005; Meroni and Diez-Roux, 2005). Many TRIM proteins have been reported as E3 ubiquitin ligase on the basis of the RING domain to elicit host antiviral innate immunity in the ubiquitylation process (Joazeiro and Weissman, 2000; Trockenbacher et al., 2001; Uchil et al., 2008). The B-boxes are cysteine-rich domains that bind zinc atoms, suggesting an important determinant of the TRIM family (Freemont, 2000; Torok and Etkin, 2001). The Coiled-coil region has been shown to be necessary for homo-interactions in several TRIM proteins, resulting in the generation of high-molecular-mass complexes (Short and Cox, 2006; Maillard et al., 2010). Additionally, almost all TRIM proteins contain an additional variable C-terminal domain, such as PRY (ryanodine receptor), SPRY (SplA and ryanodine receptor), FN3 (Fibronectin type III), PHD (plant homeo domain), ARF (ADP-ribosylation factor), MATH (meprin and tumor necrosis factor receptor-associated factor homology) and TM (transmembrane) domain etc., of which the B30.2 domain (also known as PRY/SPRY domain) is the most frequent one (Boudinot et al., 2011; Short and Cox, 2006). The B30.2 domain is constituted by the juxtaposition of a PRY (approximately 60 residues) and a SPRY (approximately 140 residues) domain, which can mediate protein–protein interaction (Sardiello et al., 2008; Meyer et al., 2003). Human TRIM proteins have been classified into 12 subfamily members based on their different C-terminal domains (Carthagena et al., 2009; Hatakeyama, 2017).

Despite their common domain architecture, TRIM proteins play multiple roles in diverse cellular processes, including differentiation, development, apoptosis, oncogenesis and antiviral immunity in mammals (van der Aa et al., 2009; Nisole et al., 2005; Gack et al., 2007). Interest in TRIM proteins has grown extensively in the last few years as these proteins have been demonstrated to act as potent antiviral proteins. TRIM5, one of the best studied members of the family, has been shown to restrict post-entry of diverse retroviruses, including HIV-1 and N-MLV (Schaller et al., 2007; Stremlau et al., 2004; Pertel et al., 2011). TRIM5 also displays a species-specific anti-retroviral activity which affects HIV infection stronger in rhesus macaques than that in human. It

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can bind to the retrovirus capsids via the C-terminal SPRY domain, causing the capsid to be unwound failure (Sawyer et al., 2007, 2005). TRIM22 has been reported to inhibit a wide range of viruses, including block the release of HIV Gag-only particles, inhibit EMCV replication through degrading 3C proteinase, and inhibit HBV replication by blocking RNA synthesis (Barr et al., 2008; Eldin et al., 2009; Gao et al., 2009). The anti-retroviral activity has also been reported for other TRIM proteins such as TRIM11, 19, 25, 26 and 56, which can interfere with various stages of HIV-1 or MLV replication by different mechanisms (Uchil et al., 2008; Gack et al., 2007; Yuan et al., 2016; Dutrieux et al., 2015; Kane et al., 2016).

In order to identify TRIM proteins with potential antiviral activity, researchers thought TRIM proteins that have been under positive selection throughout evolution suggesting that they directly interface with ever evolving pathogens (Sawyer et al., 2005; Malfavon-Borja et al., 2013). Studies also showed that TRIM proteins containing RBCC-B30.2 domains were younger, evolving faster, and often under positive selection (Han et al., 2011). Since several RBCC-B30.2 genes, such as TRIM21, 25 and 38, are implicated in antiviral immunity, which evolutionary features may be responsible for species-specific battles against viral infection (Uchil et al., 2008; Gack et al., 2007; Mallery et al., 2010). Moreover, innate immunity is the first line of defense against pathogens. Type I interferons (IFN-α/β) are the main antiviral cytokines inducing the expression of hundreds of interferon-stimulated genes (ISGs). In recent studies, it has been found that most TRIM proteins are induced by interferon in human or mouse macrophages, which makes them resistant to pathogens (Carthagena et al., 2009; Rajsbaum et al., 2008). Therefore, the identification, evolution and expression analysis of TRIM family are the basis of studying its antiviral function. However, no comprehensive study has been performed so far for the entire porcine TRIM (pTRIM) family.

In this study, we firstly characterized the entire pTRIM family, including classification, evolution and genomic location *etc*. Then, molecular evolutionary analysis was conducted to gain further insight into the evolutionary characteristics of TRIM genes in mammalian. We identified interferon-sensitive pTRIM genes by detecting the changes of pTRIMs expression in porcine alveolar macrophages (PAMs) after interferon-beta (IFN-β) stimulation. To further elucidate possible implication of pTRIMs in the antiviral activities, we assessed the effect of porcine reproductive and respiratory syndrome virus (PRRSV) and porcine epidemic diarrhea virus (PEDV) on the pTRIMs in PAMs by real-time quantitative PCR (RT-qPCR) (Butler et al., 2014; Opriessnig, 2015). Finally, we investigated the tissue-specific distribution of these implicated restriction factors. These findings have provided insights into implication of pTRIMs in anti-PRRSV or anti-PEDV activities.

2. Materials and methods

2.1. Gene sequences

All porcine (Sus scrofa), human (Homo sapiens), mouse (Mus musculus), and green monkey (Chlorocebus sabaeus) TRIM sequences were obtained from the National Center for Biotechnology Information (NCBI) database. The related information of pTRIM family was collected from NCBI. Then, pTRIM gene sequences were used as queries to search novel other mammalian TRIM gene sequences when available, including dog (Canis lupus familiaris), horse (Equus caballus), killer whale (Orcinus orca), rabbit (Oryctolagus cuniculus), panda (Ailuropoda melanoleuca) and bat (Pteropus vampyrus), using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) at NCBI genome browsers.

2.2. Structural and phylogenetic analysis

The conserved domains of pTRIM proteins were predicted using SMART program (http://smart.embl-heidelberg.de/). Multiple sequence alignment of pTRIM nucleotide sequences was carried out using ClustalW. A Neighbor-joining tree was constructed with Molecular Evolution Genetics Analysis (Kumar et al., 2016). Tree nodes were critically evaluated by performing 1,000 bootstrap replicates.

2.3. Molecular evolutionary analysis

For each mammalian TRIM gene, 5˜10 orthologs were aligned using ClustalW and a NJ tree with 1000 bootstrap trial was constructed with MEGA7. Based on these alignments and trees, the COMEML program from the PAML package (Yang, 2007) was used to analyze the molecular evolution of TRIM genes. Constrained model M7 (model = 7) and unconstrained model M8 (model = 8) were utilized at the same time to detect the recurrent positive selection. Simulations were run with the follow parameters: f 61. For each pair of codon models, a likelihood ratio test (LRT) was performed and twice the difference of log-likelihood value ($2\ln\lambda$) was calculated to test the significance (P value were calculated using a Chi-squared test assuming two degrees of freedom). Moreover, the average ratio of non-synonymous (dN) to synonymous (dS) substitution rates, $\omega = dN/dS$, for those selective sites of each mammalian TRIM gene were calculated. Sites allocated under the class with $\omega > 1$ are considered as being under positive selection and their posterior probabilities were identified by a Bayes Empirical Bayes (BEB) analysis. Tests were considered positive when $p < 0.05$. Sites identified by BEB with a posterior probability higher than 95 percent were considered significant.

