

1 ***Streptococcus suis* encodes multiple allelic variants of a phase-variable Type III DNA**
2 **methyltransferase, ModS, that control distinct phasevarions**

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11 **Running title:** Characterisation of *modS* phasevarions in *S. suis*

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16

17 **Abstract**

18 *Streptococcus suis* is a significant cause of bacterial meningitis in humans, particularly in S.E.
19 Asia, and is a leading cause of respiratory and invasive disease in pigs. Phase-variable DNA
20 methyltransferases, associated with Restriction-Modification (R-M) systems, are a source of
21 epigenetic gene regulation, controlling the expression of multiple genes. These systems are
22 known as phasevarions (phase-variable regulons), and have been characterised in many
23 host-adapted bacterial pathogens. We recently described the presence of a Type III DNA
24 methyltransferase in *S. suis*, ModS, which contains a simple sequence repeat (SSR) tract
25 within the open reading frame of the *modS* gene, and which varied in length between
26 individual strains. We also observed multiple allelic variants of the *modS* gene were present
27 in a population of *S. suis* isolates. Here, we demonstrate that a biphasic ON-OFF switching of
28 expression occurs in the two most common ModS alleles, ModS1 and ModS2, and that
29 switching is dependent on SSR tract length. Further, we show that ModS1 and ModS2 are
30 active methyltransferases in *S. suis* using Single-Molecule, Real Time (SMRT) sequencing.
31 ON-OFF switching of each ModS allele results in the regulation of distinct phasevarions, with
32 the ModS2 phasevarion impacting growth patterns and antibiotic resistance. This is the first
33 demonstration of a phase-variable Type III DNA methyltransferase in a Gram-positive
34 organism that controls a phasevarion. Characterising the phenotypic effects of
35 phasevarions in *S. suis* is key to understanding pathogenesis and the development of future
36 vaccines.

37 **Importance**

38 *Streptococcus suis* is a causative agent of meningitis, polyarthrititis and polyserositis in swine,
39 and is a major cause of zoonotic meningitis in humans. Here we investigate epigenetic gene

40 regulation in *S. suis* by multiple phasevarions controlled by the phase-variable Type III DNA
41 methyltransferase ModS. This is the first characterised example of a Type III R-M system
42 regulating a phasevarion in a Gram-positive organism. We demonstrate that biphasic ON-
43 OFF switching of ModS expression results in differences in bacterial growth and antibiotic
44 resistance. Understanding the effects of ModS phase variation is required to determine the
45 stably expressed antigenic repertoire of *S. suis*, which will direct and inform the
46 development of antimicrobial treatments and vaccines against this important pathogen.

47 Introduction

48 *Streptococcus suis* is an important veterinary pathogen that contributes a substantial
49 disease burden to the swine industry (1). *S. suis* is also a significant pathogen in humans,
50 causing zoonotic meningitis, most commonly associated with occupational exposure (2) and
51 consumption of contaminated pork products (3). *S. suis* frequently colonises the upper
52 respiratory tract of pigs, where it is considered a commensal organism (4). Virulent strains of
53 *S. suis* are able to adhere to and invade epithelial cells of the airway allowing access to the
54 bloodstream. In both pigs and humans, *S. suis* is able to breach the blood brain barrier,
55 resulting in the development of meningitis and septic shock (4).

56 Phase variation is the rapid and reversible switching of gene expression, and plays an
57 important role in the pathogenesis of many organisms, where it is usually associated with
58 expression of surface factors such as capsules (5), adhesins (6, 7) and lipooligosaccharide
59 biosynthesis (8). Randomised gene expression as a result of phase variation can complicate
60 vaccine development as it results in an unstable antigenic repertoire, and is potentially a
61 major cause of vaccine escape. Many bacterial pathogens also encode cytoplasmically
62 located phase-variable DNA methyltransferases, that are associated with restriction-
63 modification (R-M) systems (9). Variable methyltransferase expression results in genome
64 wide methylation differences, resulting in differential gene regulation by epigenetic
65 mechanisms in systems called phasevarions (phase variable regulons) (9, 10). Many
66 phasevarions are controlled by the ON-OFF switching of Type III DNA methyltransferases,
67 encoded by *mod* genes. Phasevarions controlled by Type III *mod* genes have been described
68 in *Haemophilus influenzae* (11, 12), *Moraxella catarrhalis* (13), *Neisseria* spp. (14),
69 *Helicobacter pylori* (15) and *Kingella kingae* (16). The phasevarions in these human adapted

70 pathogens all control expression of genes involved in the pathogenesis of these organisms
71 (17-19). All these phase variable *mod* genes contain simple DNA sequence repeat (SSR)
72 tracts within their open reading frame. These SSRs tracts are highly unstable and prone to
73 variation in length due to slipped-strand mispairing during DNA replication, resulting in the
74 biphasic ON-OFF switching of gene expression; the *mod* gene is either in-frame and Mod is
75 expressed (ON), or a variation in SSR tract length results in a frameshift and premature stop
76 codon, and Mod is not expressed (OFF) (20).

77 The methyltransferase specificity of a Type III Mod protein is dictated by the central target
78 recognition domain (TRD) of the encoding *mod* gene (9, 10). This TRD varies between the
79 alleles of individual *mod* genes, with the 5' and 3' regions of individual *mod* genes being
80 highly conserved between alleles (9, 10). For example, there are twenty-one *modA* alleles
81 encoded by non-typeable *Haemophilus influenzae* (NTHi) and *Neisseria* species; six *modB*
82 and seven *modD* alleles have been identified in *Neisseria gonorrhoeae* and *Neisseria*
83 *meningitidis*; and nineteen *modH* alleles are present in *H. pylori* (10, 21). Our previous
84 analysis of the restriction enzyme database REBASE demonstrated the presence of a *mod*
85 gene in *S. suis*, which we have named *modS*, that contained a GAGCA_(n) SSR tract (22). A
86 follow up study analysing of a large collection of *S. suis* isolates determined the presence of
87 three distinct *modS* alleles, each of which methylated an adenine in distinct DNA sequences.
88 ModS1 methylated 5'-GCG^(m6)ADT-3' (D is either A,G or T), ModS2 methylated 5'-
89 VTC^(m6)ATC-3' (V is either A,G or C), and ModS3 (present in a single strain identified in
90 GenBank) methylated 5'-GTTC^(m6)ANNNB-3' (B is either C, G or T; N is any nucleotide) (23).
91 These specificities were determined through heterologous expression of the
92 methyltransferase in *E. coli*. Whilst variable length GAGCA_(n) SSR tracts were present in

93 different strains of *S. suis* encoding the same *modS* allele, it was not determined whether
94 *modS* is phase variable, or whether the ModS protein is an active methyltransferase in *S.*
95 *suis* when expressed.

