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Mining the 99 Lives Cat Genome Sequencing Consortium database implicates genes and variants for the *Ticked* locus in domestic cats (*Felis catus*)

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Summary

Tabby patterns of fur coats are defining characteristics in wild and domestic felids. Historically, three autosomal alleles at one locus (*Tabby*): *Abyssinian* (T^a; a.k.a. *ticked*), *mackerel* (T^m; a.k.a. *striped*) and *blotched* (t^b; a.k.a. *classic, blotched*) were thought to control these patterns in domestic cats and their breeds. Currently, at least three loci influence cat tabby markings, two of which are designated *Tabby* and *Ticked*. The *Tabby* locus is *laeverin* (*LVRN*) and affects the mackerel and blotched patterns. The unidentified gene for the *Ticked* locus on cat chromosome B1 was suggested to control the presence or absence of the ticked pattern (Tabby – *Abyssinian* (T^a; a.k.a. *ticked*). The cat reference genome (Cinnamon, the Abyssinian) has the ticked phenotype and the variant dataset and coat phenotypes from the 99 Lives Cat Genome Consortium (195 cats) were used to identify candidate genes and variants associated with the *Ticked* locus. Two strategies were used to find the Ticked allele (s), one considered Cinnamon with the reference allele or heterozygous (Strategy A) and the other considered Cinnamon as having the variant allele or heterozygous (Strategy B). For Strategy A, two variants in *Dickkopf Wnt Signaling Pathway Inhibitor 4* (*DKK4*), a p.Cys63Tyr (B1:41621481, c.188G>A) and a less common p.Ala18Val (B1:42620835, c.53C>T) variant are suggested as two alleles influencing the *Ticked* phenotype. Bioinformatic and molecular modeling analysis suggests that these changes disrupt a key disulfide bond in the Dkk4 cysteine-rich domain 1 or Dkk4 signal peptide cleavage respectively. All coding variants were excluded as *Ticked* alleles using Strategy B.

Keywords Abyssinian, coat pattern, *Dickkopf Wnt Signaling Pathway Inhibitor 4*, *DKK4*, *Tabby*

Introduction

The stripes, swirls and spots of cat coat patterns are well recognized and often defining characteristics in felids. Both wild felids and domesticated cat breeds display a wide range of coat patterning phenotypes. Lions (*Panthera leo*), pumas (*Puma concolor*), Abyssinians and Singapuras display basically no pattern, whereas tigers (*Panthera tigris*) and many mackerel tabby cat breeds present with stripes. Leopards (*Panthera pardus*), Egyptian maus and ocicats have

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distinctive spots, and clouded leopards (*Neofelis nebulosa*), marbled Bengals and American shorthairs (blotched, classic tabbies) can present with intriguing blotches and swirls (Fig. 1). In the wild, undoubtedly, these patterns confer a camouflage tailored to the felid's ecological niche and have been a focus of artificial selection in the domesticated cat breeds. Considering species taxonomy, the blotched tabby cat was considered the 'voucher' specimen for the domestic cat (Abdel-Malek *et al.* 2001), hence, this pattern is also known as a 'classic' tabby (Fig. 1c; Linneaus 1758).

For over a century of domestic cat breeding, the *Tabby* locus, which was originally thought to control all of the domestic felid pattern and markings, was defined by three autosomal alleles: *Abyssinian* (T^a; a.k.a. *ticked*), *mackerel* (T^m; a.k.a. *striped*) and *blotched* (t^b; a.k.a. *classic*, *blotched*; Lomax & Robinson 1988). Traditional patterns were represented by three phenotypes governed by the following genotypes: T^a, which produces few markings and possible stripes on the head, legs and tail but not on the torso; T^mT^m *or* T^mt^b, which produces stripes on the head, legs, tail and torso; and t^bt^b, which produces stripes on the head, legs and tail but circular patterns on the torso. Cats homozygous for the *Abyssinian* allele (T^aT^a) may have less barring on the legs and can often be distinguished from heterozygotes. Hence, the T^a allele can be considered co-dominant to T^m and t^b when considering the leg barring, but this phenotype has variable expression and is difficult to clearly distinguish. The allelic series $T^a > T^m > t^b$ was suggested; however, this series came under dispute because the distinctive spotted patterns of Egyptian maus, ocicats and other cats could not be explained by this single, locus multi-allelic model (Cat Fanciers' Association 2004; Kaelin & Barsh 2010; Eizirik *et al.* 2010).

