A Genetic Model of Common, Complex Disease Hints at Genomic Architecture:
Brindle Coat Color in Canines

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ABSTRACT

Background: One of the greatest challenges facing scientists today is an understanding of genetic factors associated with the development of common, complex diseases such as diabetes, depression, and cancer. In addition to symptom burden (effecting quality of life), premature death, and disability caused by such diseases, the economic burden to society is vastly overwhelming. Last year, Major Depression alone cost nearly $211 Billion [USD] to US taxpayers. Yet, these diseases have been incredibly arduous to study due to the hundreds of genes that contribute very small effects that lead to disease development. We suggest that an alternative model of complex diseases is needed to elucidate the causative genetic mechanisms. We exploit a naturally occurring clear phenotype (brindle color) present on a simplified genetic background (pet dogs) and analyze large-scale genomic variation (CNVs) effecting gene expression (epigenetics – DNA methylation).

Significance: Common, complex disease is a tremendous burden for patients, families, and clinicians alike. Elucidation of the genetic mechanisms leading to disease development would result in personalized therapies and improved patient outcomes.

Purpose: To identify large-scale genomic variations present in Brindle color dogs and determine if epigenetic regulation is leading to phenotype development.

Conceptual Framework: We used the genetic single gene-two hit model (Hussian, 2015) to guide this work. This framework combines rare and common variant theories with the addition of mutator/anti-mutator modulation to lead to disease development.

Methods: Using a custom-designed aCGH to interrogate genomic structural variation, we analyzed 12 dogs of 3 different coat colors (Black, yellow, and brindle). Then, we performed genome-wide DNA methylation analysis on a subset of 8 dogs to determine the effect genomic variation has on epigenetic silencing of gene expression.

Results: We identified a 67 Kb complex genetic variation (10 probes, p=0.001) that disrupts gene expression and is epigenetically silenced in certain skin cells producing pigment differences (p=0.04).

Conclusion: Brindle coat color in canines is a complex genetic mechanism involving structural changes leading to epigenetic effects. Identifying this mechanism provides the first tractability for understanding complex diseases and is particularly exciting as a model for identifying such features in human diseases.

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INTRODUCTION

The Problem of Common, Complex Disease

One of the greatest challenges facing clinical scientists in the 21st century is understanding the genetic basis for complex human diseases.[1] One prevalent paradigm, coined the 'common disease-common variants' (CDCV) paradigm, states that genetic susceptibility to common disease is due to genetic variants that are relatively frequent in general populations and have low penetrance.[2] In the early 2000s, this resulted in a then-revolutionary technique - the Genome-wide Association Study (GWAS) based on single nucleotide polymorphism (SNP) frequencies in large populations.[3] Yet, GWAS methods have not been able to identify the majority of causative variation associated with common, complex diseases.[4] In fact, within complex diseases, all of the common variants (taken together) only explain a very limited proportion of the disease heritability.[5] For example, schizophrenia has a heritability of 85%, but of the 100 SNPs that have been associated with the disease, only a very small percentage of the heritability can be explained with ORs on the order of 1.1-1.2.[6,7] This has led to the inevitable question, "where is the missing heritability?"

Finding the "Missing Heritability"

More recently, scientists have begun to believe that this "missing heritability" can be explained by copy number variations (CNVs) and epigenetic modifications that are

currently unknown.[6,8] CNVs are defined as a DNA fragment with variable copies compared to a reference genome and can be present in duplication, deletion, inversion and translocation and are present in more than 20% of the genome.[9,10] The number of studies linking CNVs with common complex diseases has increased dramatically in recent years.[10]

In addition to the role CNVs have in disease liability, science is just beginning to elucidate the role of epigenetics in complex disease. The modern definition of epigenetics covers heritable information of gene expression mediated by dynamic mechanisms that include DNA-methylation, histone modifications, and noncoding RNAs [11]. DNA methylation at the 5′ position of cytosine (5-mC) is a key epigenetic mark that is critical for various biological and pathological processes and is now considered the "fifth base".[12] In fact, the "loss of 5-hmC" is considered an epigenetic hallmark of melanoma, with diagnostic and prognostic implications in multiple cancer types.[13-15]