2.4. Cells and viruses

African green monkey kidney (Marc-145 and Vero) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO). Porcine alveolar macrophages (PAMs) were obtained from lung lavage samples of 4- to 6-week-old PRRSV-negative piglets and cultured in RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). All cells were maintained in an incubator at 37 ℃ with 5% CO_2. The highly pathogenic PRRSV strain Li11 and PEDV strain GDS01 were propagated in Marc-145 and Vero cells respectively. All animal experiments were performed in accordance with the regulation and guidelines of Institutional Animal Care and Use Committee of Sun Yat-sen University (IACUC DD-17-0403).

2.5. Expression profiles of pTRIMs after IFN-β treatment or viral infection in PAMs

For IFN-β treatment experiment, PAMs were seeded in 12-well plates. Six hours later, cells were treated with or without human IFN-β (100 ng/mL, Sangon Biotech, China), and collected in 750 μL of TRIzol reagent (Invitrogen, USA) at 12 h post-stimulation.

For viral infection experiment, PAMs were seeded in 12-well plates. Six hours later, cells were infected with or without PRRSV or PEDV at a MOI of 0.1, and harvested in 750 μL of TRIzol reagent at 24 h post-infection (hpi).

Real-time quantitative PCR (RT-qPCR) was used to detect the relative mRNA level of pTRIMs in PAMs after IFN-β stimulation (non-treated PAMs served as the control) or PRRSV/PEDV infection (non-infected PAMs served as the control). *ISG15* was used as the positive control, whose expression is known to be up-regulated by IFN-β (Supplementary table S1).

2.6. Real time quantitative PCR

Total RNA was isolated from PAMs using TRIzol reagent, and reverse transcribed using a ReverTra Ace® qPCR RT Master Mix with gDNA Remover reagent kit (TOYOBO, Osaka, Japan) in accordance with the manufacturer's instructions. SYBR green (Yeasen, China) real-

Table 1
Porcine TRIM genes included in our study.

Gene name	Domains*	Accession No.	Chromosome (start..end)	Protein length (aa)
TRIM1	R-B1-B2-CC-FN3-PRY-SPRY	XM_005657908	X (88294032..88394885)	715
TRIM2	R-B2-CC-IG_FLMN-NHL (6)	XM_021101488	8 (75770344..75939812)	794
TRIM3	R-B2-CC-IG_FLMN-NHL (6)	XM_021062339	9 (3256915..3281846)	744
TRIM4	R-B2-CC-PRY-SPRY	XM_003124333	3 (7815188..7837587)	516
TRIM5	R-B2-CC-SPRY	NM_001044532	9 (4157290..4170233)	492
TRIM6	R-B2-CC-PRY-SPRY	XM_021062360	9 (4197922..4204688)	487
TRIM7	R-B2-CC-PRY-SPRY	XM_021083408	2 (57310530..57322427)	510
TRIM8	R-CC	XM_001928904	14 (113637437..113652577)	551
TRIM9	R-B1-B2-CC-FN3-SPRY	XM_013993396	1 (180723281..180830555)	795
TRIM10	R-B2-PRY-SPRY	XM_013977547	7 (22718000..22731445)	482
TRIM11	R-B2-CC-PRY-SPRY	XM_021083380	2 (51729333..51743256)	468
TRIM13	R-B2-TM	XM_021065576	11 (17785031..17850005)	407
TRIM14	B2-CC-PRY-SPRY	XM_003122031	1 (239912126..239945082)	445
TRIM15	R-B2-CC	XM_013977549	7 (22730372..22742941)	579
TRIM16	B2-CC-PRY-SPRY	XM_021067910	12 (58926083..58940642)	582
TRIM17	R-B2-CC-PRY-SPRY	XM_021083381	2 (51726854..51732075)	475
TRIM18	R-B1-B2-CC-FN3-PRY-SPRY	XM_021080485	X (7235386..7906049)	667
TRIM21	R-B2-CC-PRY-SPRY	XM_005656620	9 (5782133..5790628)	459
TRIM23	R-B1-B2-CC-ARF	XM_003134013	16 (44103339..44146291)	574
TRIM24	R-B1-B2-CC-PHD-BROMO	XM_021079186	18 (11055211..11151755)	1082
TRIM25	R-B2-CC-PRY-SPRY	XM_005656971	12 (33184039..33212440)	632
TRIM26	R-B2-PRY-SPRY	NM_001123209	7 (22757794..22767662)	545
TRIM27	R-B2-CC-PRY-SPRY	XM_003128235	Un#	513
TRIM28	R-B1-B2-CC-PHD-BROMO	XM_021097344	6 (63094382..63101532)	861
TRIM29	B2-CC	XM_021062976	9 (47256314..47284104)	617
TRIM31	R-B2-CC-PRY-SPRY	XM_021098028	7 (22665150..22681616)	592
TRIM32	R-B2-CC-NHL (3)	XM_005660379	1 (257051421..257066911)	680
TRIM33	R-B1-B2-CC-PHD-BROMO	XM_021090006	4 (106058197..106195145)	1127
TRIM34	B2-CC-PRY-SPRY	XM_013979110	9 (4175660..4188528)	403
TRIM35	R-B2-CC-PRY-SPRY	XM_001928380	14 (11031667..11051903)	493
TRIM36	R-B2-CC-FN3	XM_021084641	2 (119304476..119352465)	739
TRIM37	R-B2-CC-MATH	XM_021067282	12 (35259292..35393464)	1010
TRIM38	R-B2-CC-PRY-SPRY	XM_021098422	7 (20682536..20694340)	465
TRIM39	R-B2-CC-PRY-SPRY	XM_005653455	7 (23047048..23062974)	488
TRIM40	R-CC	XM_021098154	7 (22702891..22717850)	300
TRIM41	R-B2-CC-PRY-SPRY	XM_021083399	2 (57285401..57295771)	629
TRIM42	R-B2-CC-FN3	XM_013982083	13 (81537589..81567393)	724
TRIM44	B2-CC	XM_003122865	2 (25275193..25378738)	345
TRIM45	R-B1-B2-CC-IG_FLMN	XM_001929343	4 (103613182..103623228)	580
TRIM46	R-B2-CC-FN3	XM_003125718	4 (94632073..94642756)	759
TRIM47	R-B2-PRY	XM_003131190	12 (5535259..5539721)	646
TRIM50	R-B2-CC-PRY-SPRY	NM_214186	3 (10647690..10655345)	486
TRIM54	R-B2	NM_001244359	3 (111836038..111852468)	364
TRIM55	R-B2-CC	XM_021088506	4 (68487249..68530927)	551
TRIM56	R-B2	XM_021086255	3 (8834260..8842524)	755
TRIM58	R-B2-CC-PRY-SPRY	XM_021083395	2 (55822361..55843923)	480
TRIM59	R-B2-TM	XM_005669954	13 (100118824..100133117)	403
TRIM62	R-B2-CC-PRY-SPRY	XM_003127763	6 (89406470..89442794)	475
TRIM63	R-B2-CC	NM_001184756	6 (83493200..83507471)	354
TRIM64	R-B2-SPRY	XM_005667218	9 (23009082..23015287)	451
TRIM65	R-B2-CC-PRY-SPRY	XM_003131202	12 (5523056..5528884)	522
TRIM66	B1-B2-CC	XM_021062260	9 (711571..769799)	1123
TRIM67	R-B1-B2-CC-FN3-PRY-SPRY	XM_021072335	14 (59260070..59303244)	782
TRIM68	R-B2-PRY-SPRY	XM_003482550	9 (5502931..5516936)	485
TRIM69	CC-PRY-SPRY	XM_021096339	1 (126775856..126788628)	351
TRIM71	R-B1-B2-CC-IG_FLMN-NHL (6)	XM_021071552	13 (18762043..18835788)	869
TRIM72	R-B2-CC-PRY-SPRY	XM_003124494	3 (17286471..17297841)	478