To localize genes associated with *Tabby*, a genome scan was performed on a large pedigree of cats segregating for tabby coat markings, specifically for the Abyssinian (T^a) and the blotched (t^bt^b) phenotypes (Lyons *et al.* 2006). A genetic linkage between the phenotypes and eight short tandem repeat (STR) markers on cat chromosome B1 was indicated, the most significant linkage was between cat short tandem repeat marker FCA700 and '*Tabby*' phenotype ($Z = 7.56$, $\theta = 0.03$). The linked markers covered a 17 cM region and flanked an evolutionary breakpoint, suggesting that the *Tabby* gene has a homolog on either human chromosome 4 or 8, which share synteny with cat chromosome B1.

A second linkage analysis confirmed the B1 location of a patterning locus in cats and identified a second locus controlling patterning on cat chromosome A1 (Eizirik *et al.* 2010; Kaelin *et al.* 2012). Using cross-species analyses, five SNPs showed significant association (P range = 9×10^{-4} to 3.2×10^{-9}) within a 180 kb interval on cat chromosome A1. The associated gene and variants were refined and the candidate gene was identified as laeverin (*LVRN*; *a.k.a. transmembrane aminopeptidase Q*, *Taqpep*), which encodes a membrane-bound metalloprotease and plays a regulatory role in extravillous trophoblast migration (Maruyama *et al.* 2007; Horie *et al.* 2012). Three variants, p.S59X (c.176C>A), p.D228N (c.682G>A) and p.W841X (c.2522A>G), were suggested to cause the blotched tabby patterns across breeds (Kaelin *et al.* 2012). An additional variant was suggested to be the cause of the rare king cheetah phenotype, in which spots coalesce into blotches and stripes (Buckley *et al.* 2020b).

The tabby markings of cats are also influenced by the pigment switching signaling pathway including *agouti-signaling protein* (*ASIP*) and *melanocortin 1 receptor* (*MC1R*; Barsh *et al.* 2000; Abdel-Malek *et al.* 2001; Baxter, Loftus & Pavan 2009; Baxter & Pavan 2013). Cats with tabby patterning have solid-colored hairs as part of the fur composing the stripes, swirls and spots, whereas the fur in between the pattern is 'ticked' with bands of pheomelanin and eumelanin (i.e. agouti – named after the South American rodent which also has bands of both pigment types), giving an illusion of brown coloration. Ticked tabbies, such as an Abyssinian, do not have any pattern, but have ticked, agouti hairs throughout the coat. These hairs can have limited ticking, with one band of eumelanin at the tip and a base band of pheomelanin, or the fur can have several alternating bands of eumelanin and pheomelanin. The number and extent of alternating bands of pigment is postulated as a separate locus suggested known as *Wide-band* (Robinson 1991).

After the publication of the variants in *LVRN* on cat chromosome A1 for the now defined *Tabby* locus (OMIA 001429-9685), the associated locus identified on cat chromosome B1 was termed the '*Ticked*' locus (*Ti*) (Eizirik *et al.* 2010; Kaelin *et al.* 2012). The *Ticked* locus is more akin to an absence of pattern common to the Abyssinian and Singapura cat breeds, and probably interacts with the *ASIP-MC1R-LVRN* pathway in a manner influencing the presence or absence of coat pattern, regardless of the type of pattern dictated by the *Tabby* locus. Thus, the *Ticked* locus is epistatic to the *Tabby* locus. The *Ticked* allele (*Ti*/−), which has no pattern, is considered autosomal dominant; however, as patterning is highly prevalent in domestic cats and present in the progenitor wildcat species, the wt allele for the *Ticked* locus is the presence of pattern (i.e. non-ticked (ti^+ti^+)).

Analyses of the single nucleotide variant and phenotypic data of the 99 Lives Cat Genome Sequencing dataset was used to refine the associated region on cat chromosome B1 and identify putative functional variants that cause the lack of patterning in cats and probably the gene for the *Ticked* locus.

Materials and methods

The STRs from the published linkage analyses (Lyons *et al.* 2006) were remapped to the Felis catus V 9.0 genome assembly (Buckley *et al.* 2020a) using the basic local alignment search tool (BLAST; Altschul *et al.* 1990). An extended area around the 17 cM linked region on cat chromosome B1 was analyzed for candidate variants.

Figure 1 *Tabby* patterns of cats and the *Ticked* cats in the 99 Lives Cat Genome database. (a) The wt domestic cat is a brown mackerel (striped) tabby with relevant color genotypes *A*−, *B*−, *C*−, *D*−, *E*−, *gg*, *ii*, *ss*, $Ta^{M}-$, $ti^{+}ti^{+}$ and $X^{o}X^{o}$. (b) The Somali (longhaired Abyssinian) is the hallmark breed with a *Ticked* phenotype ($Ti^{A}-$). (c) An American shorthair with the blotched (a.k.a. classic) tabby ($ti^{+}ti^{+}$, $ta^{b}ta^{b}$), which represents the voucher specimen for a domestic cat although not the common wt specimen, which is a mackerel tabby. (d) The Egyptian mau breed has been selected for spots. The ticked cats in the data analyses include (e) Art Deco – red ticked Oriental shorthair (image courtesy of Winter Trussell, DVM); (f) Nina – blue ticked random bred; (g) Cali – obligate heterozygote bred from a Somali; and (h) Monkey – obligate heterozygote bred from a Somali. Images of Somali, the American shorthair and the Egyptian mau courtesy of Chanan Photography, Richard Katris.