96 In this study we investigated phase variation of the two most prevalent *modS* alleles, *modS1*
97 and *modS2*, by enriching populations of *S. suis* strains for specific GAGCA_(n) SSR tract lengths
98 in each encoding *modS* gene. We then determined if differential protein expression
99 occurred in enriched ON-OFF populations of *S. suis* using SWATH-MS. Clinically relevant
100 phenotypes were assessed *in vitro* to determine if phase variation of the ModS
101 methyltransferase could result in relevant phenotypic changes or advantages *in vivo*.
102 Studying the impact of these systems provides an understanding of not only disease
103 pathogenesis, but will also guide the development of vaccines by defining the stably
104 expressed antigenic repertoire of *S. suis*.

105

106 **Results**

107 ***ModS is a phase variable DNA methyltransferase***

108 In all previous examples of phase-variable Type III *mod* genes, SSR tract length variation
109 results in a biphasic ON-OFF switching of expression. In order to determine if variation in
110 length of the GAGCA_(n) repeat tract (Figure 1A) located in the *modS* open reading frame led
111 to phase variable expression of the gene, isogenic strains were generated containing
112 defined lengths of this SSR tract. The isolation of three consecutive repeat tract lengths
113 would theoretically result in one strain where the respective *modS* gene is ON, (GAGCA_(n)
114 repeat tract length will place the gene in frame and expressed), and two strains of the triplet

115 will be OFF (GAGCA_(n) repeat tract length will place the gene out of frame and not
116 expressed). These enriched populations were generated in *S. suis* strains LSS89 (*modS1*) or
117 SS1056 (*modS2*), as our analysis of the TRDs of these two alleles showed they were highly
118 variable, and therefore predicted to methylate different target sequences (Figure 1B) as we
119 demonstrated with heterologous over-expression of ModS1 and ModS2 previously (23).
120 Strains were enriched for SSR tract lengths containing either 19, 20 or 21 repeats in *modS1*
121 and 17, 18 or 19 repeats in *modS2*. Fragment analysis of each strain confirms enrichment of
122 each SSR tract length to above 80% (Figure 1C).

123 Western blotting of whole cell lysates of these enriched strains with antisera generated
124 against the conserved region of all ModS proteins demonstrate that variation in SSR tract
125 length led to a biphasic ON-OFF switching of expression (Figure 1D; full blot in
126 Supplementary Figure 1). The ModS1 protein was only produced in the LSS89 strain which
127 contained 21 GAGCA repeats in the *modS1* gene. ModS2 was only produced in the SS1056
128 strain enriched for 18 GAGCA repeats. This matches the prediction from the annotated start
129 codon of both genes (Supplementary Figure 2).

130 To demonstrate methyltransferase activity of the expressed ModS protein, Pacific
131 Biosciences (PacBio) Single-Molecule, Real-Time (SMRT) sequencing was carried out on
132 genomic DNA isolated from our triplet sets of enriched isogenic strains (19, 20, 21 repeats
133 for *modS1* in strain LSS89; 17, 18, 19 repeats for *modS2* in strain SS1056). We detected a
134 separate, distinct Type I methyltransferase motif in strain LSS89 compared to strain SS1056
135 (Table 1), with these motifs being fully methylated in all three strains of each enriched
136 triplet set. When comparing the methylomes of enriched LSS89 strains, we detected a Type
137 III methyltransferase motif, 5'-GCG^(m6)AT, only present in the strain where ModS1 was

138 expressed (21 repeats ON; Table 1). This methylation by ModS1 occurred at 2859 5'-
139 GCG^(m6)AT sites throughout the genome, and was not detected in strains enriched for 19 or
140 20 GCACA repeats in the *modS1* gene. SS1056 enriched strains exhibited a different Type III
141 motif, 5'-VTC^(m6)ATC, which was only present when ModS2 was expressed (18 repeats ON;
142 Table 1). This motif was methylated 4810 times in the SS1056 genome, and again was not
143 methylated in strains enriched for 17 or 19 repeats (OFF) in the *modS2* gene. These motifs
144 are the same as detected previously when these ModS alleles were heterologously over-
145 expressed in *E. coli* (5).

146

147 ***Biphasic switching of ModS1 and ModS2 results in the expression of distinct phasevarions***

148 Quantitative proteomics was carried out to determine if distinct protein expression profiles
149 occurred as a result of ModS expression in *S. suis*, i.e., if these phase-variable
150 methyltransferases control phasevarions. The expression profiles of an ON-OFF ModS1 pair
151 from strain LSS89 (21 repeats ON and 19 repeats OFF) and an ON-OFF ModS2 pair from
152 strain SS1056 (18 repeats ON and 17 repeats OFF) were assessed using SWATH-MS. Both
153 strain pairs exhibited unique changes in protein expression dependent on phase variation of
154 their respective *modS* gene, with SWATH-MS covering ~23% of total proteins in LSS89
155 strains and ~22% of total in SS1056 strains. Significant differences in protein abundance
156 between the ON and OFF populations are represented as volcano plots in Figure 2. In strain
157 LSS89, ModS1 ON resulted in higher expression of several proteins involved in a range of
158 cellular processes compared to strains where ModS1 was OFF. Multiple transcriptional
159 regulators were upregulated by expression of ModS1 such as the replication initiator protein
160 DnaA (24), and a YlbF/ YmcA type protein (25), as well as transcription factors and

161 repressors. Many ribosomal subunits were upregulated as well as those involved in general
162 metabolism such as a nitroreductase (26), glucokinase (27) and a protein involve in
163 alkylphosphonate utilisation (28) (Table 2). Conversely, in strains where ModS1 was not
164 expressed, proteins such as ribosomal proteins, several components of ABC transporters,
165 and the fatty acid binding protein DegV (29) were all increased in expression (Table 2).