Studies on the domesticated canine have recently provided new insight on their potential as a genetic model. Dogs have many similarities to humans, including genetics and certain disease predispositions.[16,17] Dogs also have some intriguing differences, such as a reduced genetic heterogeneity within breeds combined with increased heterogeneity across breeds. This underlies many of the breed-to-breed differences in disease risks; yet other traits seem likely to have been purposely introduced by breeders, such as chondroplasia [18,19]. The unique genetic power of canines can be seen in brindle coat color,

a coat color characterized by a pattern of alternating black/brown and red/yellow coat

color that forms an

irregular pattern,

typically a "v" over

the dorsum and "s"

over the flanks and

ventrum(See Figure

1).[20]

Here, we

circumvent the

traditional

problems

Figure 1: Coat Color in Greyhounds. (A) Light Brindle (K^{br}/K^y, K^{br}/K^{br}) (B) Dark Brindle (K^{br}/K^y, K^{br}/K^{br}) (C) Yellow(K^y/K^y) (D) Black(K^B/K^y, K^B/K^{br}, K^B/K^B). Photos courtesy of www.greyhound-data.com.

encountered by studies of common, complex disease by using a novel animal model (the

dog), a novel methodological approach (DNA methylation and CNVs), and use a simple

phenotype (brindle coat color) to understand complex genetic variation.

Coat Color Basics

In most mammals, melanocytes synthesize red/yellow (pheomelaninic) or

black/brown (eumelaninic) color that is governed by two genes: *Agouti*, which encodes a

paracrine-signaling ligand secreted by cells adjacent to hair follicle melanocytes, and

Melanocrtin 1 receptor (*MC1R*), which encodes a seven trans-membrane receptor that,

when active, causes melanocytes to produce eumelanin [20]. Agouti coat color effects are mediated by competitive inhibition of alpha-melanocyte-stimulating hormone binding at MC1R. Thus, *MC1R* loss of function or *Agouti* gain of function results in production of pheomelanin (red/yellow), while *MC1R* gain of function or *Agouti* loss of function results in production of eumelanin (black/brown).[21] However, it was recently revealed that the pigment type-switching mechanism in the dog has additional complexity that was previously unknown in any species, including the presence of a black coat color that is dominant to yellow.[20,22]

Candille et al. demonstrated that dominant black is the result of a mutation in the beta Defensin 3 gene *CBD103*.[22] Specifically, a three base pair deletion results in loss of a single glycine residue ("ΔG23"). That variation has two effects: i) increased extracellular levels (mechanism unknown) and ii) increased affinity of *CBD103* for *MC1R*. The dominant black effect is mediated by *CBD103* inhibition of Agouti binding at *MC1R*. A linkage study first implicated chr16 as the locus for brindle coat color, and as part of this analysis Kern et al. were able to define a dominance order of $K^{Black} > K^{Brindle} > K^{Yellow}$ (hereafter K^B, K^{br}, K^y, see Figure 1).[20] They postulated that brindle likely results from an unstable allele that switches from yellow to black through some epigenetic process. Chen et al. subsequently identified common structural variation at the *CBD103* locus – directly affecting its copy number and having a breakpoint proximal to the gene – and proposed

that a structural variant could be responsible for such a Mendelian trait with an epigenetic readout.[23]

To verify $\Delta G23$ as the causal mutation, Candille et al. generated a transgenic mouse model.[23] While the $\Delta G23$ mouse was black, unexpectedly 21/23 wildtype K^Y/K^Y also had black coat color.[23] This suggests the possibility that the $\Delta G23$ mutation is not necessarily the dominant black mechanism but could instead be a marker of a *CBD103* allele that is expressed at increased levels over wild type. In the same study, brindle coat color was mapped to a 1.85 Mb region overlapping *CBD103*.