Variant filtering

The details of the 99 Lives dataset, including sequence data processing and the variant calling workflow have been previously published (Buckley *et al.* 2020a; Buckley *et al.* 2020b; Yu *et al.* 2020). The 99 Lives VCF was filtered for candidate variants using VarSeq (Golden Helix). The VCF was annotated using both Ensembl 101 release (20 August 2020) and NCBI *Felis catus* annotation release 104 (10 December 2019). The ticked (*Ti*) allele is considered dominant (absence of pattern); therefore, a ticked cat can

be heterozygous or homozygous for the ticked allele and hence the variants controlling the allele. In addition, as the *Ticked* locus has not been examined genetically in detail; phenocopies may be present or more than one allele may produce the same phenotype. For example, four variants are known to cause long hair in cats (Drögemüller *et al.* 2007; Kehler *et al.* 2007). A independent WGS of the reference cat, Cinnamon, is available in the 99 Lives dataset. Because the cat reference genome represents an Abyssinian cat (Linneaus 1758; Lomax & Robinson 1988; Lyons *et al.* 2006; Kaelin & Barsh 2010), Cinnamon, who has a ticked phenotype, Cinnamon can be either homozygous or heterozygous for the variant controlling the ticked allele (Buckley *et al.* 2020a; Li *et al.* 2016; Montague *et al.* 2014; Pontius *et al.* 2007). If Cinnamon is homozygous, then the ticked allele would be the reference allele, which is not the wt allele in cats as ticked is rare. However, if the reference cat (Cinnamon) is heterozygous, either the ticked or non-ticked allele could have been randomly selected as the reference allele. Therefore, two filtering strategies (A and B) were considered to identify candidate variants: (B) Abyssinians (including Cinnamon's WGS data) are heterozygous or homozygous for the variant allele; and (B) Abyssinians (including Cinnamon's WGS data) are heterozygous or homozygous for the reference allele. The cats used for the filtering steps within the two strategies are outlined in Table 1.

Only cats in the 99 Lives dataset with known phenotypes (ticked or non-ticked), documented by the investigators by visual inspection and or images, were used to filter the variants for candidates (Table 1), but all cats were considered in the final variant considerations and discussions. Breeds defined by their patterning, such as spotted cats (Egyptian mau, ocicat), striped cats (toyger) or other tabby markings (Bengal and savannah) were considered non-ticked. Except for one solid Bengal, cats of solid coloration (File S1) determined by the 2 bp deletion in the *Agouti* locus (*ASIP* A3:25086566; XM_019826162.2 c.264_265delCA, p.Met89Glufs*59) causing melanism or listed as white were not included in the variant filtering as the ticked phenotype would be masked (Eizirik *et al.* 2003).

The variant filtering steps used the same cats for both strategies A and B. The two strategies differed by changing the expected variant zygosity of the cats for either the reference or the variant allele. Filtering steps included: (i) eliminate intergenic, intronic and synonymous variants; (ii) consider the variant zygosity for the WGS entry for Cinnamon; (iii) consider the variant zygosity for the tabby breeds ($n = 9$); (iv) consider the variant zygosity for the known tabby cats ($n = 40$); (v) consider the variant zygosity for the known Abyssinians, which are ticked ($n = 3$); (vi) consider the variant zygosity for the two known heterozygous ticked cats; and (vii) consider the variant zygosity for the two additional cats with the ticked phenotype (not listed as a specific breed).

Table 1 Coding variant analysis of the *Ticked* linked region at B1: 35–65 Mb.

	Number of variants
Strategy A	
Filter 1 – without intergenic, intronic, synonymous	10 675
Filter 2 – Cinnamon as heterozygous or homozygous variant	372
Filter 3 – tabby breeds ($n = 9$) (Ocicat, E. mau, Toyger, Bengal, Savannah) – as homozygous reference	38
Filter 4 – known tabbies (40) – as homozygous reference	8
Filter 5 – Abyssinian as heterozygous or homozygous variant (PennyLane, Dot, CVB15215)	1
Filter 6 – Cali and Monkey set as heterozygous	1
Filter 7 – ticked cats (Nina, Art Deco) as heterozygous or homozygous variant	0
Strategy B	
Filter 1 – without intergenic, intronic, synonymous	10 675
Filter 2 – Cinnamon as heterozygous or homozygous reference	10 590
Filter 3 – tabby breeds ($n = 9$) (Ocicat, E. mau, Toyger, Bengal, Savannah) – as homozygous variant	44
Filter 4 – known tabbies (40) – as homozygous variant	3
Filter 5 – Abyssinian as heterozygous or homozygous reference (PennyLane, Dot, CVB25215)	0
Filter 6 – Cali and Monkey as heterozygous	0
Filter 7 – ticked cats (Nina, Art Deco) – as heterozygous or homozygous reference	0