166 In strain SS1056, ModS2 ON-OFF switching resulted in varied expression of a distinct set of
167 proteins (Table 3). ModS2 expression increased the levels of proteins involved in amino acid
168 metabolism such as cysteine synthase and aminopeptidase D (30). Proteins involved in
169 general metabolism also showed increased expression in ModS2 ON, such as
170 dihydroxyacetone kinase (31). The DNA biosynthesis enzymes ribonucleotide-diphosphate
171 reductase and ribonucleotide-triphosphate reductase (32) were increased in expression in
172 ModS2 ON, as was an acyl carrier protein involved in fatty acid biosynthesis (33). Three
173 proteins were upregulated in strains which did not express ModS2 (OFF); an ATP-dependent
174 Clp protease, the nucleotide exchange factor GrpE and a glyoxalase/bleomycin
175 resistance/extradiol dioxygenase family protein involved in resistance to antimicrobials.

176

177 ***ModS switching results in differences in growth***

178 Our proteomic analysis of ModS switching demonstrated several proteins which could
179 potentially impact growth rate exhibited differential regulation in both ModS alleles. These
180 included proteins involved in general metabolic enzymes, DNA transcription factors and
181 repressors as well as biosynthesis enzymes (Tables 2 & 3). In order to determine if these
182 altered protein expression levels could affect *S. suis* growth rates, we conducted standard
183 growth curves of our enriched ModS ON-OFF pairs in rich media. These growth curves

184 showed a small but repeatable ($n = 3$) difference in growth rate for both strain pairs (Figure
185 3): when ModS1 was ON, growth was to a lower final OD (P value <0.05), and conversely,
186 when ModS2 was ON, growth was to a higher final OD (P Value <0.05).

187

188 ***ModS2 phase variation results in differences in antibiotic resistance***

189 SWATH-MS also demonstrated differential expression of an antibiotic resistance protein in
190 ModS2 (WP_002935876.1), annotated as a glyoxalase/bleomycin resistance/extradiol
191 dioxygenase family protein, which exhibited higher expression in strains which did not
192 express ModS2 (OFF) compared to ModS2 ON. We therefore carried out a minimum
193 inhibitory concentration (MIC) analysis using several beta lactam antibiotics (ampicillin,
194 penicillin and amoxicillin) and a glycopeptide antibiotic (vancomycin) (Table 4). Although
195 there was no change in MIC to amoxicillin, vancomycin and penicillin, there was a two-fold
196 increase in resistance to ampicillin when ModS2 was OFF compared to ModS2 ON (0.32
197 $\mu\text{g}/\text{mL}$ vs 0.16 $\mu\text{g}/\text{mL}$ respectively).

198

199 **Discussion**

200 Over the last 15 years, phasevarions (phase-variable regulons) controlled by ON-OFF
201 switching of Type III *mod* genes have emerged as an important gene regulation strategy in a
202 diverse range of host-adapted pathogens (9, 12, 13, 15, 16, 21). In all these examples, phase
203 variable ON-OFF switching of *mod* expression occurs via changes in the length of locus
204 encoded SSR tracts. In this study, using a combination of strain enrichment, Western
205 blotting, and SMRT sequencing, we conclusively demonstrate that *S. suis* contains multiple

206 allelic variants of a new phase-variable Type III *mod* gene, *modS*, with this representing the
207 first example of a phasevarion controlled by biphasic ON-OFF switching of a Type III DNA
208 methyltransferase in a Gram-positive organism (Figure 1). The antisera used to detect ModS
209 was generated against the conserved regions of ModS, and should recognise all ModS
210 alleles with equal affinity, as sequence variation of Mod proteins only occurs in the central
211 TRD region (9, 34). Both ModS alleles were only expressed in strains with specific SSR tract
212 lengths; ModS1 was only present in strains with 21 GAGCA repeats and ModS2 was only
213 expressed in strains with 18 GAGCA repeats. In both cases, the repeat length placed the
214 *modS* gene in the correct reading frame and demonstrates that the expression of the ModS
215 protein is dependent on the SSR tract length.

216 SMRT sequencing was used to determine methyltransferase activity and specificity of
217 ModS1 and ModS2. LSS89 strains of *S. suis* enriched for ModS1 expression (21 GACAC
218 repeats ON) exhibited methylation of the adenine in 5'-GCG^{m6}AT motifs which was not seen
219 in strains in which ModS1 was OFF (19 or 20 GACAC repeats). ModS2 expressing strains
220 (SS1056) of *S. suis*, exhibited methylation of a Type III Mod motif of 5'-VTC^{m6}ATC motifs
221 (where V can be either A, G or C) when ModS2 was predicted to be ON (18 GACAC repeats).
222 This confirmed our previous findings when we over-expressed ModS1 and ModS2 using a
223 recombinant *E. coli* system (23). SMRT sequencing also demonstrates that both ModS1 and
224 ModS2 are active methyltransferases in *S. suis*, methylating at thousands of sites in their
225 respective genomes (in strain LSS89, 2861 5'-GCG^{m6}AT sites are present; in SS1056, 4812 5'-
226 TC^{m6}ATC sites are present; Supplementary Data 1).

227 SWATH-MS proteomic analysis confirmed that the altered methylation resulting from ModS
228 phase-variation results in a change in the expression of numerous proteins in distinct

229 phasevarions. These changes in protein expression were seen across many families of
230 protein, including those involved in central metabolism, gene regulation, and transporters.
231 Phase-variable switching of ModS resulted in differences in growth rate, but it is unclear
232 whether this is a result of an alteration of regulatory genes due to ModS switching, or
233 another, as yet uncharacterised effect of ModS phase variation. Although the final
234 differences in final OD between enriched ON and OFF strain pairs were small, they are
235 statistically significant (P value <0.05), and could have an effect on both long-term carriage
236 and disease, particularly if one population has an advantage resulting from differential
237 protein expression over time. ModS2 phase variation also resulted in a difference in
238 antibiotic resistance. A protein repressed by ModS2 expression, annotated as a glyoxalase,
239 bleomycin resistance and extradiol dioxygenase family protein, described as involved in
240 resistance to beta-lactam and glycopeptide antibiotics (35), which are commonly used to
241 treat bacterial infections in humans as well as commercial pig farms (36). The MIC of *S. suis*
242 was assessed against three commonly used beta lactams (ampicillin, amoxicillin and
243 penicillin) and a glycopeptide from the same antibiotic class as bleomycin (vancomycin). A
244 strain in which ModS2 was OFF, where the expression of this resistance protein was
245 increased, showed a small (2-fold) increase in MIC towards ampicillin, compared to the
246 isogenic strain in which ModS2 was ON. Although small, there may be a cumulative effect,
247 particularly as many beta lactam antibiotics are extensively used prophylactically in
248 commercial pig farms (37-40). This has the potential to result in a long-term increase in
249 ampicillin resistance in *S. suis* populations, although this would need to be studied in a
250 model system and is beyond the scope of this work.

