Genome wide association analysis identifies genetic variants associated with reproductive variation across domestic dog breeds and uncovers links to domestication

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Abstract

The diversity of eutherian reproductive strategies has led to variation in many traits, such as number of offspring, age of reproductive maturity, and gestation length. While reproductive trait variation has been extensively investigated and is well established in mammals, the genetic loci contributing to this variation remain largely unknown. The domestic dog, *Canis lupus familiaris* is a powerful model for studies of the genetics of inherited disease due to its unique history of domestication. To gain insight into the genetic basis of reproductive traits across domestic dog breeds, we collected phenotypic data for four traits – cesarean section rate (n = 97 breeds), litter size (n = 60), stillbirth rate (n = 57), and gestation length (n = 23) – from primary literature and breeders' handbooks. By matching our phenotypic data to genomic data from the Cornell Veterinary Biobank, we performed genome wide association analyses for these four reproductive traits, using body mass and kinship among breeds as co-variates. We identified 14 genome-wide significant associations between these traits and genetic loci, including variants near *CACNA2D3* with gestation length, *MSRB3* with litter size, *SMOC2* with cesarean section rate, *MITF* with litter size and still birth rate, *KRT71* with cesarean section rate, litter size, and stillbirth rate, and *HTR2C* with stillbirth rate. Some of these loci, such as *CACNA2D3* and *MSRB3*, have been previously implicated in human reproductive pathologies. Many of the variants that we identified have been previously associated with domestication-related traits, including brachycephaly (*SMOC2*), coat color (*MITF*), coat curl (*KRT71*), and tameness (*HTR2C*). These results raise the hypothesis that the artificial selection that gave rise to dog breeds also shaped the observed variation in their reproductive traits. Overall, our work

establishes the domestic dog as a system for studying the genetics of reproductive biology and disease.

Introduction

Mammals exhibit wide variation in traits associated with reproduction (1-3). For example, gestation length can range from 12 days in the Gray dwarf hamster, *Cricetulus migratorius*, to 21 months in the African bush elephant, *Loxodonta africana* (4-6); neonate size can range from less than one gram in the shrew family (Soricidae), to more than a metric ton in the baleen whales (Balaenopteridae) (4,6); and neonates can be either precocial (e.g., cricetid rodents, rabbits, and canids) or altricial (e.g., hystricomorph rodents, ungulates, and cetaceans) (1). This variation in reproductive traits also extends to methods of implantation (7), structure of the placenta (8,9), and lactation strategies (10,11). Not surprisingly, many reproductive traits also exhibit substantial intra-specific variation (5). For example, many mammals exhibit intraspecific variation in gestation length, including primates (12), rat and rabbits (13), as well as the domesticated cattle (14) and thoroughbred horses (15). Similarly, body fat percentages, which are associated with the energetics of reproduction, vary greatly between wild and captive baboons, and intraspecific variation among captive lemurs can vary from 8 – 41% (16).

The existence of phenotypic variation in reproductive traits is well established, and can inform our understanding of the factors that shape patterns of survival and reproduction in both agricultural (17-20) and human populations (21). Not surprisingly, most genome wide association (GWAS) studies of reproductive traits focus on economically important traits in domesticated species, such as reproductive seasonality in rabbits (17), infertility in pigs (18), and dairy traits in cattle (19). GWAS studies focused on understanding human reproductive biology and its associated pathologies have also shed light on the genetic

basis of reproductive traits, including birth weight (22) and gestational duration or length (23-25). For example, maternal variation in six genomic loci (*ADCY5*, *AGTR2*, *EBF1*, *EEFSEC*, *RAP2C*, and *WNT4*) is associated with gestational duration and preterm birth (25). While these studies contribute to our understanding of the genetic architecture of reproductive traits, we still understand very little about the molecular pathways underlying this variation and are unable to explain the majority of the heritability in reproductive traits (26-29).

To address this challenge, we studied the genetics of reproductive traits in a powerful new model system: the domestic dog. The dog is well-suited to this question, because the domestication bottleneck followed by intense artificial selection and inbreeding imposed over the past 300 years has led to the generation of more than 340 recognized breeds that exhibit dramatic morphological variation (30-32). Domestic dog breeds also show substantial variation in their reproductive traits. For example, Pomeranians and Norfolk Terriers typically have only 2 pups per litter, whereas Dalmatians and Rhodesian Ridgebacks typically sire 8-9 pups per litter (33). Similarly, 80 – 90% of French Bulldogs and Boston Terriers are born via cesarean section due to cephalopelvic disproportion, whereas only 2 – 3% of Australian Shepherds and Shar Peis require cesareans (34). Recent analyses have begun to study the genetic mechanisms that underlie the remarkable morphological variation between modern dog breeds in diverse traits such as snout length, ear erectness, and tail curliness (35-38), as well as genetic disease (39).