Two phenotypically ticked cats were known to be heterozygous (obligate heterozygous) for the ticked allele as they were produced by a breeding of a tabby cat (Bengal) mated with a pedigreed ticked Somali (Fig. 1g,h; Cogne *et al.* 2020). The cats used in each filtering step are presented in Table 1.

A previous analysis of structural variants was conducted on the 99 Lives WGS dataset containing 54 cats, which included two of the Abyssinians for this study (Buckley *et al.* 2020b https://doi.org/10.1371/journal.pgen.1008926.s025). This dataset was examined for consistent structural variants found in the two Abyssinians but absent in the other non-ticked cats, which are present in the current dataset.

Bioinformatic analysis of the impact of Dkk4 variants

Signal peptide cleavage was predicted using the SIGNALP 5.0 server (Almagro Armenteros et al. 2019) for both wt Dkk4 protein and the p.Ala18Val variant. Disulfide bonds in the Dkk4 CRD1 domain were visualized using the structure of residues 19–224 of human Dkk4 (Patel *et al.* 2018) in the ChimeraX molecular graphics system (Pettersen *et al.* 2021). The p.Cys63Tyr variant was modeled with the swapaa command using the Dunbrack backbone-dependent rotamer library and taking into account the lowest clash score, highest number of H-bonds and highest rotamer probability.

Results

The 99 Lives dataset includes 195 domestic cats. Cat signalment, short read archive (SRA) submission identifiers and investigator contacts are presented in File S1. The 99 Lives dataset included an independent ILLUMINA-based sequence for the reference cat, listed as Cinnamon, thus, both alleles for the reference cat can be determined in the 99 Lives data. Considering the *ASIP* 2 bp deletion (Eizirik *et al.* 2003), 79 cats were identified as solid and one as white, thus, their ticked – tabby phenotype could not be determined (File S1). Three Egyptian maus, one ocicat, one savannah, one toyger and three Bengals represented breeds known for their tabby markings and 40 additional cats were known tabbies. Six additional cats were hairless or pointed; therefore the ticked phenotype could not be accurately determined. Fifty-three cats did not have a definitive phenotype as no images were available.

The dataset included two obligate heterozygous ticked cats (Cali and Monkey; Fig. 1g, h) identical by descent for their ticked allele as they are parent–offspring bred from a ticked Somali. Three known ticked Abyssinians (Cinnamon, Dot and Penny Lane) and three cats with the ticked phenotype (CVB15215, Nina (Fig. 1f) and Art Deco (Fig. 1e)) were also in the dataset; therefore, at least eight cats were known to have the ticked phenotype.

Eight STRs from the published linkage analysis were remapped to cat chromosome B1q within the region of positions 49–58 Mb (Table S1; Lyons *et al.* 2006). The linkage region is near the centromere and has known recombinants, thus the region was extended to include genes on the centromeric portion of B1p, including single variant analyses for all sequences (genic and intergenic) from 35 to 65 Mb on B1. This region contained 897 372 variants, with approximately 53% as intergenic variants, 45% as intronic variants and 0.5% as synonymous variants (Table 2, File S2a). After applying filter 1, the intergenic, intronic and synonymous variants were excluded and 10 675 variants remained to be filtered (File S2b); however, all variants were considered in the analyses. The reductions of the non-coding and coding variants at each step of the analyses are summarized in Table 2.

Five additional filtering steps were used to eliminate variants not associated with the ticked phenotype (Table 1, Files S2b and S3a,b). Two strategies were considered to identify candidate variants: (A) Cinnamon and other Abyssinians (the reference genome cat) as heterozygous or homozygous for the variant allele; and (B) Cinnamon and other Abyssinians as heterozygous or homozygous for the reference allele. Therefore, the zygosities for the remaining cats with known phenotypes were altered accordingly, depending on which of the two strategies was being considered. For strategy A (Table 1, File S2b), Cinnamon must be heterozygous or homozygous for the variant allele (Filter 2), then only 372 variants remained as candidates. After filter 3 (Tabby breeds ($n = 9$) must be homozygous reference), only 38 variants remained. Filter 4 set the zygosity for the 40 known tabby cats to homozygous reference and reduced the possible variants to 8. After applying filters 5 and 6, which considered the three known Abyssinians and the two obligate heterozygous cats (Cali and Monkey), only one variant remained, namely a variant in *DKK4*. This variant was at position B1:41621481 and was a guanine to alanine change (XM_023252567.1; ENSFCAT00000034752: c.188G>A), causing a p.Cys63Tyr amino acid change. The allele count for this variant was consistent with the ticked allele being rare in the domestic cat population and identified in seven cats, two as a homozygote and five as a heterozygote. The only two homozygous cats were the two pedigree Abyssinians, and five heterozygous cats were identified, comprising the two known heterozygotes (Cali and Monkey; Fig. 1g,h), Nina (Fig. 1f), CVB15215 and Cinnamon.