251 Although many of the changes in protein expression determined by our SWATH-MS analysis
252 of enriched ON-OFF population in each ModS allele were small (1.5-fold to 2-fold), SWATH-

253 MS is a technique which quantitates protein expression based on abundance (41).
254 Differential expression of proteins will therefore only identify differences in highly
255 expressed proteins, and expression differences of proteins beyond the limit of detection will
256 be missed. We recognise this is a limitation of our approach, but a SWATH-MS proteomics
257 approach has been previously used to characterise phasevarion mediated changes to
258 protein expression (13, 42), with similar small differences in expression also reported in
259 these phasevarions. Therefore, whilst we have determined that both ModS1 and ModS2
260 affect expression of small distinct sets of proteins, it is likely that further changes to gene
261 and protein expression remain undetected. In order to thoroughly characterise each
262 phasevarion, further analysis will be needed. It will also be important to characterisation the
263 phasevarions controlled by other *modS* alleles; in previous work we showed that a third
264 *modS* allele – *modS3* – was present in *S. suis*. The ModS3 allele methylated a distinct
265 sequence to ModS1 and ModS2 (23), and is therefore highly likely to control a different
266 phasevarion. The presence of additional *modS* alleles should also be determined, and
267 studied, in order to detail all gene and protein expression changes mediated by
268 phasevarions in *S. suis*.

269 This characterisation of ModS is the first described instance of a phasevarion in a Gram-
270 positive organism controlled by a Type III methyltransferase. Different alleles of ModS
271 methylate distinct motifs and result in distinct phasevarions. These distinct ModS
272 phasevarions result in gross differences in growth rate, and in the case of ModS2,
273 differential resistance to antibiotics. Both these phenotypes could affect disease and
274 pathology, and this remains to be studied using both *in vitro* models and ideally, an *in vivo*
275 challenge using the natural host (pigs). A thorough understanding of phase variation of gene
276 expression, and in particular phasevarions, is required in order to determine the stably

277 expressed antigenic repertoire of a bacterial species. The prevalence of phase variable
278 methyltransferases across the bacterial domain demonstrates that phasevarions are a
279 widespread contingency strategy (22, 43, 44), and that characterisation of these systems is
280 imperative in order to rationally design effective vaccines that only target stably expressed
281 antigens in the organisms where they are present.

282

283 **Materials and methods**

284 ***Bacterial strains and growth***

285 *S. suis* strains LSS89 and SS1056 (45) used in this study were grown in THB broth (Oxoid)
286 supplemented with 2% yeast extract (THB-Y) or on THB-Y plates (THB-Y broth supplemented
287 with 1.5% w/v agar). Cultures were incubated overnight at 37°C.

288 ***Strain enrichment***

289 Fragment length analysis of the SSR tract in each *modS* allele was conducted to determine
290 the length of the GAGCA_(n) SSR tract. A PCR was performed using a fluorescently labelled
291 forward primer SsuT3-F-FAM (5'-FAM-CAT CAA AAA CGG CTT GAC AGC C) and the reverse
292 primer SsuT3-R (5'-GCA ATG TTG TCT GAT AAA ACA TCT TTT G) as described previously (23).
293 DNA fragment length analysis was carried out at the Australian Genome Research Facility
294 (AGRF; Brisbane, Australia). This technique was used to enrich populations of *S. suis* strains
295 for defined SSR lengths through a combination of fragment length analysis and subculturing.
296 *S. suis* populations of strain LSS89 (encoding *modS1*) were enriched for 19, 20 and 21
297 GAGCA repeats, and strain SS1056 (encoding *modS2*) were enriched for 17, 18, 19 GAGCA

298 repeats. Populations containing >80% of each single tract length were considered to be
299 enriched, and were used in subsequent studies.

300 ***SDS-PAGE and Western blot***

301 Cell lysates of each enriched strain were prepared from overnight liquid cultures of *S. suis*
302 grown in THB-Y broth. Samples were normalised to O.D.₆₀₀ 30.0, pelleted at 12,000xg for 1
303 minute and resuspended in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% SDS, 50 mM
304 Tris HCl, pH 8). Samples were sonicated twice for 30 seconds at a probe intensity of 15
305 amplitude microns, on ice. Lysates were clarified by centrifugation at 14,000xg for 20
306 minutes to remove cellular debris.

307 Supernatants were prepared for SDS-PAGE using BOLT 4x loading dye containing 100 mM
308 dithiothreitol (Sigma) and boiled for 30 minutes, then loaded on precast 4-12% BOLT Bis-Tris
309 gel (Thermo Fisher) and run for 45 minutes at 165V in MOPs 1x running buffer according to
310 manufacturer's instructions (Thermo Fisher). Proteins were visualised by Coomassie brilliant
311 blue staining. Twice the amount of lysate was loaded onto gels for use in Western blotting
312 and proteins were transferred to pre-equilibrated polyvinylidene difluoride (PVDF)
313 membranes (BioRad) at 20 volts for 60 minutes using the BOLT transfer system (Thermo
314 Fisher). Following transfer, membranes were blocked with 10% skim milk in TBS-T for 60
315 minutes. Membranes were incubated with a 1:1,000 dilution of anti-ModS antisera (raised
316 as detailed below) overnight at 4°C, followed by a 1:10,000 anti-mouse alkaline-
317 phosphatase secondary antibody (Sigma). Blots were developed using NBT/BCIP (Roche)
318 according to manufacturer's instructions.