To gain insight into the genetic basis of reproductive traits across domestic dog breeds, we collected phenotypic data for four reproductive traits, namely cesarean section rate, litter size, stillbirth rate, and gestation length. We synthesized data from the primary literature and breeders' handbooks to obtain coverage of between 23 (trait) and 97 (trait) dog breeds, as well as body mass data from 101 dog breeds. By matching our phenotypic data to genome-wide genotypic data from the Cornell Veterinary Biobank, we performed GWAS analyses and identified 14 genetic loci that are significantly associated with these reproductive traits (using body mass as a co-variate). Several of these variants are in or near genes previously implicated in human reproduction-related pathologies. The majority of the variants that we discovered to be significantly associated with reproductive trait variation are also associated with domestication-related traits. For example, we found that: variation in a gene previously identified to be involved in brachycephaly is also significantly associated with rates of cesarean sections; variation in a gene previously associated with docility is also associated with stillbirth rates; and variation in genes previously linked to coat phenotypes, such as color and curliness, is also associated with several reproductive traits. These results suggest that selection for breed-specific morphological traits during dog domestication may have also directly or indirectly influenced variation in reproductive traits. More broadly, our results establish the domestic dog as a tractable system for studying the genetics of reproductive traits and underscore the potential for cryptic interactions between reproductive and other traits favored over the course of adaptation.

Results

117 To identify SNPs that are significantly associated with four reproductive traits in domestic
118 dog breeds, we conducted across-breed GWAS analyses using a multivariate linear mixed
119 model implemented in the program GEMMA (Zhou & Stephens, 2012). Number of
120 individuals and distribution of breed varied with analysis. After filtering for MAF (MAF <
121 0.05; 10,804 SNPs were excluded) and linkage disequilibrium (34,240 additional SNPs
122 were excluded), 115,683 SNPs were included in the GWAS analysis for each reproductive
123 trait. To validate our GWAS approach and analytical choices, we first used our collected
124 values for body mass, a trait whose genetic associations have been previously extensively
125 studied in dogs (36,37). As expected, our analysis recovered the major genes associated
126 with dog breed body mass variation, including *IGF1* ($P = 2.1 \times 10^{-31}$), *SMAD2*
127 ($P = 1.2 \times 10^{-17}$) and *IGF2BP2* ($P = 5.1 \times 10^{-11}$) (Supplementary Figure 1,
128 Supplementary Table 2).
129
130 **Four genetic loci significantly associate with cesarean section rate**
131 To examine whether there is variation in cesarean section rate among breeds, we first
132 identified cesarean section rate values for a total of 97 of the 162 dog breeds with
133 genotypic data (Supplementary Table 1). The cesarean section rate values were derived
134 from a British survey across 151 breeds covering 13,141 bitches, which had whelped
135 22,005 litters over the course of a 10 year period (34). The frequency of cesarean sections
136 was estimated as the percentage of litters reported to be born by cesarean section. Among
137 the 97 breeds with overlapping genetic data, the median cesarean section rate is 17.1%,
138 with a minimum of 0% in Curly Coated Retrievers and Silky Terriers and a maximum of
139 92.3% in Boston Terriers (Supplementary Figure 3A).

140

141 To identify SNPs that are significantly associated with the observed variation in cesarean
142 section rate across domestic dog breeds, we conducted an across-breed GWAS analysis
143 using 115,683 SNPs and cesarean section values across 95 dog breeds (Figure 1A,
144 Supplementary Figure 2A). We identified four significant SNPs, three of which mapped to
145 genes, namely paralemmin 3 (*PALM3*, uncorrected $P = 1.4 \times 10^{-9}$), sparc-related
146 modular calcium-binding protein 2 (*SMOC2*, uncorrected $P = 2.0 \times 10^{-7}$), and keratin 71
147 (*KRT71*, uncorrected $P = 2.9 \times 10^{-7}$), and a fourth that mapped to the intergenic region
148 between the *CD36* glycoprotein and a lincRNA (uncorrected $P = 9.7 \times 10^{-8}$; Figure 1A).
149

150 The first significantly associated SNP (chromosome 1: 55,983,871) is found in the intron
151 between exons 13 and 14 of *SMOC2*, a gene that is associated with brachycephaly in dogs
152 (38,40); variation in *SMOC2* accounts for 36% of facial length variation in dogs (41). In
153 humans, *SMOC2* is highly expressed in endometrium as well as other reproductive tissues,
154 including the fallopian tubes, ovaries and cervix (Figure 2) (42). The 3' intronic location of
155 the SNP raises the possibility that it might be regulatory (43).
156

157 The second SNP is found in the 3' UTR of *PALM3*, which is a member of the paralemmin
158 gene family that also includes *PALM1*, *PALM2*, and *PALMD* (palmdelphin); members of this
159 family are implicated in plasma membrane dynamics and as modulators of cellular cAMP
160 signaling in the brain (44,45). The function of *PALM3* may be slightly different from the rest
161 of the genes in the family, with recent work suggesting that PALM3 is a binding protein of
162 the single immunoglobulin IL-1 receptor-related molecule (SIGIRR), which is a negative

163 regulator of Toll-Interleukin-1 receptor signaling (46). In humans, *PALM3* is primarily
164 expressed in the membranes of the stomach, kidney, parathyroid gland and epididymis
165 (Figure 2) (42). The SNP (chromosome 20: 48,454,259) that is significantly associated with
166 cesarean section rate is found in the first intron of the *PALM3* gene, suggesting that it might
167 be involved in regulatory actions typically observed in 5' introns (43).
168
169 The third SNP (chromosome 27: 2,539,211) results in a missense mutation of exon 2 of
170 *KRT71*, which belongs to a family of keratin genes specifically expressed in the inner root
171 sheath of hair follicles (47). Prior analysis in dogs identified variation in gene *KRT71*, along
172 with variation in genes *RSPO2* and *FGF5*, accounting for most coat phenotypes (48), such as
173 curliness.
174
175 The fourth significant SNP (chromosome 18: 20,272,961) is found in the intergenic region
176 between the *CD36* gene and a lincRNA (ENSCAFG00000034312). The protein product of
177 CD36 is the fourth major glycoprotein of the platelet surface and serves as a receptor for
178 thrombospondin in platelets (49). Other known functions include transport of long chain
179 fatty acids (50).
180
181 **Six genetic loci significantly associate with litter Size**
182 To examine whether there are SNPs that are significantly associated with variation in litter
183 size among breeds, we retrieved litter size data from 10,810 litters of 224 breeds
184 registered in the Norwegian Kennel Club (33). For these data, we were able to obtain
185 average number of pups per litter values for 60 of the 162 dog breeds with overlapping