After applying filter 7, which meant two ticked cats had to be heterozygous or homozygous for the variants, one ticked Oriental cat (Art Deco; Fig. 1e) did not have the p.Cys63Tyr variant, suggesting a misidentification of this cat or possible heterogeneity. However, an obtained photograph confirmed the phenotype. As identified in filter step 4, this cat had a second, slightly more common missense variant in *DKK4*, a p.Ala18Val that was also identified in Cinnamon and consistent with Cinnamon being a compound heterozygote for two *DKK4* variants (see the discussion below). This variant was identified in 15 cats, 11 as a homozygote and four as a heterozygote.

Considering the second strategy B and filter 2 (File S3a,b), when Cinnamon must be heterozygous or homozygous for the reference allele, 10 590 variants remained for consideration. Filter 3 (tabby breeds ($n = 9$) as homozygous variant) reduced the candidates to 44 variants. Filter 4 set the zygosity for the 40 known tabby cats to a homozygous variant, which reduced the possible number variants to five. Considering the three Abyssinian cats as heterozygous or homozygous for the reference allele (filter 5), no variants remained, except for intergenic and intronic. No structural variants were identified that were specific to the two Abyssinians but absent from the other subset of 52 non-ticked cats (File S4).

DKK4 had 51 variants within the 99 Lives dataset (File S5). However, 30 variants had an allele count of three or less and had poor genotyping qualities, and thus, were not considered valid variants. Four *DKK4* variants were synonymous changes and were not further considered. Five additional missense variants were present (p.Ile86Met, p.Arg71Lys, p.Lys132Arg, p.Ille201Thr and p.Ser208Gly), which were excluded as candidates along with the additional variants as they were present in several tabby cats. The p.Cys63Tyr variant was concordant in all ticked cats,

Table 2 Variant summary of the *Ticked* linked region (B1: 35–65 Mb) for 195 cats from the 99 Lives dataset.

Strategy		Number of variants												
		A							B					
Variant type	None	1[1]	2	3	4	5	6	7[2]	2	3	4	5	6	7[2]
3′ UTR	4919	4919	190	21	4	0	0	0	4880	24	3	0	0	0
5′ UTR start gain	55	55	1	0	0	0	0	0	55	0	0	0	0	0
5′ UTR	1650	1650	79	6	1	0	0	0	1641	6	0	0	0	0
Disruptive inframe deletion	2	2	0	0	0	0	0	0	2	0	0	0	0	0
Disruptive inframe insertion	6	6	0	0	0	0	0	0	6	0	0	0	0	0
Downstream gene	13	13	4	0	0	0	0	0	13	0	0	0	0	0
Frameshift	152	152	9	2	0	0	0	0	139	0	0	0	0	0
Inframe deletion	20	20	0	0	0	0	0	0	20	0	0	0	0	0
Inframe insertion	32	32	3	0	0	0	0	0	32	0	0	0	0	0
Initiator codon	4	4	0	0	0	0	0	0	4	0	0	0	0	0
Intergenic	477 950	–	26 001	375	0	9	9	9	467 917	3226	581	71	71	48
Intron	404 352	–	19 314	468	0	32	31	31	396 052	2013	218	38	31	20
Missense	1813	1813	18	2	3	1	1	1	1810	5	1	0	0	0
Non-coding exon	1308	1308	30	0	0	0	0	0	1303	5	1	0	0	0
Splice acceptor	24	24	1	0	0	0	0	0	23	0	0	0	0	0
Splice donor	22	22	1	0	0	0	0	0	21	0	0	0	0	0
Splice region	618	618	35	0	0	0	0	0	605	4	0	0	0	0
Stop gained	31	31	1	0	0	0	0	0	30	0	0	0	0	0
Stop lost	4	4	0	0	0	0	0	0	4	0	0	0	0	0
Stop retained	2	2	0	0	0	0	0	0	2	0	0	0	0	0
Synonymous	4395	–	42	0	0	0	0	0	4395	7	0	0	0	0
Total B1: 35–65 Mb	897 372	10 675	45 729	4138	750	41	40	40.0	878 954	5290	804	109	102	68.0