* R, Ring finger domain; B1, B-box1 domain; B2, B-box2 domain; CC, Coiled-coil domain; FN3, Fibronectin type III motif; PRY, PRY domain; SPRY, SPRY domain; IG_FLMN, Filamin type immunoglobulin domain; NHL, NHL repeats; TM, Transmembrane domain; ARF, ARF domain; PHD, Plant Homeodomain; BROMO, Bromodomain; MATH, MATH domain.

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time PCR was performed using a Light-Cycler 480 PCR system (Roche, Switzerland). Briefly, amplification reactions were performed in a 10 μL volume containing 1 μL cDNA, 5 μL Hieff™ qPCR SYBR® Green Master Mix, 0.5 μL primers and 3.5 μL ddH2O. The primers used are listed in Table 1. The cycling conditions was as follows: 95 ℃ for 5 min, followed by 40 cycles of 95 ℃ for 10 s, 58 ℃ for 10 s, and 72 ℃ for 30 s, with subsequent incubations at 95 ℃ for 5 s, 60 ℃ for 1 min and 95 ℃ for 15 s. GAPDH was used as a reference gene. Data were analyzed using the $2^{-\Delta\Delta CT}$ method.

2.7. Tissue-specific distribution of pTRIMs in healthy pigs

The heart, liver, spleen, lung, kidney, stomach, jejunum, colon, bladder, muscle, and lymph node were collected from three healthy pigs for RNA isolation with TRIzol reagent. 1 μg of total RNA was used for RT-qPCR to detect the relative mRNA expression of pTRIM5, 14, 21, 25 and 38 in eleven tissues as described above. The quantity of pTRIMs in these tissues was normalized as compared to that in the muscle.

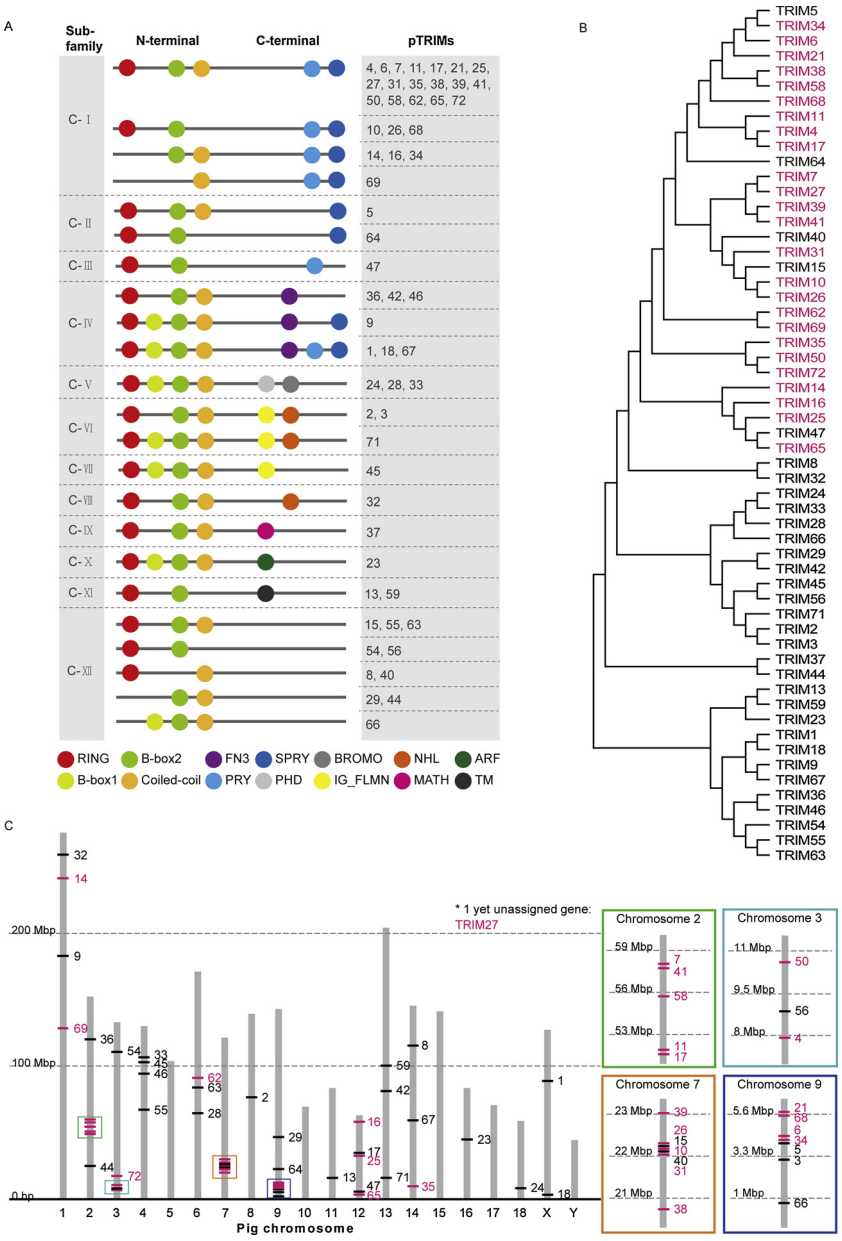

Fig. 1. Porcine TRIM family.
A. Classification of porcine TRIM proteins based on C-terminal domains. Domains are depicted in different colors. B. Phylogenetic tree of porcine TRIM genes. TRIM genes belonging to C-I sub-family are depicted in red color. C. Genomic location. Global distribution on the porcine genome is presented on the left, while the detailed views of multigenic loci are presented on the right. TRIM genes belonging to C-I sub-family are also depicted in red color.