Honor's Project Purpose

Characterize DNA methylation patterns of the genomic locus Chr16:58,975,216 58,976,773 in canine blood and skin tissue.

Conceptual Framework

We used the genetic single gene-two hit model to guide this work.[24] This framework combines rare and common variant theories with the addition of mutator/anti-mutator modulation to lead to disease development.

METHODS

Ethics Statement

All research activities were approved by The Ohio State University Animal Use and Care Committee (IACUC; Protocol 2010A0025). Protocols were based on the statutes

of the Animal Welfare Act and the guidelines of the Public Health Service as issued in the Guide for the Care and Use of Laboratory Animals (revised 1996).

Samples

The samples used as part of this study were collected under a larger study of breed variation and disease association in pure breed dogs. Samples were collected in collaboration with The Ohio State University Veterinary Medical Hospital and Greyhound Health and Wellness Program. Interested owners were screened for inclusion, and subsequently informed consent for blood collection was obtained from the owner, and sample collected by a trained veterinary technician in 1-2 7 mL BD lavender top tubes. All samples were classified according to breed and known phenotypes. Dogs were selected for use in this study based on coat color pattern listed on their registration, regardless of known disease status. Dog breeds were excluded from the study if their breed was known to have e/e genotypes. Coat colors were subsequently placed into of the phenotype categories: K^B, K^{br}, or K^y. Consequently, all Greyhounds used in further analysis were genotype and sequenced. It is noted that Greyhounds are not known to have an e/e genotype (that is, dominant melanocortin mutation that would make the dog appear to be yellow/red when it may possess the underlying K^{br} genotype). Registration and pedigree information was collected and confirmed.

DNA/RNA Isolation

Blood: Genomic DNA was isolated from lleukoctyes using 5 Prime ArchivePure DNA Blood Kits (Gaithersburg, MD) as per protocol with an additional ethanol precipitation step. Samples were tested for quality using a 2% pre-stained ethidium bromide agarose gel with electrophoresis and subsequent fluorescence. Additional quality control included measurements with the Nanodrop 2000 and Qubit 3.0 Fluorometer readings per manufacturer's protocols. Results demonstrated visualized DNA as compact, high-molecular-weight bands with no low-molecular-weight smears and OD260/280 ratios ≤1.8 and OD260/230 ratios ≤ 2.0, thus indicating intact high-quality genomic DNA.

RNA: Total RNA was isolated from 5mm skin punches; whenever possible the same subject used to generate gDNA was used to generate RNA (with a different skin punch). We used the RNeasy Fibrous Tissue Mini Kit (Qiagen) with a tissue pulverizer to isolate RNA. When necessary, some samples were placed in RNAlater-ICE frozen tissue transition solution. After isolation, SuperScript III First-Strand Synthesis kit (Invitrogen) was used to generate cDNA as per their protocol.

Microarray-based Comparative Genomic Hybridization (aCGH; Agilent) on gDNA from Whole Blood

Platform: Our initial CNV observation was made on the Nimblegen 385K array. [23] In that study, we identified a 611kb CNV on chr16 which overlapped the known K locus.[23]