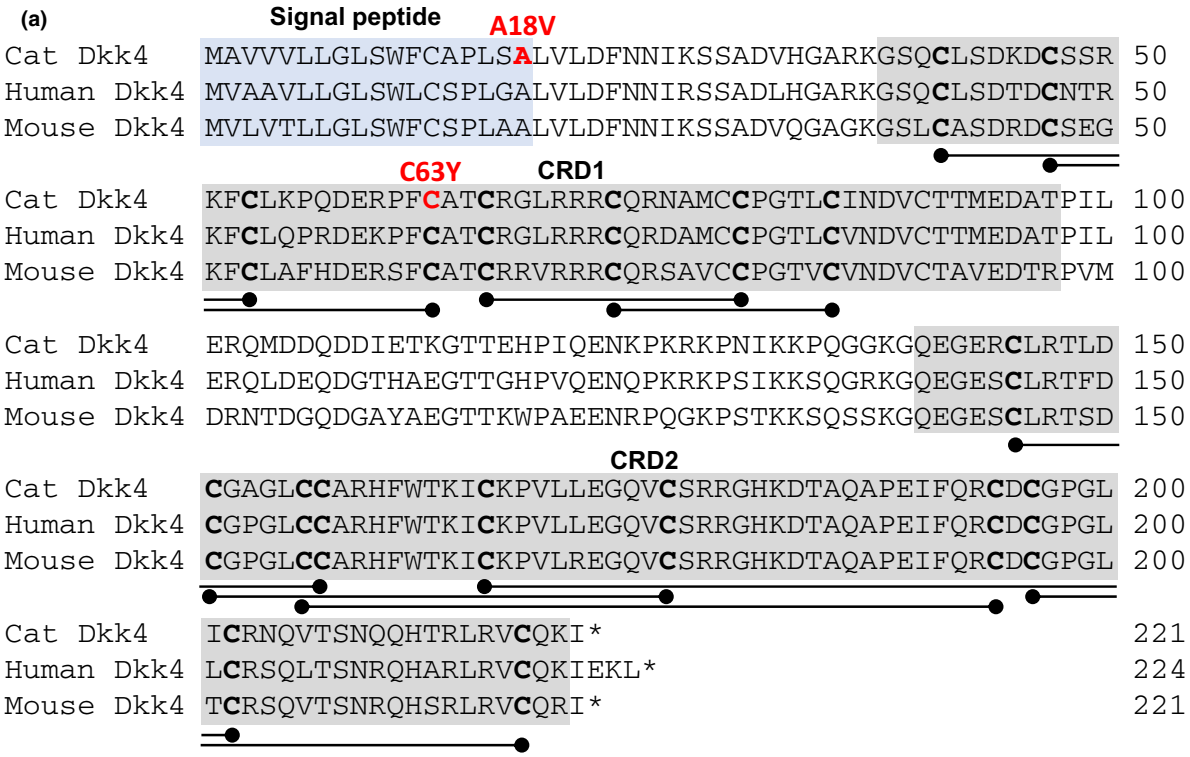
[1]Filter 1 eliminated intergenic, intronic and synonymous variants, thus the values were the same for strategies A and B. However, for this table only, filters 2–7 include all variants.
[2]Additional of the ticked cat Art Deco eliminates all variants in strategies A and B for filter 7.

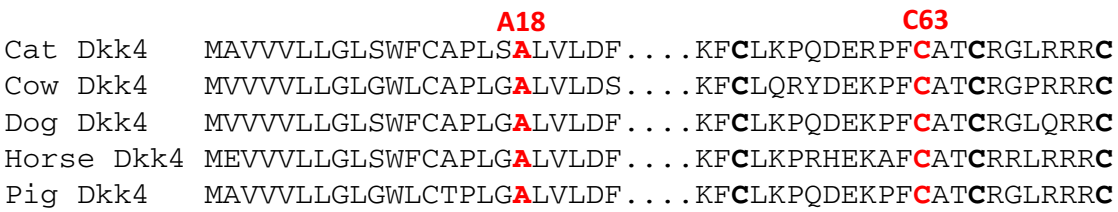
except for the ticked Oriental shorthair cat (Art Deco; Fig. 1e) and not identified in any other cats.