2.8. Immunofluorescence assay (IFA)

The viral replication was detected by immunofluorescence assay. PAMs were infected with or without PRRSV or PEDV at a MOI of 0.1. Twenty-four hours later, PAMs were rinsed with PBS three times and fixed with 4% paraformaldehyde for 15 min at room temperature (RT). After being washed with PBS, PAMs were blocked with 4% bovine serum albumin (BSA) for 1 h at RT, then incubated with anti-PRRSV N mAb or anti-PEDV N mAb for 1 h at 37 ℃. After three washes with PBS, cells were further incubated with Cy3-labeled Goat Anti-Mouse IgG (Beyotime, China) for 1 h at 37 ℃. Cell nuclei were stained with DAPI (Beyotime, China) and detected using fluorescence microscopy (Carl Zeiss, Germany).

2.9. Statistical analysis

Samples were assayed in triplicate and data are representative of three independent experiments. Data are represented as mean ± SD. Data analysis were performed using GraphPad Prism 5 software. The significance of the differences between the treatment group and control in the mRNA expressions was determined by the two-tailed unpaired *t*-test Differences between groups were considered statistically significant when the P value was less than 0.05 (*, $P < 0.05$; **, $P < 0.01$).

3. Results

3.1. Characterization of pTRIM family

In order to perform a comprehensive study of pTRIM genes, the sequences and the related information of totally 57 pTRIMs were

Table 2
Mammalian TRIM genes evolving under positive selection.

TRIM Gene	M7vsM8 (2lnλ)	P value	dN/dS for Selected Sites (ω)	No. of Mammalian Taxa
TRIM3	11.59	< 0.005	1.43	10
TRIM4	13.12	< 0.005	1.77	9
TRIM5	62.62	< 0.005	6.61	6
TRIM11	6.19	0.045	2.02	10
TRIM14	7.02	0.030	1.72	9
TRIM15	12.66	< 0.005	2.59	9
TRIM16	10.65	< 0.005	1.91	10
TRIM17	8.24	0.016	1.57	10
TRIM21	36.91	< 0.005	2.32	10
TRIM25	37.44	< 0.005	1.95	10
TRIM29	10.27	0.006	1.84	10
TRIM31	19.46	< 0.005	2.31	5
TRIM38	22.37	< 0.005	1.85	9
TRIM40	10.93	< 0.005	3.65	9
TRIM44	29.46	< 0.005	2.77	9
TRIM45	8.27	0.016	1.99	10
TRIM54	8.40	0.015	1.20	10

obtained from the NCBI (Table 1). After predicted using SMART program, most of the pTRIMs are characterized by a conserved N-terminal domain architecture (known as RBCC) and possess diverse C-terminal domains. Twelves sub-families, from C-I to C-XII, are classified based on the variable domains of C-terminus (Fig. 1A). A complete B30.2 domain is found in pTRIMs belonging to the C-I, consisting of 25 members. C-II and C-III pTRIMs contain the SPRY and PRY domain, respectively. C-IV pTRIMs are characterized by the FN3 domain. Other domains are present in members of the pTRIM sub-family in the C-terminus: the PHD and BROMO domain in C-V; the IG_FLMN domain and the NHL repeats in C-VI to C-VIII; the MATH domain in C-IX; the ARF domain in C-X; and the TM domain in C-XI. Whereas, pTRIMs belonging to the C-XII do not possess a defined C-terminal domain. Some TRIM genes are not found in porcine, such as pTRIM12, 22 and 30 *etc*.

A topological phylogenetic tree was constructed. As shown in Fig. 1B, the pTRIMs can be distinctly separated into two main groups. One group is mainly composed of 25 pTRIMs, which belong to C-I subfamily with a few exceptions: pTRIM5, 64, 40, 15 and 47. These exceptions are likely to share the same dynamics during the evolution as the C-I sub-family. The other group is composed of the remaining 27 pTRIMs belonging to C-II to C-XII sub-families. In addition, the information about genomic distribution of pTRIMs is shown in Fig. 1C. Interestingly, pTRIMs belonging to C-I sub-family have a tendency to be arranged in clusters, localized on chromosome 2, 3, 7 and 9.

3.2. Molecular evolution analysis

To gain further insight into the TRIM gene family in mammalian (pig, human, mouse, green monkey, dog, horse, killer whale, rabbit, panda and bat) evolution, the CODEML program from the PAML package was conducted. We used a likelihood-ratio test (LRT) to detect whether the given TRIM gene has evolved under positive selection or not. When positive selection has acted on a gene, several amino acids will change due to the non-synonymous mutations at a higher rate than the synonymous ones, and the ratio will fix more than 1 ($\omega > 1$). In our study, model M8 (allowing codons evolving under positive selection, $\omega > 1$) was used to test the positive selection hypothesis against model M7 (disallowing positive selection, $\omega \leq 1$). The results showed that 17 out of 57 TRIM genes passed the test with $p < 0.05$, considered as having evolved under positive selection (Table 2). While the rest 40 TRIM genes did not show evidence of positive selection (Supplementary table S2).

The model M8 was also used to identify the positively selected sites with the posterior probability more than 0.95. TRIM5 was reported as an antiviral gene that has evolved under positive selection in primates (Pertel et al., 2011; Sawyer et al., 2005). As shown in Fig. 2, we also found that TRIM5 had a cluster of sites under positive selection near and within the SPRY domain. Moreover, 9 TRIM genes under positive selection contain the B30.2 domain, belonging to the C–I subfamily. Of them, TRIM21, 25 and 38 showed strong evidence of robust positive selection, which were also reported as known restriction factors previously (Uchil et al., 2008; Gack et al., 2007; Mallery et al., 2010). TRIM21 had multiple positively selected sites in various domains, such as B-Box, coiled-coil and B30.2 domains. For TRIM25, the positively selected sites clustered near and within the B30.2 domain. Similarly, TRIM38 had a cluster of sites under positive selection in its RING domain associated with E3 ubiquitin ligase activity. Only a single site of positive selection was detected in TRIM4, 11, 16 and 31. However, we didn't find any significant site in TRIM14 and 17.