Subsequent to this finding, we custom designed our own ultra-high-resolution Canine CGH array (Agilent) using Genotypic bioinformatics core to develop probes that covered the CanFam2 genome. To avoid cross-hybridization, each probe was aligned to the CanFam2 genome using BLAST; any probe that did not map uniquely was removed except those targeting segmental duplications. This array includes 1 Million oligonucleotide spots with an average spacing of <1.7 kb genome-wide. Additionally, our array includes known Segmental Duplications based on Akey's map of segmental duplications, with an average spacing per probe of <1.5 kb.[25] Essential for an oligo array, this platform uses 50–75mers with the design of the probes to have TM within +/- 3 $^{\circ}$C of each other. Also, this platform uses 3 μg of DNA without requiring complexity reduction or amplification (which generate incomplete sampling and signal noise, respectively). The advantage of using the Agilent array compared to other commercially available arrays is that Agilent has an excellent low background with increased signal to noise ratio. This ratio allows for adequate CNV detection at 3 probes or less on a Agilent 1M probe array, compared to >10 probes needed for Nimblegen's 2.1 M probe.[26] All samples were two-color comparatively hybridized against the gDNA from a single reference dog, a Labrador Retriever. Hybridization to arrays was completed by the BioGenomics Core at The Research Institute at Nationwide Children's Hospital (TRINCH; Columbus, OH). The protocol for DNA digestion, labeling, purification and hybridization to the arrays followed the manufacturers' instructions.

Data Analysis: Microarray image files were quantified using the Agilent Feature Extraction software and then imported into Partek. We set the criteria for calling CNVs intentionally low to minimize false negatives and accept false positives. First, a filtering procedure was used to flag low-intensity features. Probes with a combined Cy3 and Cy5 intensity value of more than 3 SD below the mean of the high-intensity mode were flagged and excluded from further analysis. Next, segmentation of the remaining data was performed using a circular binary algorithm with post-processing to ensure that regions had at least three genomic coordinate consecutive probes with the same sign of deviation in the log 2 ratio as well as the median log 2 ratio which exceeded 0.3 in absolute value on the log scale. A subset of CNVs that had been previously reported were manually confirmed within the dataset.

Call Validation: We validated a subset of the CNV calls made from the 1M Agilent array by Southern blotting. We selected 3 CNV overlapping known genes (*HMGCS2, ZFHX3, SOX9*) and 1 reference gene (VEGF, as used previously[23] and described [27]). Briefly, probes were designed to target unique sequence in HindIII fragments that did not overlap predicted breakpoints, generate fragments of different sizes to allow multiplex hybridizations, and have similar melting temperatures, to result in comparable signal in multiplex format. Probes were generated by PCR, confirmed by agarose gel electrophoresis, purified using PCR Purification kit (QIAGEN), and random primed

labeled with [32]P. Signal was quantified by PhosphorImaging (Storm, GE Healthcare Life Sciences).

DNA Methylation with MeDIP

MethylCap-seq library generation and sequencing: For DNA methylation analysis, we selected MethylCap-seq. This is a cost-efficient, genome-wide, highly reproducible, high-throughput, less cumbersome method then other traditional techniques used for interrogating methylated regions.[28-30] After fragmentation, methylated DNA is captured with the high affinity methyl-CpG binding domain of human MBD2 protein and eluted in a step-wise manner indicative of methyl-CpG density.[29,30] Consequent analysis is performed on the enriched fragments by massively parallel sequencing.[28,29] MethylCap-Seq was performed at The Ohio State University Comprehensive Cancer Center's Genomics Shared Resource. Briefly, gDNA isolated from leukocytes in whole blood was quantified by Qubit flurometric quantitation (Life Technologies, Grand Island, NY). 1-1.3 µg DNA was subjected to fragmentation using a Covaris S2 Adaptive Acoustic instrument (Woburn, MA) to average fragment size of 100 – 250 bp. Methylated DNA enrichment fragments were enriched using the Diagenode AutoMethylCap Kit customized for the Diagenode SX-8G IP-Star Compact Automated System (Denville, NJ). 1 ng of the resultant enriched and ethanol precipitated methylated DNA was used to generate an Illumina-compatible sequencing library using the Kapa Hyper Prep Kit (Fremont, CA). Library fragments (average fragment size is 300-400 nt, including

adapters) were amplified using Phusion High-Fidelity PCR Master Mix with HF Buffer (NEB) for 8 PCR cycles. Library material was purified with Agencourt AMPure XP beads (1:1 volume; Beckman Coulter, Inc., Indianapolis, IN). Sequencing was performed on the Illumina HiSeq 2500 (San Diego, CA) using the single-end 50-bp approach (Version 3 chemistry) to a depth of at least 40 million passed filtered reads.