genetic data (Supplementary Table 1). Among these 60 breeds, median litter size is 5.55 pups, with a maximum 8.9 in Rhodesian Ridgebacks and a minimum of 2.4 in Pomeranians (Supplementary Figure 3B).

To identify SNPs, and genes proximal to them, that are significantly associated with the observed variation in litter size across domestic dog breeds, we conducted an across-breed GWAS analysis using 115,683 SNPs and litter size data from 60 dog breeds (Figure 1B, Supplementary Figure 2B). We identified three significant SNPs intersecting three genes, namely keratin 71 (*KRT71,* uncorrected $P = 2.2 \times 10^{-8}$), RNA Terminal Phosphate Cyclase-Like 1 (*RCL1*, uncorrected $P = 2.6 \times 10^{-8}$) and microphthalmia-associated transcription factor (*MITF*, uncorrected $P = 3.5 \times 10^{-7}$). The *KRT71* SNP is the same variant that associated with variation in cesarean section rate described above. Another three significant SNPs were found in intergenic regions; two were nearby genes *MSRB3* (methionine sulfoxide reductase B3, uncorrected $P = 1.3 \times 10^{-7}$) and *MSANTD1* (Myb/SANT DNA binding domain containing, uncorrected $P = 1.5 \times 10^{-9}$), respectively. The final variant was near an RNA of unknown function (ENSCAFG00000021196, uncorrected $P = 3.8 \times 10^{-10}$).

The *RCL1* SNP (chromosome 1: 93,189,363) is found in the intron between exons 7 and 8. *RCL1* functions in the maturation of 18s RNA (51) and is associated with cervical cancer; one role of the gene in this cancer pathology is thought to involve the regulation of insulin receptors (51). Additionally, a rare missense variation in *RCL1* was recently associated with depression (52).

The *MITF* SNP (chromosome 20: 21,848,176) is found in the intron between exons 4 and 5. *MITF* plays an integral role in the development of neural crest-derived melanocytes and optic cup-derived retinal pigment epithelial cells. In human melanocytes, *MITF* is a regulator of *DIAPH1*, a member of the formin gene family whose members are highly expressed in reproductive tissues and have been associated with a variety of reproductive phenotypes (53-57). *DIAPH1* expression is increased in spontaneous term and preterm labor myometrial tissues (58). In domesticated animals, *MITF* is a well characterized gene associated with coat color (36,59). In humans, *MITF* is expressed in melanocytes, as well as reproductive tissues including the endometrium and cervix (Figure 2)(42).

Another SNP (chromosome 10: 8,114,328) significantly associated with litter size is found in the intergenic region downstream of *MSRB3*, whose protein product catalyzes the reduction of methionine-R-sulfoxides to methionine and repairs oxidatively damaged proteins (60,61). In humans, mutations in *MSRB3* are associated with deafness (62). Epigenetic changes of *MSRB3* in the fetus during pregnancy may affect length of gestation, with increased DNA methylation correlated with increased gestational age (63,64). Furthermore, *MSRB3* shows an increase in mRNA expression in ripe (at term) versus unripe human uterine cervix, implying that *MSRB3* functions to ripen the cervix before the onset of labor (65). In previous morphological studies in dogs, *MSRB3* is associated with ear erectness (36).

231 The last SNP (chromosome 6: 61,062,626) that is significantly associated with litter size is
232 located downstream of *MSANTD1*, which is part of a gene network believed to aid in cell-to-
233 cell signaling and interaction, hematological system development and function, and
234 immune cell trafficking (66). *MSANTD1* has been identified in two independent studies as a
235 candidate gene for the determination of black coat color in goats (67,68).
236
237 **Five genetic loci significantly associate with stillbirth rate**
238 To examine whether there are SNPs that are significantly associated with variation in
239 stillbirth rate among breeds, we retrieved data for stillbirth rates for 57 of the 162 dog
240 breeds (Supplementary Table 1). The data covers 10,810 litters of 224 breeds registered in
241 the Norwegian Kennel Club and defines perinatal mortality as the sum of stillborn puppies
242 and puppies that died during the first week after birth (69). Among these 57 breeds with
243 overlapping genomic data, the median stillbirth rate is 4.2 pups, with a maximum rate of
244 12.3% in Saint Bernards and a minimum of 0% in Basenjis and Italian Greyhounds
245 (Supplementary Figure 3C).
246
247 To test if any SNPs are significantly associated with the observed variation in stillbirth rate
248 across domestic dog breeds, we conducted an across-breed GWAS analysis using 115,683
249 SNPs and stillbirth rate data from 56 dog breeds (Figure 1C, Supplementary Figure 2C). We
250 identified five significant SNPs; four intersecting 4 genes, namely nuclear protein body
251 SP140 (*SP140*, uncorrected $P = 2.8 \times 10^{-8}$), 5-Hydroxtryptamine receptor 2C (*HTR2C*,
252 uncorrected $P = 2.0 \times 10^{-7}$), keratin 71 (*KRT71*, uncorrected $P = 3.2 \times 10^{-9}$), and
253 microphthalmia-associated transcription factor (*MITF*, uncorrected $P = 1.4 \times 10^{-7}$), and

one in an intergenic region near a snoRNA (ENSCAFG00000027305, uncorrected $P = 1.3 \times 10^{-7}$) of unknown function. The *KRT71* SNP associated with variation in stillbirth rate is the same one as that associated with variation in cesarean section rate and litter size described above. Similarly, the *MITF* SNP associated with variation in stillbirth rate is the same as that associated with litter size.