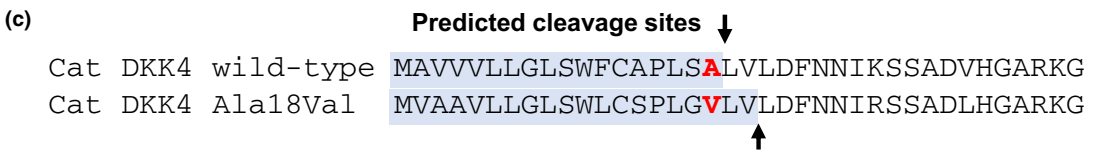
The p.Ala18Val variant had a low allele count in the data and was identified as heterozygous in four cats and homozygous in 11 cats. The two obligate heterozygous cats (Cali and Monkey; Fig. 1g,h) both excluded this variant as these cats were homozygous for the reference allele. However, if a second allele accounts for ticking, Art Deco (Fig. 1e) was heterozygous for the p.Ala18Val variant, as was one Burmese from Australia and Gizmo, a Maine coon cat from Cornell, which was listed with a coloration of black, smoke and white but was not a solid cat as determined by the *ASIP* data. This coloration was reported in the patient information and no image was available. Eleven cats were homozygous for the p.Ala18Val allele (B1:42620835; ENSFCAT00000034752:c.53C>T; XM_023252567.1:c53C>T), all Burmese from Australia.

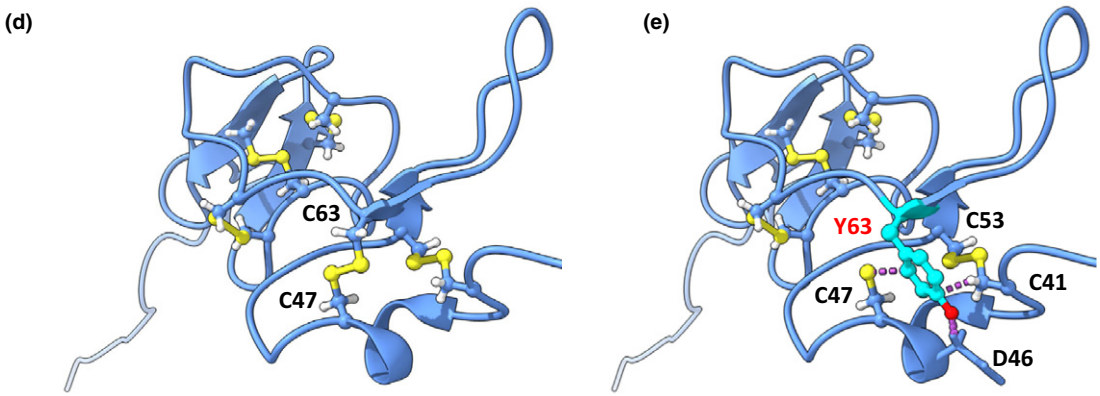
Further bioinformatic analysis of the *DKK4* variants suggest these missense changes are likely to influence the function of Dkk4. Dkk4 is secreted Wnt antagonist, and the precursor protein consists of an *N*-terminal signal peptide (amino acids 1–19) that consists of two independent folded cysteine-rich domains (CRD1 and CRD2) joined by a highly flexible, non-structured linker (Patel *et al*. 2018; Fig. 2a). Both Ala18 and Cys63 are highly conserved residues in mammalian Dkk4 sequences (Fig. 2a,b). Using the SignalP 5.0 server (Almagro Armenteros et al. 2019), the p.Ala18-Val variant is suggested to influence predicted signal peptide cleavage (Fig. 2c). The wt Dkk4 is normally cleaved between amino acids 18 and 19: LSA-LV with a probability of 0.6007. However, for Dkk4 p.Ala18Val, cleavage was predicted between amino acids 20 and 21, VLV-LD, with a much lower probability of 0.3275. Using the structure of the Dkk4 *N*-terminal cysteine-rich domain (CRD1;

Figure 2 Bioinformatic analysis of *DKK4* variants. (a) Alignment of cat, mouse and human Dkk4 proteins showing conservation of the *N*-terminal signal peptide (blue shading) and cleavage site and cysteine-rich domains CRD1 and CRD2. Key disulfide bonds in CRD1 and CRD2 are indicated. (b) Ala18 and Cys63 are highly conserved residues in key mammalian Dkk4 sequences. (c) Signal peptide cleavage analyzed using the SignalP 5.0 server (Lomax & Robinson, 1988). The wt Dkk4 is typically cleaved between amino acids 18 and 19 with a probability of 0.6007. For the Dkk4 variant p.Ala18Val, signal peptide cleavage was predicted between amino acids 20 and 21 with a lower probability of 0.3275. (d) Structural analysis reveals that the Dkk4 p.Cys63Tyr variant is predicted to disrupt a key disulfide bond between amino acids Cys47 and Cys63, one of five disulfide bonds in CRD1 that play a major role in stabilizing the Dkk4 tertiary structure. (e) Substitution of p.Cys63Tyr also results in clashes with residues Cys41, Asp46 and Cys47. [Correction added 7 April 2021, after first online publication: The figure 2 has been updated in this version.]