MethylCap-seq read alignment & assignment of methylation values from reads: Sequencing reads were received in fastq format. Duplicates (i.e., all reads with the same sequence data) among passed filter (using default Illumina settings) sequencing reads were collapsed. Collapsed reads were quality trimmed using a quality score cutoff of 33 using the fastx toolkit. Reads were then aligned to canine genome (CanFam3) using bowtie version 0.12.7.[29] DNA fragment profiles were extracted for every sample from Bioanalyzer data using a custom python script. These fragment profiles and the aligned reads were employed to assign methylation values to every CpG in the human genome using PrEMeR-CG.[31] We focused on the methylation of first exons of annotated protein coding genes, which has been shown to be especially relevant for regulation of gene expression.[32] Given that this is the first analysis of human DNA methylation changes in less than 48 hours in critical care patients, we applied stringent thresholds to the data. We removed any regions in which MethMAGE failed (due to absence of methylation data), required that the maximum of the methylation signal in the two groups was above

its median, and applied Benjamini Hochberg FDR (BHFDR)[33] with a q-value cutoff of 0.01.

DNA Methylation Validation with Bisulfite sequencing: We used the EZ DNA Methylation-Gold kit (Zymo Research) to Bisulfite covert gDNA according to their protocol. We then PCR amplified the converted gDNA with primers designed for bisulfite converted gDNA to result in amplicons ~400bp with ≥2 CPG islands within amplicon using the online database Bisearch.[34,35] We then used CloneJET PCR cloning kit (Fermentas, Thermo Scientific) with Z-competent *DH5α E. Coli* cells (Zymo Research). After colonies were identified based on the PCR screening, the PCR product was purified (QIAGEN), and then sequenced at the DNA sequencing core (Eurofins, MWG, Operon). Sequence was then analyzed and figures created using Bisulfite Sequencing DNA Methylation Analysis (BISMA) tool available from Bisulfite sequencing Data Presentation and Compilation (BDPC).[36-38]

Polymerase Chain Reaction (PCR) for Fine-Mapping Structure on Chromosome 16

PCR: Using the JumpStart REDTaq DNA Polyermase protocol (Sigma-Aldrich), PCR was performed with additional optimizations of each different primer pair reactions. PCR primers were custom designed using Oligo Calc Nearest Neighbor based melting temperature (TM) calculations,[39] and then uniqueness confirmed using the UCSC Genome Browser of CanFam2/CanFam3.[40,41] Oligos were generated by Integrated DNA Technologies (IDT). All sequencing analysis was performed by the DNA sequencing core

(Eurofins, MWG, Operon).

Digestion, Ligation, Amplification (DLA): DLAPCR walking method, we followed a

previously described protocol.[42] We generated four unique random adaptors not present

within the genome, and then paired these with rare-cutter restriction enzymes. Final

Adaptor gDNA was then combined with unique primers from the locus of interest in two

reactions; the first was an external primer pair amplification followed by an internal PCR

of the same locus.

RESULTS

Previous Work on the Brindle Locus

Previously, the presence of a 611kb CNV-rich region overlapping KB locus from chr16:61,902,802-62,514,014 within 8 dogs analyzed using a Nimblegen 385k aCGH[23] and

Figure 2: New CNV overlapping *K-locus* in only brindle dogs. The two dogs with a CNV where both registered AKC dogs with brindle listed in their coat pattern, from two different breeds **(A)** Akita **(B)** Bulldog. **(C)** New partitioning of the CNV and segmental duplication in all 8 dogs. Note that the 6 dogs share a portion of the segmental duplication.

then confirmed to be a 76kb CNV in Brindle dogs (n = 15) using the ultra-high density 1