The *SP140* SNP (chromosome 25: 42,482,266) resides in the intro between exons 4 and 5. *SP140* is the lymphoid-restricted homolog of *SP100* expressed in mature B cells, as well as some T cells (70). High levels of *SP140* mRNA are detected in human spleen and peripheral blood leukocytes, but not other human tissues (Bloch et al., 1996). *SP140* expression has been implicated in innate response to immunodeficiency virus type 1 (71). Finally, *SP140* was the gene showing the largest difference in expression level between normal and preeclamptic placentas (72) .

The *HTR2C* SNP (chromosome X: 87,378,551) is located in the intron between exons 3 and 4. *HTR2C* is one of the most important and extensively studied serotonin receptors (73). *HTR2C* has ten fixed SNP differences between dogs and wolves, and also belongs to the behavioral fear response (74). Additionally, *HTR2C* is differentially expressed in the brain between tame and aggressive mice and foxes (75), providing additional evidence for its involvement in the tame behaviors of domesticated dogs (74).

Four genetic loci significantly associate with gestation length

To examine whether there is variation in gestation length among breeds, we identified individual gestation length averages by breed predominantly in breeder handbooks. Utilizing breeders' handbooks, we were able to identify gestation length means for a total of 23 of the 162 dog breeds that we had genotypic data for (Supplementary Table 1). Among these 23 breeds, the median gestation length is 62.2 days, with a maximum length of 65.3 in beagles and a minimum of 60.1 in the Alaskan Malamute (Supplementary Figure 3D).

To identify SNPs, and genes proximal to them, that are significantly associated with the observed variation in gestation length across domestic dog breeds, we conducted an across-breed GWAS analysis using 115,683 SNPs and gestation length data from 23 dog breeds (Figure 1D, Supplementary Figure 2D). Our analysis identified six significantly associated SNPs that mapped to 4 genes, namely solute carrier family 9 (*SLC9A8*, uncorrected $P = 3.7 \times 10^{-11}$), calcium channel, voltage-dependent, alpha-2/delta Subunit 3 (*CACNA2D3*, uncorrected $P = 3.1 \times 10^{-7}$), microtubule associated tumor suppressor candidate 2 (*MTUS2*, uncorrected $P = 3.6 \times 10^{-7}$), and helicase family member 1 (*HFM1*, uncorrected $P = 4.0 \times 10^{-7}$), and two lincRNAs (ENSCAFG00000037743, uncorrected $P = 4.4 \times 10^{-7}$, and ENSCAFG00000039067, uncorrected $P = 1.6 \times 10^{-7}$) whose function is unknown.

The first significantly associated SNP (chromosome 24: 36,399,705) resides in intron 78 of *SLC9A8*, an integral transmembrane protein that exchanges extracellular Na+ for intracellular H+. *SLC9A8* serves multiple functions, including intracellular pH homeostasis,

cell volume regulation, and electroneutral NaCl absorption in epithelia (76). Knockout male mice have impaired luteinizing hormone-stimulated cAMP production and are infertile, despite normal morphology of their reproductive system and normal behavior (77). *SLC9A8* is expressed ubiquitously (Figure 2) (42), an expression pattern suggestive of involvement in housekeeping functions.

The second SNP (chromosome 20: 35,206,774) is found in the intron between exons 26 and 27 of *CACNA2D3*. This gene is one of four members of the alpha-2/delta subunit three family of the voltage-dependent calcium (Ca2+) channel complex, regulating the influx of Ca2+ ions entering the cell upon membrane polarization (78). The regulation of calcium is a fundamental process relevant to life at fertilization, and subsequent control of development and differentiation of cells (79). In previous studies in humans, *CACNA2D3* is differentially methylated in the amnion between normal and preeclamptic pregnancies (80) and in blood between extreme preterm and term infants at birth (55,81). Additionally, *CACNA2D3* is one of four genes recently described as influencing cranial morphology in human populations (82). In other domesticated animals, *CACNA2D3* is downregulated by Colony Stimulating Factor 2 (*CSF2*) in the trophoectoderm of pregnant cattle, which increases the ability of the preimplantation embryo to advance to the blastocyst stage (83). In the closely related wolf, *CACNA2D3* is under diversifying selection associated with environmental adaptations to altitude (84-86).