319 ***Construction of a TRD-less ModS protein***

320 The *modS1* gene was amplified from *S. suis* strain LSS89 excluding the CAGCA_(n) SSR tract
321 using primers SsuT3-oE-F (5'-AGTCAG CATATG AGC AGA GCA AAG CAA AAG CTT GGA GAA
322 TAC ACT CAA G) and SsuT3-oE-R (5'-AGTCAG GGATCC CTA CAC CAC CTT CAC TTT GGT ACC)
323 with KOD Hot Start DNA polymerase (Merck Millipore) according to manufacturer's
324 instructions. The resulting product was then cloned into the NdeI-BamHI site of the
325 expression vector pET15b (Novagen) with the gene in-frame with the N-terminal His-tag to
326 generate vector pET15b:SsuT3-His_tag. The TRD coding region of *modS* was then removed
327 and the conserved 5' and 3' coding regions were fused. This was generated by using the
328 pET15b::SsuT3-His_tag vector as a template via inverse PCR with primers SsuT3-TRD-
329 remove-F (5'-CCCA AAT ATC TCA TCA CAG ATA GCT TTG AGG TTG G) and SsuT3-TRD-
330 remove-R (5'-GCT GTT ATG CAG CTG AAT GCA GAA GAT GG) binding either side of the TRD,
331 using KOD Hot Start DNA polymerase (Merck Millipore) according to manufacturer's
332 instructions. His-tagged TRD-less ModS was over-expressed in *E. coli* BL21 using isopropyl β-
333 d-1-thiogalactopyranoside (IPTG) induction (0.5 mM) overnight at 37°C with 200 rpm
334 shaking, and protein purified using TALON resin (Takara Bio) using standard protocols in 50
335 mM phosphate buffer pH7.4 containing 300mM NaCl.

336 **Generation of ModS Antisera**

337 A cohort of five 6-8 week old female BALB/c mice (Animal Resources Centre, WA, Australia)
338 were immunized subcutaneously with 25 µg of recombinant TRD-less ModS protein in 25µL
339 PBS, mixed with 25 µL Freund's Adjuvant (Merck, Darmstadt, Germany; Freund's Complete
340 Adjuvant (FCA) on day 0 and Freund's Incomplete Adjuvant (FIA) subsequently on days 14,
341 21, 28 and 42. Terminal bleeds were collected on day 58 and serum separated via
342 centrifugation. Pre-immune (naïve) serum was collected from cohorts prior to immunization

343 via tail bleed. Serum was stored in 50% glycerol at -20°C. This antisera recognises all ModS
344 alleles with equal affinity as it was raised against the conserved regions of ModS shared by
345 all alleles. All animal work was carried out according to the Australian Code for the Care and
346 Use of Animals for Scientific Purposes, with approval from the Griffith University Animal
347 Ethics Committee (GLY/16/19/AEC).

348 ***Single-Molecule, Real-Time (SMRT) sequencing and methylome analysis***

349 Genomic DNA from our enriched triplet sets of *S. suis* strains LSS89 (*modS1* 19, 20, 21
350 repeats) and SS1056 (*modS2* 17, 18, 19 repeats) was prepared from an overnight culture in
351 THB-Y broth and high-molecular-weight genomic DNA was isolated using the Sigma
352 Genelute kit (Sigma Aldrich) according to the manufacturer's instructions. SMRT sequencing
353 and methylome analysis was carried out as previously described (46, 47). Briefly, DNA was
354 sheared to an average length of approximately 5-10 kb (genomic DNA) using g-TUBEs
355 (Covaris, Woburn, MA, USA) and SMRTbell template sequencing libraries were prepared
356 using sheared DNA. DNA was end repaired, then ligated to hairpin adapters. Incompletely
357 formed SMRTbell templates were degraded with a combination of Exonuclease III (New
358 England Biolabs; Ipswich, MA, USA) and Exonuclease VII (USB; Cleveland, OH, USA). Primer
359 was annealed and samples were sequenced on the PacBio Sequel system (Menlo Park, CA,
360 USA) using standard protocols for long insert libraries. SMRT sequencing and methylome
361 analysis was carried out at SNPSaurus (University of Oregon, USA).

362 ***SWATH-MS proteomics***

363 Overnight cultures of each *S. suis* strain (10^7 CFU/ml) were harvested, lysed in guanidium
364 buffer (6 M guanidium chloride, 50 mM Tris-HCl pH8, 10 mM dithiothreitol) and incubated
365 at 30°C for 30 minutes with shaking (500 rpm). Cysteines of the total protein were alkylated

366 by addition of acrylamide to a final concentration of 25 mM and incubated at 30°C for 60
367 minutes with shaking (500 rpm). Concentration of samples was assessed using a Nanodrop
368 2000 (Thermo Fisher). A 100 µg aliquot of the protein was then precipitated by addition of
369 1:1 methanol: acetone at -20°C overnight. The protein was pelleted at 18,000xg for 10
370 minutes and supernatant was removed before the pellet was resuspended in 50 µL trypsin
371 reaction buffer and 1 µg trypsin (New England Biolabs) added and the suspension incubated
372 overnight at 37°C. Tryptic digested peptides were then desalted and purified using a Ziptip
373 (Millipore) as per manufacturer instructions. SWATH-MS was performed as previously
374 described (48). Briefly, tryptic peptides were analyzed by LC-ESI-MS/MS using a Prominence
375 nanoLC system (Shimadzu) and Triple TOF 5600 mass spectrometer with a Nanospray III
376 interface (SCIEX). Peptides were separated on a Vydac EVEREST reversed-phase C18 HPLC
377 column at a flow rate of 1 µL/min. A gradient of 10–60% buffer B over 45 min, with buffer A
378 (1% acetonitrile and 0.1% formic acid) and buffer B (80% acetonitrile and 0.1% formic acid)
379 was used. An MS-TOF scan was performed from an m/z range of 350– 1800 for 0.5 s
380 followed by information dependent acquisition of MS/MS of the top 20 peptides from m/z
381 40–1800 for 0.05 s per spectrum, with automated CE selection. Identical LC conditions were
382 used for SWATH-MS. SWATH-MS of triplicate biological replicates was performed with the
383 same MS-TOF scan, followed by high sensitivity information-independent acquisition with
384 m/z isolation windows with 1 m/z window overlap each for 0.1 s across an m/z range of
385 400–1250. Collision energy was automatically assigned by Analyst software (AB SCIEX) based
386 on m/z window ranges. Proteins were identified by searching against *S. suis* Lss89 and
387 SS1056 genomes (NCBI Accession GCA_900059105.1 and GCA_900051945.1 respectively)
388 and common contaminants with standard settings using ProteinPilot 5.0.1 (AB SCIEX). False
389 discovery rate analysis was performed on all searches. ProteinPilot search results were used

390 as ion libraries for SWATH analyses. The abundance of proteins was measured automatically
391 using PeakView (AB SCIEX) with standard settings. Comparison of protein relative
392 abundance was performed based on protein intensities or ion intensities using a linear
393 mixed-effects model with the MSstats package in R. Proteins with greater than X changes in
394 abundance and with adjusted P-values. The mass spectrometry proteomics data have been
395 deposited to the ProteomeXchange Consortium via the PRIDE (49) partner repository with
396 the dataset identifier PXD023726.