M oligonucleotide array CGH (See Figure 2). Using a Southern blot, we were able to

further define allelic differences in *CBD103* (establishing heterozygosity and

homozygosity); these results suggested 4 different alleles corresponding to two
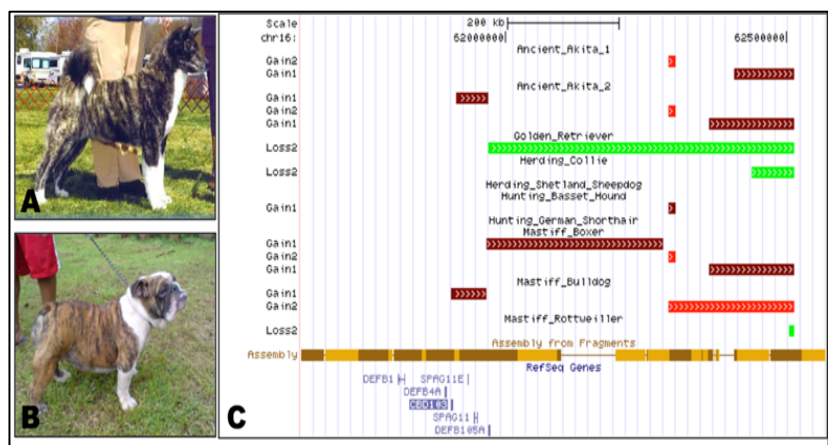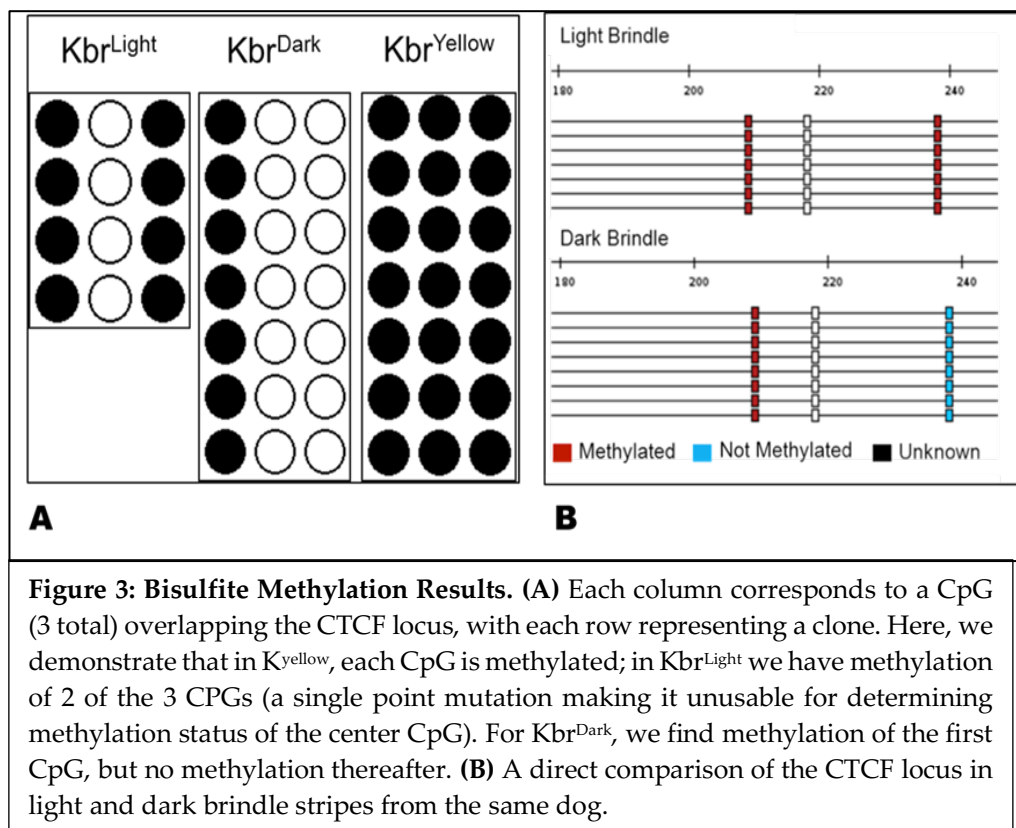
different alleles in black, one for brindle, and one for yellow. We then used overlapping

PCR assays to scan the full 6.6 kb region within the restriction enzyme cleavage sites

and computationally identified a statistically significant purported promoter, CTCF

epigenetic insulator, and open reading frame. This leads to the possibility of an

antisense transcript affecting the type switching in brindle coat color.