The third significantly associated SNP (chromosome 25: 10,481,606) falls in a large intronic region of the *MTUS2* gene. The protein product of *MTUS2* is cardiac zipper protein

322 (CAZIP), a member of a class of proteins that interact with angiotensin II receptor
323 interacting proteins (ATIP) (87). *MTUS2* plays a role in the development and function of the
324 heart and nervous system in vertebrates (88).
325
326 The fourth SNP (chromosome 6: 57,457,184) is located in the 3' intron of *HFM1*, a DNA
327 helicase that confers genome integrity in germline tissues (89). *HMF1* plays a role in
328 meiotic recombination implying a major evolutionary role through the creation of diverse
329 offspring. In mice, deletion of *HFM1* eliminates a major fraction of cross over events (90),
330 whereas in cattle *HMF1* is associated with both fertility and milk production in Holstein
331 cattle (91), as well as with alteration of global recombination rates in Holstein, Holstein-
332 Friesian, Jersey, and crossbred individuals (92).
333
334 **Discussion**
335 Mammals exhibit a great deal of variation in their reproductive traits, yet remarkably little
336 is known about the genetic basis of these traits. To begin to address this, we used GWAS
337 analyses to examine the genetic basis of four reproductive traits (cesarean section rate,
338 stillbirth rate, litter size, and gestation length) across up to 97 domestic dog breeds. We
339 identified several significant genetic associations for each trait (Figure 1).
340
341 Six of the 14 genetic variants that we found to be associated with reproductive trait
342 variation have been previously identified to be involved in diverse traits associated with
343 dog domestication (Table 2), such as brachycephaly and coat curl and color, suggesting that
344 selection for signature traits of dog breeds may have also directly or indirectly influenced

345 variation in reproductive traits. For example, one of the variants that we found to be
346 associated with cesarean section rate is in an intron of *SMOC2*, a gene previously associated
347 with brachycephaly in dogs (40,41). Brachycephaly, the shortening and widening of the
348 muzzle and skull, is present in several "fighting" breeds such as Boxer, Boston Terrier, and
349 Bulldog, and is thought to have been originally artificially selected on the basis that a
350 shorter and wider cranial shape would enhance the dog's biting power (93). Interestingly,
351 one of the traits that associated with brachycephaly is cephalopelvic disproportion (94), a
352 significant medical condition that can result in the death of both the litter and the bitch due
353 to the inability of the pups to pass through the pelvic canal. The negative effects of
354 cephalopelvic disproportion are alleviated by cesarean section, which not only allows these
355 breeds to reproduce but also enables the continued application of artificial selection for the
356 most extreme cranial morphology (40). Whether the *SMOC2* variant identified directly
357 influences parturition and birth timing in dogs (in humans, *SMOC2* is highly expressed in
358 several reproductive tissues; see Figure 2 and Ref. (42) or indirectly leads to adverse
359 pregnancy outcomes (e.g. brachycephalic cranial morphology leading to cesarean section)
360 remains unknown. It is highly likely, however, that the association between *SMOC2* and
361 brachycephaly came first, paving the way for the subsequent association of both with
362 cesarean section rate.
363
364 Several of the significantly associated genes that we identified in dogs appear to also be
365 associated with reproductive phenotypes in humans. This suggests the possibility that the
366 artificial selection that gave rise to dog breeds may have also contributed to the observed
367 variation in their reproductive traits. For example, a member of the gene family for a

368 subunit of the voltage-dependent calcium channel complex, *CACNA2D3,* which is associated
369 with gestation length in our study, has been shown to be both differentially methylated in
370 amnion between normal and preeclamptic human pregnancies (80), and in blood between
371 extreme preterm and term infants at birth (55,81). Furthermore, expression of *MSRB3*,
372 which is associated with litter size in our study, is elevated in ripe (at term) versus unripe
373 human uterine cervix and may be involved in the onset of labor (65). Finally, a few of the
374 other genes significantly associated with reproductive traits (*SMOC2* and *MITF*) are also
375 known to be expressed in human reproductive tissues (42)(Figure 2).
376
377 **Methods**
378 **Genotypic and Phenotypic Data**. To identify SNPs that are significantly associated with
379 reproductive traits, we used a previously published data set containing 160,727 SNPs from
380 4,342 individual dogs across 162 breeds genotyped using the Illumina 173k CanineHD
381 array that were downloaded from
382 http://datadryad.org/resource/doi:10.5061/dryad.266k4 (35,95). Following the original
383 authors, SNPs with a genotyping rate (i.e., the proportion of genotypes per marker with
384 non-missing data) below 95% and heterozygosity ratios (i.e., the ratio of the number of
385 heterozygous SNPs divided by the number of non-reference homozygous SNPs) below 0.25
386 or above 1.0 were removed.
387
388 Phenotypic reproductive trait data for litter size (number of pups), cesarean rate, stillbirth
389 rate, and gestation length across 128 breeds were collected from a variety of breeder's
390 handbook and primary journal articles (33,69,94,96-104) (see also Supplementary

Material 1). We also included body mass as a control trait. Each breed was assigned the average breed value for each phenotype; the full list of the values for all four reproductive traits and body mass across the 128 breeds is provided in supplementary table 1. For the body mass control, our collected trait values overlapped with the genotypic data (35,95) for 101 breeds corresponding to 3,384 individuals (Table 1). For the reproductive traits, our collected cesarean section rate trait values overlapped with the genotypic data for 95 breeds (3,194 individuals), our litter size trait values for 60 breeds (2,617 individuals), our stillbirth rate values for 56 breeds (2,590 individuals), and our gestation length values for 23 breeds (1,908 individuals) (Table 1).