397 ***Minimum Inhibitory Concentration (MIC) assay***

398 The MIC was measured by broth microdilution in triplicate experiments based on CLSI
399 guidelines as described previously (50). Briefly, an overnight culture of *S. suis* was diluted to
400 OD=0.1 (A_{600}) and sub-cultured grown to mid log for 3 hours at 37°C. Mid log cultures were
401 diluted to OD=0.2 (A_{600}) and 50 μ l of each culture was added to 96-well plates containing
402 serially diluted antibiotic concentrations (5 μ g/mL - 0.08 μ g/mL), and plates grown at 37°C
403 with 5% CO₂ for 24 hours. The MIC (mg/L) was determined as the last dilution at which
404 turbidity was observed following overnight growth with all assays being performed in
405 triplicate.

406

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414

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578

579 **Table 1. Summary of methylomes for *S. suis* strains LSS89 (ModS1) and SS1056 (ModS2).**

580 Strains were enriched for GAGCA_(n) SSR tracts of 19, 20, or 21 GAGCA repeats in the *modS1*
 581 gene in strain LSS89, or 17, 18, or 19 GAGCA repeats in the *modS2* gene of strain SS1056.
 582 Type III methyltransferase motifs are only detected in strains enriched for an ON number of
 583 repeats (ModS1 in red in strain LSS89 21 repeats; ModS2 in blue in strain SS1056 18 repeats)
 584 and matches the protein expression detected in Figure 1. In ModS2, V represents A, G or C.
 585 % values represent motifs detected/motifs present. Full methylome data is presented in
 586 Supplementary Data 1.

motif	LSS89 <i>modS1</i>			SS1056 <i>modS2</i>		
	19 reps	20 reps	21 reps	17 reps	18 reps	19 reps
5'-G ^(m6) AAGNNNNNTTC/ 5'-G ^(m6) AAHNNNNCTTC	100%/ 100%	100%/ 100%	100%/ 100%			
5'-GCG ^(m6) AT (ModS1)	ND	ND	99.9%			
5'-GYT ^(m6) ANNNNNNNNTTC/ 5'-GA ^(m6) ANNNNNNNNTARC				100%/ 100%	100%/ 99.6%	100%/ 100%
5'-VTC ^(m6) ATC (ModS2)				ND	99.9%	ND

587 **Table 2. Differentially regulated proteins (>1.5 fold) in the ModS1 phasevarion. Fold**
 588 change presented as *modS1* ON vs *modS1* OFF

Down regulated in <i>modS1</i> ON			
Accession	Protein	Fold change (ON vs OFF)	p-value
WP_002940030.1	30S ribosomal protein S12	2.87	1.43E-8
WP_002938143.1	ABC transporter permease	2.48	6.54E-7
WP_009909754.1	50S ribosomal protein L27	1.92	9.04E-7
WP_014638653.1	ABC transporter substrate-binding protein	1.65	4.81E-7
WP_002936253.1	DegV family protein	1.62	2.53E-4
WP_023369127.1	Sugar ABC transporter substrate-binding protein	1.55	7.86E-14
WP_004195491.1	50S ribosomal protein L23	1.54	9.68E-8
Up regulated in <i>modS1</i> ON			
WP_002936659.1	50S ribosomal protein L29	9.14	2.76E-8
WP_002940438.1	Amino acid ABC transporter ATP-binding protein	2.1	8.50E-4
WP_012027640.1	Ribosome biogenesis GTPase Der	2.08	3.12E-6
WP_002942806.1	CsbD family protein	2.03	6.94E-8
WP_014636557.1	Transcriptional repressor	1.99	1.78E-15
WP_002936247.1	HU family DNA-binding protein	1.97	1.81E-6
WP_002936483.1	50S ribosomal protein L7/L12	1.89	1.82E-14
WP_002938891.1	Acyl carrier protein	1.88	≤1.00E-17
WP_002936048.1	DUF1846 domain-containing protein	1.86	≤1.00E-17
WP_014736429.1	Nitroreductase family protein	1.77	2.17E-5
WP_014735259.1	30S ribosomal protein S10	1.76	≤1.00E-17
WP_002940682.1	30S ribosomal protein S16	1.73	6.91E-8
WP_002934959.1	DUF1149 family protein	1.67	4.09E-14
WP_023368958.1	Chromosomal replication initiator protein DnaA	1.66	≤1.00E-17
WP_023369777.1	dTDP-4-dehydrorhamnose reductase	1.64	1.96E-10
WP_002936660.1	50S ribosomal protein L16	1.62	5.40E-10

WP_004194840.1	DUF2829 domain-containing protein	1.6	1.60E-4
WP_012027178.1	PTS sugar transporter subunit IIA	1.6	3.27E-3
WP_002936656.1	50S ribosomal protein L24	1.59	3.52E-6
WP_002937025.1	Response regulator transcription factor	1.59	2.89E-3
WP_011922043.1	DivIVA domain-containing protein	1.59	8.40E-4
WP_002938966.1	YlbF/YmcA family competence regulator	1.58	≤1.00E-17
WP_012775074.1	Alkylphosphonate utilization protein	1.54	8.72E-4
WP_023370402.1	ROK family glucokinase	1.53	≤1.00E-17
WP_002936622.1	30S ribosomal protein S13	1.51	1.00E-4
WP_023369315.1	Glycine cleavage system protein H	1.5	1.94E-12

589

590 **Table 3. Differentially regulated proteins (>1.5 fold) in the ModS2 phasevarion.** Fold
 591 change presented as *modS2* ON vs *modS2* OFF