Work on the

Brindle Locus

for this Project

Using

MeDIP with

Next

Generation

Sequencing,

we identified 2

Figure 3: Bisulfite Methylation Results. (A) Each column corresponds to a CpG (3 total) overlapping the CTCF locus, with each row representing a clone. Here, we demonstrate that in K^{yellow}, each CpG is methylated; in Kbr^{Light} we have methylation of 2 of the 3 CPGs (a single point mutation making it unusable for determining methylation status of the center CpG). For Kbr^{Dark}, we find methylation of the first CpG, but no methylation thereafter. **(B)** A direct comparison of the CTCF locus in light and dark brindle stripes from the same dog.

regions of the first exon of *CBD103* that were differentially methylated (Brindle Vs

Fawn, p = 0.036; Brindle Vs Black, p= 0.037).

Based on our previous work, we hypothesized that epigenetical regulation is

involved in the color type-switching and maintenance in brindle.[20] We isolated genomic

DNA from the skin of black, yellow, dark brindle and light brindle stripes of dogs. We

conducted Bisulfite Conversion and Sequencing (see Figure 3).

We custom-designed PCR primers for amplicons upstream and downstream of both the CTCF site and *CBD103*.[34,35] We cloned the bisulfite converted PCR product and sequenced the appropriately sized clones. Interestingly, we found differential patterns of methylation. In light brindle and yellow, we found methylation that existed after the CTCF site and at *CBD103*. But in dark brindle and black skin, we found no DNA methylation of the CTCF locus or *CBD103*.

DISCUSSION

Brindle coat pattern has been hypothesized to be the product of a binary (yellow/black) type-switching allele that i) manifests no later than early neural crest differentiation of melanocyte lineage cells and ii) is generated and maintained epigenetically,[20] but the exact mechanism has remained elusive. This is largely due to an abundance of highly repetitive elements in the locus, which has resulted in putative genome assembly errors and hindered determination of the genetic variation that differs between K alleles. Here, we present work that gives insight into the complex mechanism of brindle coat pattern in dogs. The nature of the genetic mechanism underlying brindle is reminiscent of X-inactivation in mammals (reviewed in [43]). There, two non-coding genes, *Tsix* and *Xist*, act in cis and their expressed RNAs are localized to the nucleus. X-inactivation is mediated by the coating of one X chromosome with *Xist* RNA. After the initiation of X-inactivation, *Tsix* – which is proximal, in an antisense position to *Xist* –

expression is downregulated, creating a permissive state for *Xist* upregulation by activators (see Figure 4.)