Genome Wide Association (GWAS) Analyses. To test SNPs for associations with the four reproductive traits of interest, we conducted a GWAS analysis for each individual trait using body mass as a covariate, and accounting for kinship, as well as for body mass as a proof of concept. All GWAS analyses were run using a linear-mixed model as implemented in the program GEMMA, version 0.94 (105). Numerous studies have shown that the vast majority of morphological, ecological and physiological traits vary as a function of an organism's body mass (106-108) as well as a function of kinship (35,36). Most notably for the purpose of this study, body mass has been previously shown to be strongly correlated with litter weight (109-111), neonate weight (109-112), and gestation length (6,109,110,113,114).

To ensure our analysis reflected the reproductive trait of interest and not SNPs associated with body mass, we used body mass as a covariate for all reproductive trait analyses. To be

able to do so, we pruned our genotypic data so that they included only dog breeds (and individuals) for which we had both body mass and reproductive trait of interest values (see Supplementary Table 1).

To account for population stratification, we calculated a kinship matrix of the included breeds using GEMMA and included it as a random effect in each association analysis. Each value of a kinship matrix describes the probability that a particular allele from two randomly chosen individuals at a given locus is identical by descent (115). Finally, to control for inflated *P* value significance from the testing of multiple hypotheses, we used a significance threshold of $P = 4.3 \times 10^{-7}$ (Bonferroni cutoff of $\alpha = 0.05, N = 115{,}574$) for all analyses. All reported P values are uncorrected Wald's P values as calculated in PLINK.

Finally, to reduce potential error stemming from SNP misidentification in our analyses, we included only SNPs with a minor allele frequency (MAF) > 0.05, since SNPs with very low minor allele frequencies are more prone to error due to the small number of samples that have the called nucleotide. Furthermore, we pruned SNPs not in complete or near-complete linkage disequilibrium using a variance inflation factor of 10, using the PLINK command --indep 100 10 10 (116).

To gain insight into the genetic elements putatively involved with the traits of interest, we mapped all SNPs found to be significantly associated with each trait of interest using custom perl and R scripts to the CanFam3.1.87 dog genome assembly (117,118). Transcript IDs were mapped to gene names using bioconductor biomaRt interface to the ENSEML

biomart (119). If the significant SNP was outside gene boundaries, we reported the nearest upstream or downstream gene. Manhattan plots and quantile-quantile plots were generated using R 3.1.2 (120) with the qqman package (121). Calculation of the λ inflation parameter, a metric of any existing systematic bias in the data set, was calculated using the GenABEL R package (122) and was used to interpret Type I error rate in the multiple testing of GWAS analyses (123).

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TABLES

Table 1. Numbers of breeds and individuals with overlapping phenotypes and genotypes included in our analysis.

Trait	Number of Overlapping Breeds	Number of Overlapping Individuals
Body Mass	101	3,384
Cesarean Section Rate	97	3,194
Litter Size	60	2,617
Stillbirth Rate	57	2,590
Gestation Length	23	1,908

460 **Table 2.** Summary of genes that contain or are adjacent to the SNPs that are significantly

461 associated with variation in reproductive traits across dog breeds.

Gene ID	Gene Name	Chr.	rs Number	Variant	Reproductive Trait(s)	Domestication-related Trait(s)
SMOC2	SPARC related modular calcium binding 2	1	rs21966904	Non-coding (Intron 13)	Cesarean section rate	Brachycephaly (Dogs)
PALM3	paralemmin 3	20	rs22853767	Non-coding (3' UTR)	Cesarean section rate	-
KRT71	keratin	27	rs23373415	Coding (exon 2)	Cesarean section rate, litter size, stillbirth rate	Coat phenotypes (Dogs)
CD36	CD36 glycoprotein	18	rs22664051	Intergenic variant (downstream)	Cesarean section rate	-
RCL1	RNA terminal phosphate cyclase	1	rs21894066	Non-coding (Intron 7)	Litter size	-

	like 1					
MITF	melanogenesis associated transcription factor	20	rs20848176	Coding (exon 5)	Litter size, stillbirth rate	Coat color (Dogs)
MSRB3	methionine sulfoxide reductase B3	10	rs22060533	Intergenic variant (downstream)	Litter size	Ear erectness (Dogs)
MSANTD1	Myb/SANT DNA binding domain containing	6	rs9084938	Intergenic variant (downstream)	Litter size	Black coat color (Goats)
SP140	nuclear protein body SP140	25	rs8856304	Non-coding (intron 4)	Stillbirth rate	-
HTR2C	5-hydroxytryptamine receptor 2	X	rs24622199	Non-Coding (intron 2)	Stillbirth rate	Tameness (Dogs, Foxes, Mice)
SLC9A8	solute carrier family 9 member A8	24	rs23219089	Non-coding (intron 7)	Gestation length	-
CACNA2D3	calcium voltage-	20	rs22853845	Non-coding	Gestation	Blastocyst

	gated channel auxiliary subunit alpha2delta 3			(intron 9)	length	development (Cattle)
MTUS2	microtubule associated tumor suppressor candidate 2	25	10481606	Non-coding (intron 6)	Gestation length	-
HFM1	ATP dependent DNA helicase homolog	6	rs24306896	Non-coding (intron 4)	Gestation length	Fertility and milk production (Cattle)

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Figure Legends

Figure 1. Significant GWAS results for reproductive traits in domestic dogs. Manhattan plots showing the statistical significance of each SNP as a function of genomic position for (A) cesarean section rate (n = 3,194 individuals, n = 97 breeds), (B) litter size (n = 2,617 individuals, n = 60 breeds), (C) stillbirth (n = 2,590 individuals, n = 57 breeds), and (D) gestation length (n =1,908 individuals, n = 23 breeds). Horizontal line indicates the significance threshold at $P = 4.3 \times 10^{-7}$. Significant SNPs are labels with the intersecting or nearest gene. Plots were generated in R using the qqman package.