Additionally, 7 candidate restriction factors were identified without the B30.2 domain (Fig. 2). Of them, TRIM15, 29, 40, 44 and 54 do not contain any domain in the C-terminal region, and few of the positively selected sites were within the known domains. TRIM3 and 45 contain the IG_FLMN domain, and the sites were found in the IG_FLMN domain

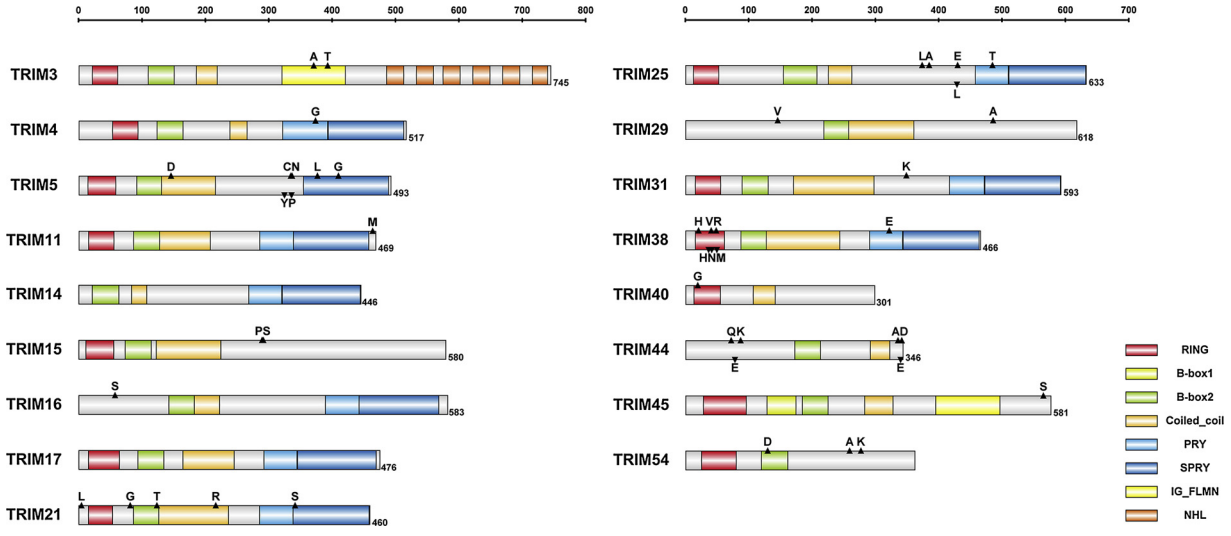

Fig. 2. Architectures of TRIM family members exhibiting positive selection in mammals.
A domain schematic of all porcine TRIM proteins under positive selection is shown. For each porcine TRIM protein, all positive selected sites (marked with triangles and identified by BEB with a posterior probability > 0.95) and their amino acids are presented.

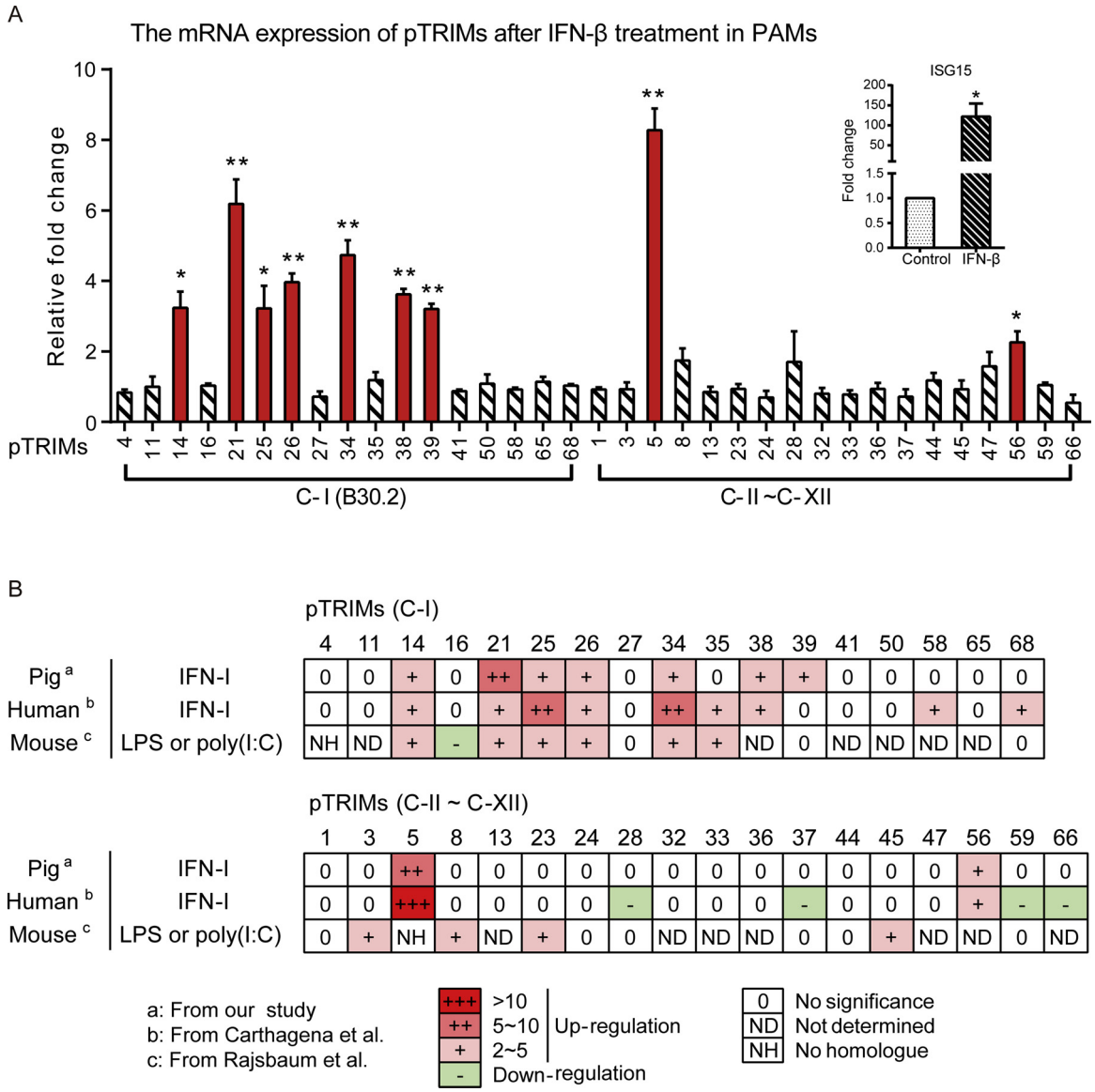

Fig. 3. Expression of TRIM genes in response to IFN-I or immune complex.
A. pTRIMs expression in PAMs upon IFN-β treatment. PAMs were treated with or without human IFN-β (100 ng/mL). RT-qPCR was used to detect the relative mRNA level of pTRIMs at 12 h post-stimulation. *GAPDH* was used as a reference gene. *ISG15* was used as the positive control. Data are represented as mean ± SD, n = 3. *, $P < 0.05$; **, $P < 0.01$. B. Summary of the mRNA expression of TRIM genes in porcine, human and mouse macrophages upon various stimuli, including PAMs upon IFN-β treatment (from our study), human macrophages treated with IFN-I (from Carthagena et al.), and mouse macrophages stimulated with LPS or poly(I:C) (from Rajsbaum et al.).

and C-terminal region, respectively. During the evolution, these sites of positive selection varied greatly from species to species (Supplementary table S3).