Tsix RNA associates with the methyltransferase DNA methyltransferase 3A (*DNMT3A*), either directly or through accessory factors, and thereby stably silences the *Xist* promoter via DNA methylation and other changes in chromatin structure. [44,45] The key mechanism that regulates this program is the binding of CTCF exclusively to the *Tsix* locus of the inactivated X chromosome. That is associated with increased expression of that copy of *Tsix* through the *Xist* locus, somehow silencing it. On the active X, the same CTCF site is
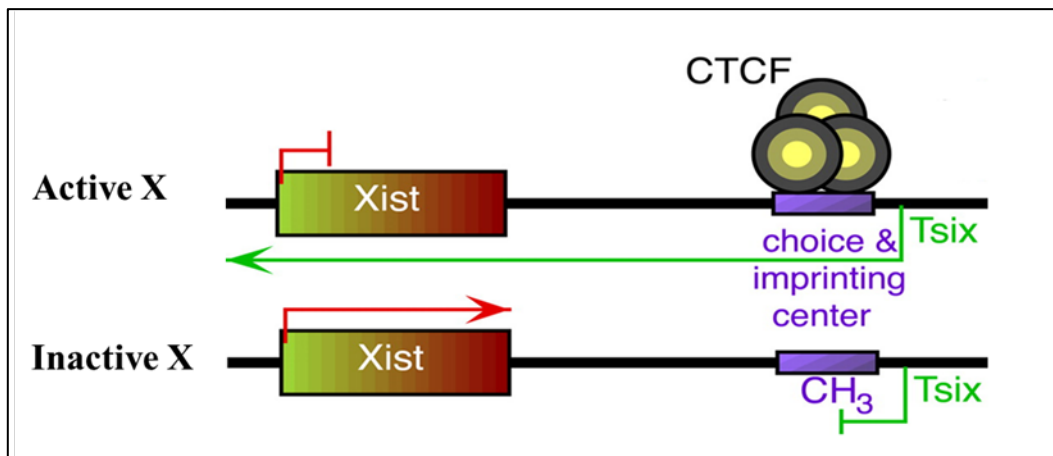

Figure 4: A model of a regulatable epigenetic switch created by CTCF and *Tsix*. Xi represents the inactive X chromosome. Xa is the active chromosome. Modified from.[43]

methylated and *Tsix* is not expressed. The evidence we have accumulated suggests the same mechanism as is seen in random X-inactivation, except that both copies of a brindle homozygote are either activated or inactivated.

Research Implications

The clearer understanding of the brindle mechanism presented here has potential implication in the current studies of common, complex diseases in the human population. Much of the previous research to date has identified low or rare minor allele frequencies (MAF) in association with such diseases rather than single-gene associations.[46] Contrarily, research focused on CNVs in the human genome has revealed that many significant genetic elements are overlapped by these structural variants, including approximately 85.01% of exons.[47]

Clearly, there was is much additional work to be completed to fully elucidate the exact mechanism at the Brindle locus (Including functional and biochemical studies). Our understanding of this mechanism demonstrates the potential effect of copy number variations on gene expression, as well as the feasibility of functional effects in the human genome. Similar genetic architecture in humans may hint at plausible new explanations of disease and trait development. It also seems likely that these phenomena can be exploited as genetic tools for diverse applications of genetic/epigenetic therapeutics.

Clinical Implications for Nursing

Nurses in the clinical setting are often responsible for medication administration in the treatment and management of common, complex diseases. As targeted therapies based on genetic profiles become more common, nursing practice must evolve to encompass not only the genetics/epigenetics of individuals during diagnoses, but also the

genetics/epigenetics that affect therapeutic responses to treatment. Appropriate advances in practice will be necessitated at the individual nurse level to guide institutional implementations of patient education related to genetically based treatment regimens.[48]

One clear way nurses can be on the forefront of this era of genomic-guided health is through increasing nurse knowledge of pharmacogenomics to build a platform for nurses to promote its use among their patient population, network with other providers, work closely with pharmacists and assess patients for eligibility.[49] Nurses play a large part in patient education, when patients are faced with making decisions based on genetic information they assist with education, counseling, and emotional support. Nurses need to be aware of trends in genetic advancements with regard to how they relate to the individual care of each patient.[50]

There is also a demonstrated need for additional education for nurses themselves regarding genetic advancements. Nursing students report a perceived lack of comfort with both knowledge and clinical applications of genetics.[51] On the other hand, practicing RNs also report benefits from additional education regarding the role of genetics and genomics in patient health outcomes. Nurses' unique position allows them great potential to translate such genomic discoveries into more personalized interventions and assessment for patients.[50]

CONCLUSION

This model of the brindle coat color mechanism in dogs serves to clarify this unique framework for potential screening, development, and treatment of disease.

ACKNOWLEDGEMENTS

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