Figure 2. Gene expression in human female reproductive tissues of genes that contain or are adjacent to SNPs significantly associated with reproductive traits in domestic dogs. Raw data were obtained from the Human Protein Atlas database (42).

Supplementary Material

Supplementary Figure 1. Recapitulation of SNPs associated with body mass in 101 domesticated dog breeds. (A) Body mass distribution for 101 breeds. (B) Manhattan plots showing the statistical significance of each SNP as a function of genomic position for body mass. Plot generated in R using the qqman package. (C) Quantile-quantile plot showing the effectiveness of the stratification correction ($\lambda = 1.17$). Plot generated in R; inflation factor was calculated using the GenABEL package implemented in R.

Supplementary Figure 2. Distribution of phenotypic values of the four reproductive traits examined in this study across dog breeds. (A) cesarean section rate (n = 97 breeds), (B) litter size (n = 60 breeds), (C) stillbirth rate (n = 57 breeds), and (D) gestation length (n = 23 breeds). Plots were generated in R using the gglplot2 package.

Supplementary Figure 3. Quantile-quantile plots for the GWAS analyses of the four reproductive traits. The range for the inflation factor (λ) for all GWAS analyses is between 1.05 – 1.09, indicating the effectiveness of the stratification correction. (A) cesarean section rate ($\lambda = 1.05$), (B) litter size ($\lambda = 1.05$), (C) stillbirth rate ($\lambda = 1.05$), and (D) gestation length ($\lambda = 1.09$). Plots generated in R, and inflation factors were calculated using the GenABEL package implanted in R.

Supplementary Table 1. Summary of raw phenotypes for breeds included in analysis.

Supplementary Table 2. Summary of top 50 SNPs associated with body mass.

Supplementary Table 3. Summary of top 50 SNPs associated with cesarean section rate.

Supplementary Table 4. Summary of top 50 SNPs associated with litter size.

Supplementary Table 5. Summary of top 50 SNPs associated with stillbirth rate.

Supplementary Table 6. Summary of top 50 SNPs associated with gestation length.