Down regulated in <i>modS2</i> ON			
Accession	Protein	Fold change (ON vs OFF)	<i>p</i>-value
WP_002935876.1	Glyoxalase/bleomycin resistance/extradiol dioxygenase family protein	1.79	≤1.00E-17
WP_044673510.1	ATP-dependent Clp protease ATP-binding subunit	1.62	4.35E-4
WP_024387015.1	Nucleotide exchange factor GrpE	1.55	2.81E-12
Up regulated in <i>modS2</i> ON			
WP_012028296.1	50S ribosomal protein L20	2.13	4.82E-9
WP_012027287.1	Peptidylprolyl isomerase	1.77	4.40E-2
WP_002938891.1	Acyl carrier protein	1.68	≤1.00E-17
WP_044754372.1	Cysteine synthase A	1.66	≤1.00E-17
WP_002936486.1	50S ribosomal protein L10	1.62	2.35E-4
WP_044680965.1	Class 1b ribonucleoside-diphosphate reductase subunit alpha	1.61	1.54E-4
WP_044771061.1	Dihydroxyacetone kinase subunit L	1.55	8.68E-3
WP_024384331.1	Aminopeptidase P family protein	1.54	3.24E-4
WP_044681922.1	Ribonucleoside-triphosphate reductase	1.54	2.97E-9
WP_079394259.1	Amino acid ABC transporter substrate-binding protein	1.52	1.07E-8
WP_044674536.1	Winged helix-turn-helix transcriptional regulator	1.5	1.70E-4

592

593 **Table 4. MICs of ModS2 ON vs ModS2 OFF enriched *S. suis* strains.**

	ModS2 ON	ModS2 OFF
Ampicillin ($\mu\text{g}/\text{mL}$)	0.16	0.31
Vancomycin ($\mu\text{g}/\text{mL}$)	1.25	1.25
Amoxicillin ($\mu\text{g}/\text{mL}$)	0.16	0.16
Penicillin ($\mu\text{g}/\text{mL}$)	0.63	0.63

594

595 **Figure Legends**

596

597 **Figure 1. Expression of ModS alleles. A)** The *modS* gene contains a variable length GAGCA_(n)
598 SSR tract (grey box) near the start of the gene, and a variable central target recognition
599 domain (TRD) represented by the hatched box. The 5' and 3' regions of the *modS* gene are
600 highly (>95% nucleotide identity) conserved (white). PCR over the SSR tract was determined
601 by FAM labelled PCR using primers SsuT3-F-FAM and SsuT3-R, and analysed using fragment
602 length analysis; **B)** Alignment of the TRD regions of ModS1 and ModS2 showing <25% amino
603 acid identity. * represents identical amino acid residues, · represents similar amino acid
604 residues (basic, acidic, neutral), TRD region underlined. Alignments carried out in ClustalW;
605 **C)** Fragment length analysis traces of the enriched *modS1* and *modS2* populations of strains
606 LSS89 and SS1056, respectively, containing three consecutive GAGCA_(n) SSR tract lengths; **D)**
607 Western blot analysis using ModS antisera demonstrates that the ModS protein is only
608 present in *S. suis* populations enriched for 21 repeats in ModS1 (LSS89) and 18 repeats in
609 ModS2 (SS1056), demonstrating phase-variable expression of this protein.

610

611 **Figure 2. Volcano plot demonstrating changes to protein expression as a result of ModS.**
612 SWATH-MS proteomics demonstrated a coverage of 450 of 1964 identified proteins (~23%)
613 in LSS89 ModS1 ON-OFF strain pair, and 411 of 1905 (~22%) identified proteins in SS1056
614 ModS2 ON-OFF strain pair. The x axis indicates relative fold difference in protein abundance
615 in ON compared to OFF; the y axis indicates statistical significance.

616

617 **Figure 3. Growth Curves of *S. suis* populations enriched for *modS1* and *modS2*.** ON-OFF
618 strain pairs for ModS1 (strain LSS89) and ModS2 (SS1056) were grown in rich media (THB-Y
619 broth) for 18 hours with shaking. Statistically significant differences (P value <0.05) in
620 absorbance at each time point are indicated by asterisks, assessed using Student's t-test.

621

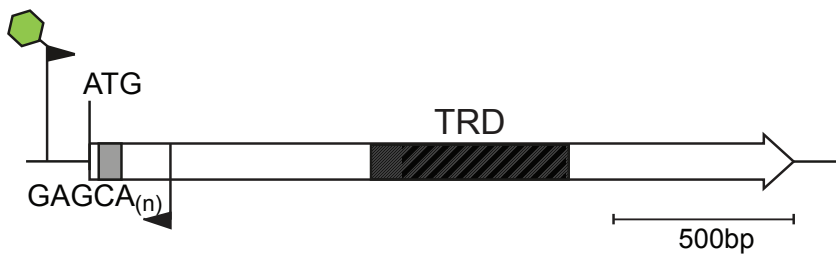
622 **Supplementary figure legends**

623 **Supplementary Figure 1. Expression of ModS is dependent on SSR tract length.** (A)
624 Coomassie staining of whole cell lysates from enriched strains of *S. suis*. (B) Western blot
625 using anti-ModS antisera demonstrates that ModS1 is only produced in strains enriched for
626 21 GAGCA repeats and that ModS2 is only produced in strains enriched for 18 repeats.
627 Dotted line represents region of blot presented in Figure 1D.

628

629 **Supplementary Figure 2.** Three open reading frames occur in the *modS* gene due to
630 variation in length of the GAGCA_(n) simple sequence repeat tract. Due to a frameshift down-
631 stream of the GAGCA_(n) SSR tract, 19 and 20 repeats result in a premature stop codon (in
632 bold, highlighted with *), and consequently no expression of the ModS protein; 21 GAGCA_(n)
633 repeats in the SSR tract results in the gene being in-frame, and therefore ModS protein is
634 produced.