3.3. Expression profiles of TRIM genes in response to IFN-I or immune complex in macrophages

To assess the capacity of IFN-β on pTRIM gene expression in PAMs, RT-qPCR was used to detect the relative mRNA level at 12 h post-stimulation (non-treated PAMs served as the control). We first analyzed the basal expression of all pTRIM genes in untreated PAMs, and defaulted that genes with ct values more than 30 were undetectable. Of the 57 analyzed pTRIM transcripts, 35 were detectable in PAMs (Fig. 3A). Next, we analyzed which genes were regulated by IFN-β (100 ng/mL) in PAMs. The results showed that the expression of pTRIM14, 25, and 56 was significantly up-regulated ($P < 0.05$), and the expression of pTRIM5, 21, 26, 34, 38, and 39 was dramatically increased ($P < 0.01$). Of them, 7 TRIM genes (pTRIM14, 21, 25, 26, 34, 38 and 39) contain the B30.2 domain, belonging to the C-I subfamily.

In addition, to explore the TRIM gene expression in different species stimulated by various stimuli, we summarized the TRIM expression in porcine (from our study), human and mouse macrophages upon IFN-I or immune complex stimuli (Fig. 3B) (Carthagena et al., 2009; Rajsbaum et al., 2008). The table showed that 5 TRIM genes (TRIM14, 21, 25, 26 and 34) expression was significantly up-regulated in all three species ($P < 0.05$). The TRIM5 expression was dramatically up-regulated by 8.2-fold and more than 10-fold in porcine and human macrophages, respectively, with no homologue gene in mouse. The expression of TRIM38 and 56 was both significantly increased in porcine and human macrophages, while the significant up-regulation of TRIM35 expression was only detected in human and mouse macrophages. Moreover, the expression of TRIM3, 8, 23, 39, 45, 58, and 68 was significantly increased in only one species. However, several TRIM genes (TRIM16, 28,

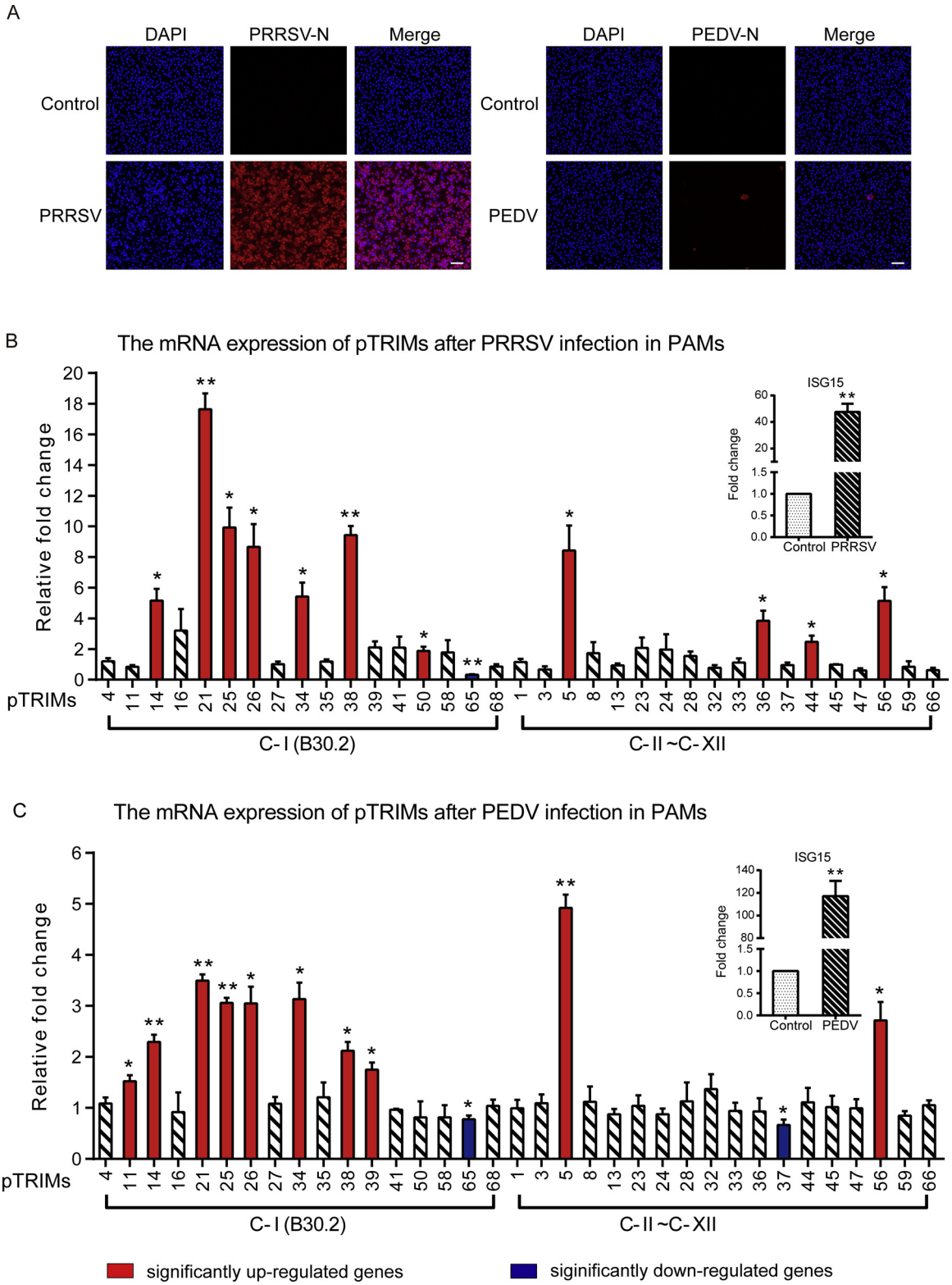

Fig. 4. Expression of porcine TRIM genes after PRRSV or PEDV infection in PAMs.
A. Infection of PAMs with or without PRRSV or PEDV at 0.1 MOI at 24 hpi. Bar, 500 μm. B. Expression of pTRIMs infected by PRRSV. C. Expression of pTRIMs infected by PEDV. RT-qPCR was used to detect the relative mRNA level of pTRIMs. *GAPDH* was used as a reference gene. *ISG15* was used as the positive control. Data are represented as mean ± SD, n = 3. *, $P < 0.05$; **, $P < 0.01$.

37, 59 and 66) expression was significantly down-regulated upon IFN-I or immune complex stimuli in human or mouse macrophages.

Taken together, we totally identified 9 pTRIM genes (pTRIM5, 14, 21, 25, 26, 34, 38, 39 and 56) as ISGs, in which TRIM39 is a porcine-specific interferon-stimulated gene.