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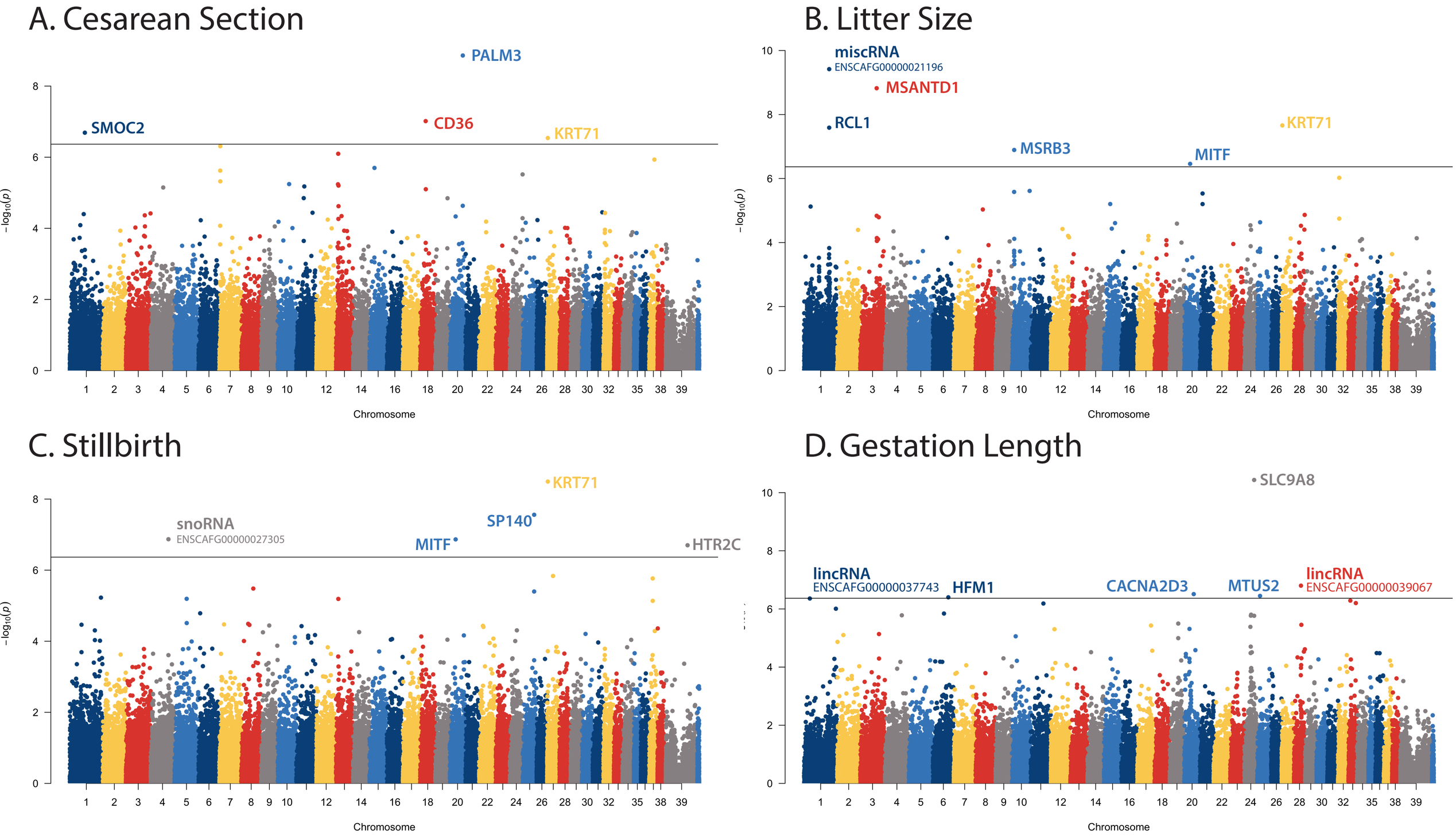
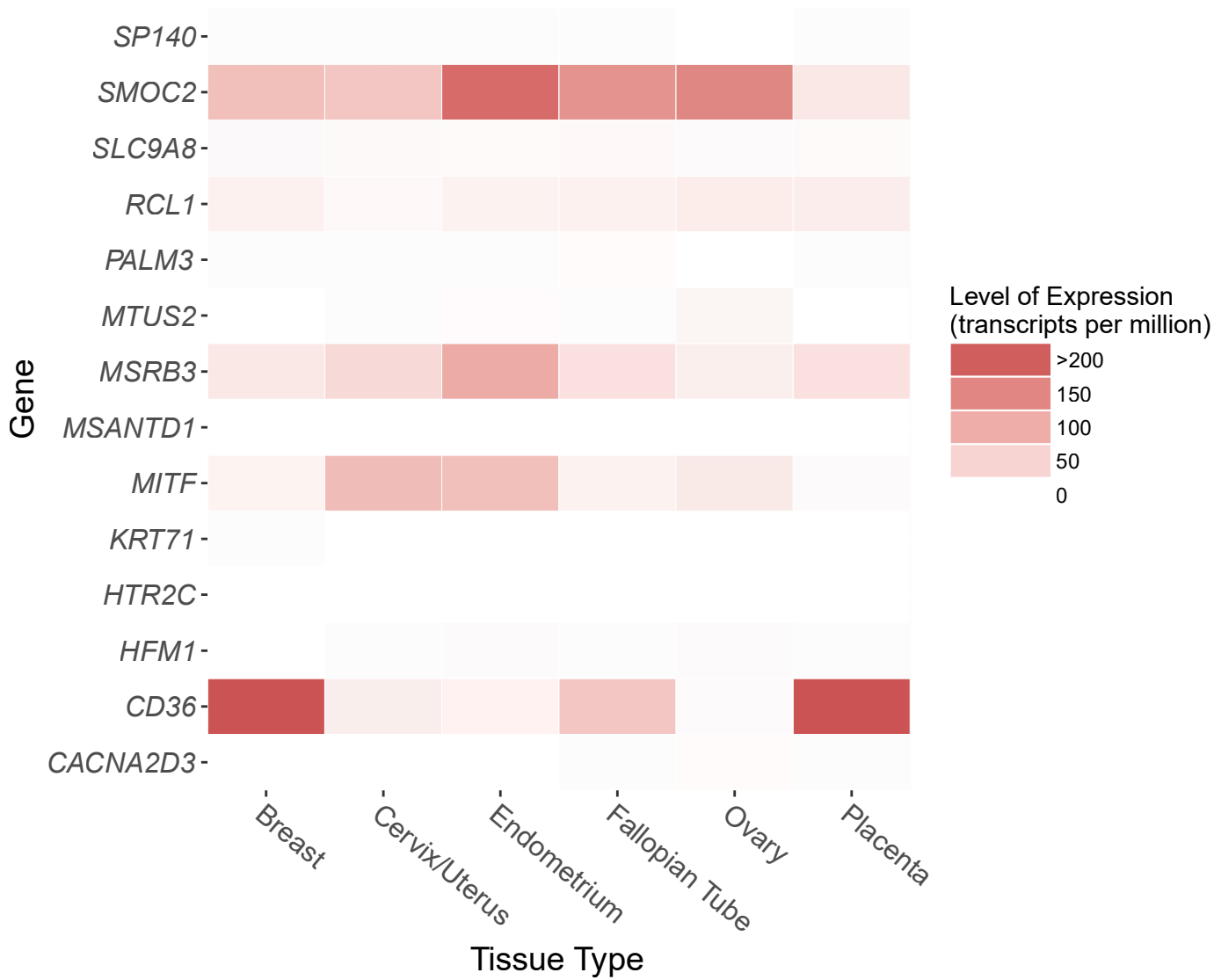

A. Cesarean Section
PALM3
SMOC2
CD36
KRT71
B. Litter Size
miscRNA
ENSCAFG00000021196
MSANTD1
RCL1
MSRB3
MITF
KRT71
C. Stillbirth
KRT71
SP140
snoRNA
ENSCAFG00000027305
MITF
HTR2C
D. Gestation Length
SLC9A8
lincRNA
ENSCAFG00000037743
HFM1
CACNA2D3
MTUS2
lincRNA
ENSCAFG00000039067
−log10(p)
Chromosome

.


SP140
SMOC2
SLC9A8
RCL1
PALM3
MTUS2
MSRB3
MSANTD1
MITF
KRT71
HTR2C
HFM1
CD36
CACNA2D3
Gene
Breast
Cervix/Uterus
Endometrium
Fallopian Tube
Ovary
Placenta
Tissue Type
Level of Expression
(transcripts per million)
>200
150
100
50
0