A



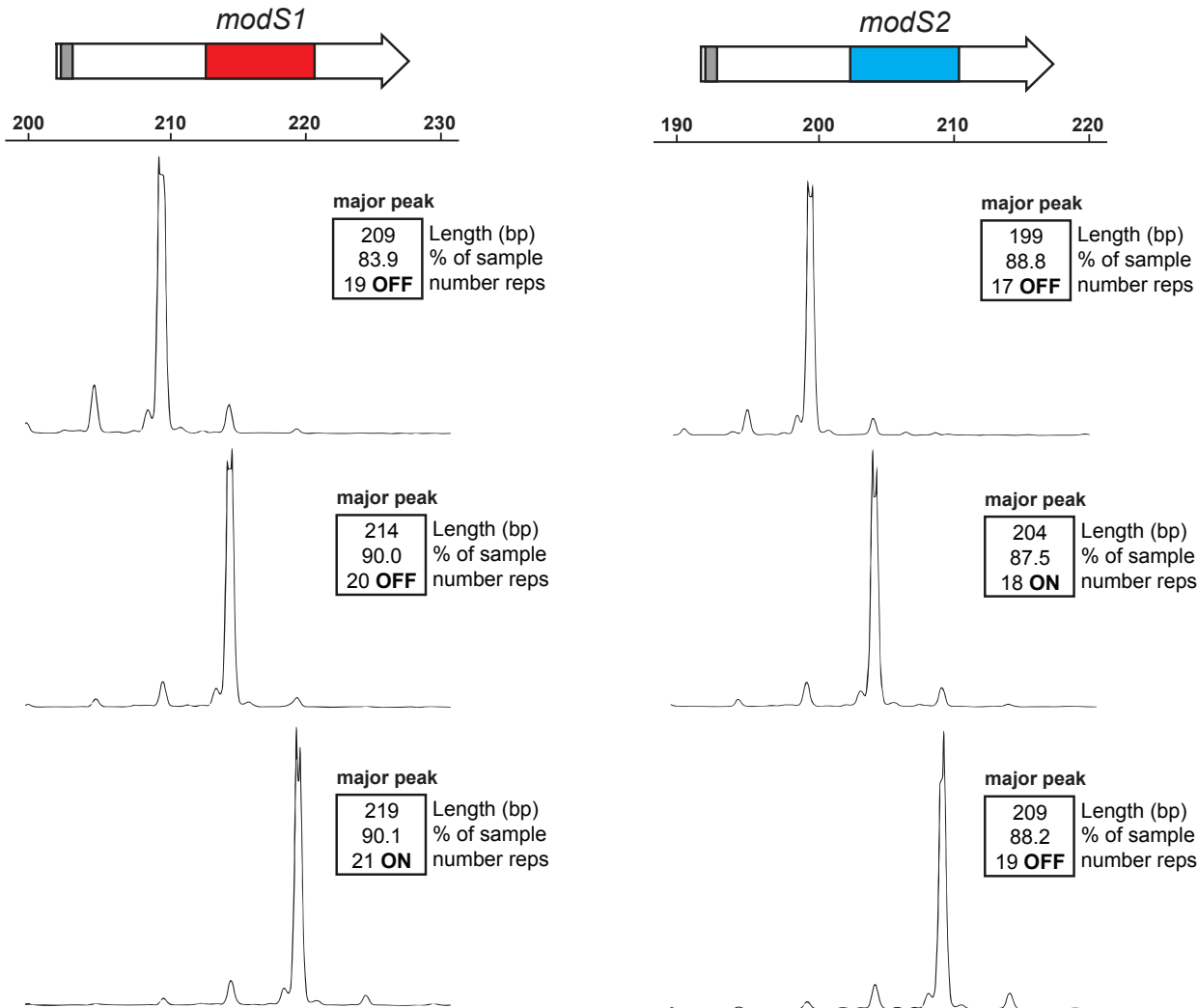
B

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ModS1 247 QANLKAICDEIFGEEENFVADVWERAFAPVNLK-----KHFSESHDIYLVYAKNVEFAVNHGIPRSEDSNSRYQNPNDVNR 322
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ModS1 323 GPWTSGLDSVGPVAVPSNIYEIISPGRSIFPPSGRSWLLSKERFEFIIADNRIWFGANGDNV-----PRIKRFLSEVK 395
ModS2 316 GSWNV--FWSNADRPMSRYDVLG-----FTPSTGQWRWSKEKADEAIENYIEFIANYSDKMTLEEYSSLNPQ-KKFIRRIE 378
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ModS1 396 NVTPTMTIWKY----ADVGHQSASQDLKFLFDGKAY---FTYKPKVPLMKQIVQLYSEKDGILIDFFAGSATTADAVM 467
ModS2 379 NGIGKNGGVQYVAPSNTSLRTSNWTDLEVSQIAKEYDLPFDNPKNKSLIREILTSKTDSDSLIDFFAGSATTADAVM 457
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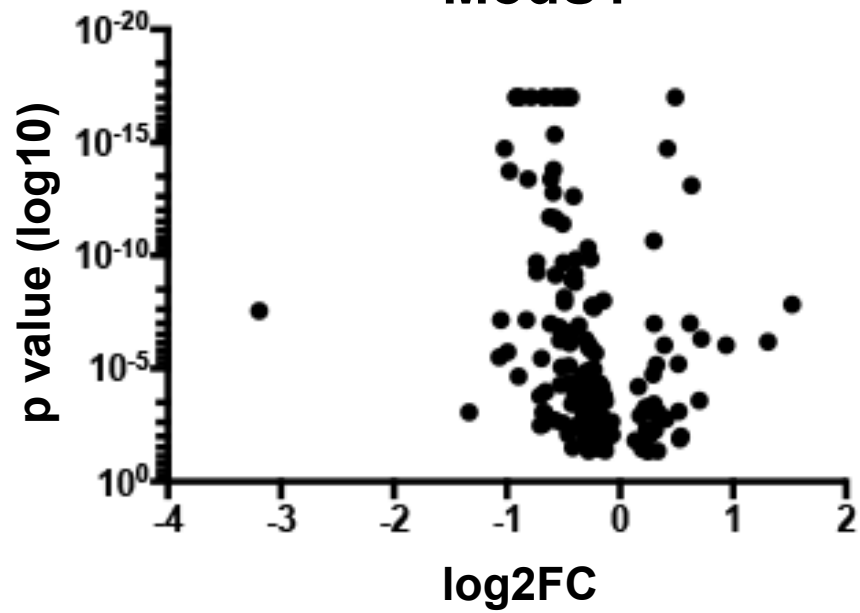
C



D



ModS1



ModS2