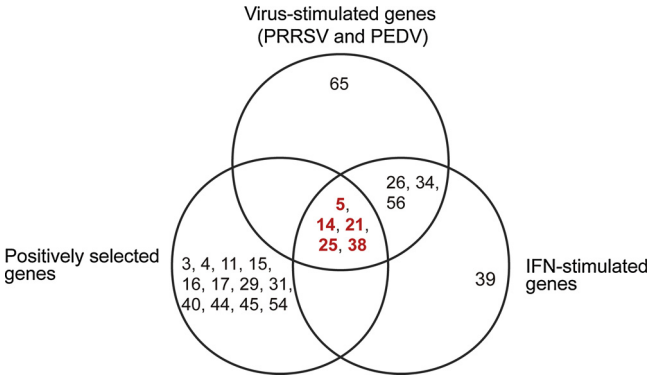

Fig. 5. Implicated restriction factors.
pTRIM5, 14, 21, 25, and 38 genes are the most potential host restriction factors against PRRSV and PEDV infection, based on the molecular evolution analysis, IFN-I stimulation experiment, and viral infection experiments.

3.4. Expression profiles of pTRIMs after PRRSV or PEDV infection in PAMs

In order to study the effect of PRRSV or PEDV (0.1 MOI) infection on pTRIM gene expression in PAMs, the relative mRNA level was detected at 24 hpi (non-infected PAMs served as the control) by RT-qPCR. As shown in Fig. 4A, PRRSV proliferated rapidly in PAMs, which are known as the target cells to PRRSV. While PEDV replication was minimal in PAMs, consistent with the previous research (Deng et al., 2019). Then, we analyzed which genes were regulated by viruses (0.1 MOI) in PAMs at 24 hpi, and *ISG15* was used as the positive control (Fig. 4). The experiments showed that the expression of pTRIM5, 14, 21, 25, 26, 34, 38 and 56 was both significantly up-regulated after PRRSV or PEDV infection in PAMs ($P < 0.05$), and the expression of pTRIM65 was both significantly reduced ($P < 0.05$). The expression of pTRIM36, 44 and 50 was significantly increased after PRRSV infection ($P < 0.05$). The pTRIM11 and 39 expression was significantly up-regulated after PEDV infection, while the pTRIM37 expression was significantly down-regulated ($P < 0.05$). Moreover, the expression pattern of pTRIMs in response to viral infection was similar to that to interferon stimulation in PAMs at indicated points.

3.5. Tissue-specific distribution of the implicated restriction factors in healthy pigs

Based on the above-mentioned evolution analysis, the interferon stimulation experiment, and the viral infection experiments, five pTRIM genes (pTRIM5, 14, 21, 25 and 38) in the middle (the overlapping region of three circles) of the venn diagram (Fig. 5) were implicated as restriction factors against viruses of pig.

In order to characterize tissue-specific distribution of the implicated restriction factors, RT-qPCR was performed to quantify the mRNA level of pTRIM5, 14, 21, 25, and 38 in eleven healthy porcine tissues including heart, liver, spleen, lung, kidney, stomach, jejunum, colon, bladder, muscle, and lymph node. As shown in Fig. 6, pTRIM5, 14, 21, 25 and 38 were detectable in all tested tissues, and the quantity of pTRIMs in these tissues was normalized as compared to that in the muscle. The results showed that the tissue-specific distribution of these 5 pTRIMs was similar. They had highest expression level in spleen. Higher expression was detected in lung, liver, stomach, kidney and lymph node, in which lung is the target organ of PRRSV infection. Expression levels of these pTRIMs in jejunum, colon, bladder, and heart were relatively low.

4. Discussion

In spite of widespread immunization with the currently marketed vaccines, PRRS and PED still persist in many swine-producing countries due to the antigens shift and drift, resulting in devastating damage to the pork producers (Butler et al., 2014; Opriessnig, 2015). Recent studies of PRRSV or PEDV infection have greatly focused on host antiviral genes, providing a significant approach to diseases prevention; therefore, identifying new antiviral host factors is of urgent need (Ke et al., 2017; Li et al., 2017; Zhang et al., 2018). Interestingly, an increasing number of TRIM proteins have been found to display antiviral activities or mediate the antiviral immunity (McNab et al., 2011; Nisole et al., 2005). Researches of TRIM family in human, mouse, primate, and zebrafish, provided some insight into the significant characteristics of TRIM proteins (Sardiello et al., 2008; Boudinot et al., 2011; Carthagena et al., 2009). However, no comprehensive study has been performed so far for the entire pTRIM family. No information is available on the regulation of pTRIM genes expression after PRRSV or PEDV infection. We therefore performed an exhaustive description of pTRIMs, including classification, evolution and expression analysis, to elucidate implication in the antiviral activities.

There are totally 57 TRIM genes in pig, and the number is less than that in human (Carthagena et al., 2009). Among them, several genes do not contain the complete RBCC architecture, but we still included them in our study, as they probably derive from a common ancestor gene. However, several known TRIMs cannot be found in pig. For instance,

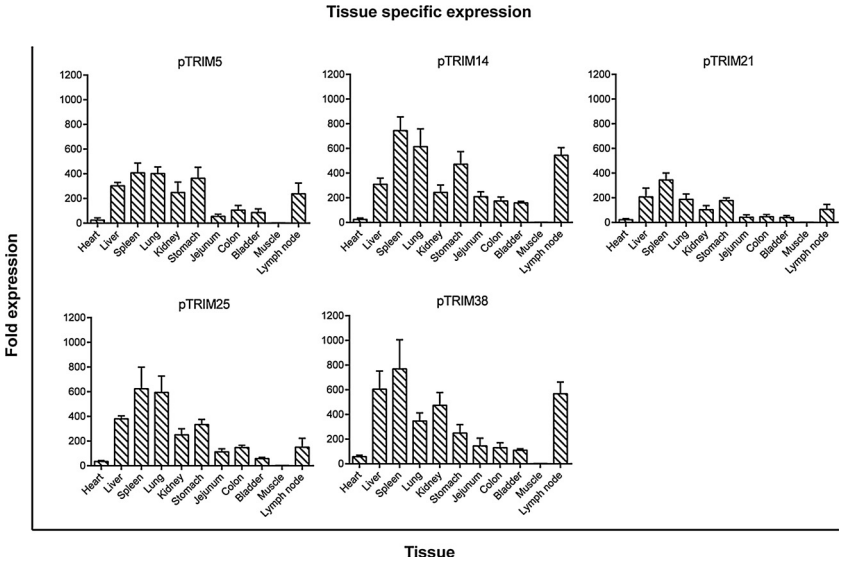

Fig. 6. Tissue distribution of pTRIM5, 14, 21, 25 and 38.
RT-qPCR was used to detect the relative mRNA expression of pTRIM5, 14, 21, 25 and 38 in eleven healthy porcine tissues. *GAPDH* was used as a reference gene. The quantity of pTRIMs in these tissues was normalized as compared to that in the muscle. Data are represented as mean ± SD, n = 3.

TRIM22 is not in our list, but it has been reported to inhibit the HIV, HBV and EMCV replication in other mammals (Barr et al., 2008; Eldin et al., 2009; Gao et al., 2009). To judge whether a gene is a restriction factor or not, one possible way is to detect whether this gene has evolved rapidly (Yang, 2007; Bamshad and Wooding, 2003). Previous studies have shown that TRIM genes with the B30.2 domain in the C-terminus has evolved much faster when compared to those that are conserved among distant species (Sardiello et al., 2008; Meyer et al., 2003). In this study, 57 pTRIM proteins were divided into 12 sub-families based on variable C-terminal domains (Fig. 1A). A total of 28 pTRIMs possess only a complete or partial B30.2 domain in the C-terminus, belonging to C-I to C-III sub-family. Besides, they are clustered together in the phylogenetic tree and on chromosomes, suggesting that they share the same dynamics during evolution (Fig. 1B & C). In addition, we identified 17 TRIM genes having evolved under positive selection in mammals, and figured out positive selection will be expected to act on genes with the B30.2 domain (Fig. 2). Indeed, we found 9 pTRIMs with the B30.2 domain, including pTRIM4, 11, 14, 16, 17, 21, 25, 31 and 38, have evolved under positive selection. Among these TRIM genes, a couple of known restriction factors were found, such as TRIM21, 25 and 38 (Uchil et al., 2008; Gack et al., 2007; Mallery et al., 2010). However, we detected that TRIM5 only contains a SPRY domain but it has undergone the intense selective pressures, accordance with other studies in primates (Sardiello et al., 2008). Moreover, seven pTRIM genes under positive selection do not possess any part of the B30.2 domain. For instance, TRIM15, a known functional factor that has the ability to inhibit the release of retroviruses, consists of the classical TRIM alone, which may reflect that the absence of the B30.2 domain does not preclude TRIM gene from being candidate restriction factors (Uchil et al., 2008). Hence, we regarded these 17 pTRIMs as the potential candidates with antiviral activity.

Innate immunity is the first line of defense against pathogens. Type I Interferons are the main mediators of innate immunity against viral infection, by upregulating the expression of many antiviral effectors. Strikingly, most of the TRIM proteins implicated in antiviral response have been found to be up-regulated by IFNs or viral infection (Carthagena et al., 2009; Gack et al., 2007; Schaller et al., 2007). Therefore, the identification of TRIM proteins up-regulated in response to IFNs/viruses may pinpoint TRIM proteins with antiviral activities. During our investigation of IFN stimulation in PAMs, 35 out of the 57 pTRIMs were detectable in PAMs. We identified 9 pTRIMs (pTRIM5, 14, 21, 25, 26, 34, 38, 39 and 56) significantly up-regulated as the ISGs (Fig. 3A). Interestingly, our data largely overlapped with the studies performed on human and mouse TRIM genes by Carthagena et al and Rajsbaum et al (Fig. 3B) (Carthagena et al., 2009; Rajsbaum et al., 2008). For instance, TRIM14, 21, 25, 26 and 34 were all significantly up-regulated in response to IFNs in porcine, human and mouse macrophages. The expression of TRIM38 and 56 was both significantly increased in porcine and human macrophages, but undetectable in mouse cells. TRIM5 expression was dramatically up-regulated by 8.2-fold and more than 10-fold in porcine and human macrophages, respectively, with no homologue gene in mouse. In addition, we identified pTRIM39 as a porcine-specific ISG, suggesting its species-specific battle against virus infection. On the contrary, the expression of several TRIM genes (TRIM16, 28, 37, 59 and 66) was significantly down-regulated upon IFN-I or immune complex stimuli in human and mouse, not in porcine. It must be noted that there are some correlation between the susceptibility to IFNs and the domain structure. The PRY/SPRY domain may confer antiviral activity on TRIM, which is why these genes are of particular interest. Of 9 porcine ISGs, 7 TRIM genes (pTRIM14, 21, 25, 26, 34, 38 and 39) present the B30.2 structure characteristic of the C-I porcine TRIM subfamily, whereas TRIM5 belongs to the C-II subfamily since it lacks the PRY domain.

PAMs are the major producers of IFN-I *in vitro* during viral infection. Moreover, PAMs are known as the target cells to PRRSV, and can be infected by PEDV *in vitro*. The innate immune response to PEDV is cell type-specific, with macrophages responding via IFN-α/IFN-β response, whereas the epithelial cell response includes IFN-λ (Deng et al., 2019). Our two viral infection experiments on PAMs showed that the significantly up-regulated expression of pTRIM5, 14, 21, 25, 26, 34, 38, and 56 was virus nonspecific, whereas pTRIM36, 44, and 50 affected PRRSV infection specifically, and pTRIM11 and 39 affected PEDV infection specifically (Fig. 4). Moreover, down-regulated expression of pTRIM65 was found after PRRSV and PEDV infection, which needs further studies.

Based on the analysis, we elucidated pTRIM5, 14, 21, 25 and 38 as implicated restriction factors (Fig. 5), the gene expressions of which are tissue-specific (Fig. 6). Studies found that TRIM5 can restrict post-entry of HIV-1 and N-MLV dependent on its SPRY domain (Stremlau et al., 2004). TRIM14 can mediate inhibition on RNP formation and influenza virus replication (Wu et al., 2019). TRIM21 plays an important role in viral neutralization with its E3 ubiquitin ligase activity (Mallery et al., 2010). TRIM25 controls RIG-I-mediated antiviral activity in response to RNA virus infection (Gack et al., 2007). TRIM38 positively or negatively regulates innate immunity by its E3 ligase (Uchil et al., 2008). However, very little is known about these 5 pTRIMs against porcine viruses. Till now, pTRIM21 was demonstrated to inhibit foot-and-mouth disease virus replication (Fan et al., 2016), and pTRIM25 was found to inhibit PRRSV replication in Marc-145 cells (Zhao et al., 2019). Whether and how pTRIM5, 14, 21, 25, and 38 induce anti-PRRSV or anti-PEDV response needs further studies.

In summary, our results revealed evolution and expression properties of entire pTRIM family. More importantly, we predicted pTRIM5, 14, 21, 25 and 38 as implicated restriction factors against PRRSV and PEDV infection, which may act via a direct host–virus interaction. These findings have provided a basis to further functional studies of pTRIM genes and the mechanism by which pTRIMs may act during viral infection.

Author contributions

YC and YW conceived and designed the experiments; YW and SZ performed the experiments; YW analyzed the data; YW and CZ prepared the figures and tables; YW and CZ wrote the paper; CX modified the manuscript. YC checked and finalized the manuscript. All authors read and approved the final manuscript.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197647.

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