Montague *et al.* 2014), the p.Cys63Tyr variant was predicted to disrupt a key disulfide bond between amino acids Cys47 and Cys63 (Fig. 2a,d), one of five disulfide bonds in CRD1 that play a major role in stabilizing the Dkk4 tertiary structure (Patel *et al.* 2018). The introduction of a tyrosine at position 63 also results in predicted clashes with residues Cys41, Cys47 and Asp46 (Fig. 2e).

Discussion

At least two different loci have been associated with coat patterning in domestic cats, *Tabby* and *Ticked* (Eizirik *et al.* 2010; Kaelin *et al.* 2012; Lyons *et al.* 2006). Only the gene and variants for the *Tabby* locus on cat chromosome A1 have been identified and the *Tabby* gene, *LVRN*, is suggested to have interactions with other genes, such as *endothelin 3* (*EDN3*), for influencing pattern (Kaelin *et al.* 2012). However, *Ticked* (previously known as *Tabby*), was the first locus to be regionally positioned to the centromeric region of cat chromosome B1q (Lyons *et al.* 2006). The ticked allele, formerly known as tabby – Aby (T^a), presents as a cat devoid of patterning, implying no stripes, blotches, swirls or spots – no tabby markings within the cat coat coloration (Fig. 1b). This allele is dominant, suppressing patterning, and hence epistatic to the *LVRN Tabby* locus, which controls the type of pattern, including mackerel (stripes) or classic (blotched) pattern (Fig. 1a,c). The control of spotted patterns common to the Egyptian mau (Fig. 1d) and ocicat breeds is probably influenced by yet unidentified loci (Eizirik *et al.* 2010; Kaelin *et al.* 2012). *Ticked* is a rare phenotype and is infrequent in random-bred cats and other breeds; however, the phenotype is fixed in the Abyssinian and Singapura cat breeds. However, breeds that have temperature-sensitive variants, such as the tyrosinase (*TYR*) variants associated with Siamese, Burmese and related breeds, could be ticked tabbies (Lyons *et al.* 2005). 'Ghost' or bleed-through tabby patterns can appear in the coats of cats with the *TYR* variants as they age, which is an undesirable presentation for these breeds with temperature-sensitive coat colorations.

The development of the 99 Lives cat genome sequencing database and improved assembly and annotation of the cat have led to vastly improved variation identification for cats and the ability to define candidate genes and variants based on cat phenotypes (Buckley *et al.* 2020a; Buckley *et al.* 2020b; Cogne *et al.* 2020; Jaffey *et al.* 2019; Mauler *et al.* 2017; Pettersen *et al.* 2021; Yu *et al.* 2020). As the cat used for the reference genome has the ticked phenotype of interest, and because this variant is autosomal dominant and a ticked cat, such as the reference cat, can be heterozygous, the wt allele (non-ticked) may or may not be the reference allele in the cat genome assembly. Therefore, two different strategies were necessary to identify candidate variants in which strategy A was successful. For strategy A, Cinnamon was considered heterozygous or homozygous for the variant allele; all variants in the region, including intergenic and intronic, were eliminated as candidates except for a p.Cys63Tyr variant in *DKK4*. This variant was at position B1:41621481, near the centromere and at an extreme end of the linked region in the linkage analyses. The allele count suggested that no other cats in the 99 Lives dataset, including 127 solid-coloration cats and 79 cats with unknown phenotypes, have a ticked phenotype. Forty intergenic and intronic variants were not excluded. Considering variant discovery strategy B, Cinnamon was considered as heterozygous or homozygous for the reference allele. All variants in the region, except approximately 68 intergenic and intronic, were eliminated as candidates. In addition, a previous study examined the structural variants of a 54-cat subset of the current WGS dataset and included two Abyssinian cats. No structural variants were identified that were unique to the two Abyssinian cats and absent from the 52 non-ticked cats; therefore, further structural variant analyses were not performed.

One cat, a ticked Oriental shorthair (Art Deco; Fig. 1e), excluded the p.Cys63Tyr as causal for all ticked phenotypes. This cat, as well as the reference cat, Cinnamon, was heterozygous for a different allele, the p.Ala18Val variant, which also had a low allele count and was identified as heterozygous in four other cats and homozygous in 11 cats. However, the two obligate heterozygous cats both excluded this variant as explaining all cases of ticked coats as these cats were homozygous for the reference allele at the same genomic position. If a second allele accounts for ticking in cats, Cinnamon was a compound heterozygous for the p.Cys63Tyr/p.Ala18Val variants. A previous study on hairlessness in the lykoi cat breed described a similar experience with new phenotypes presenting as compound heterozygotes (Buckley *et al.* 2020b). One Burmese from Australia and a cat from Cornell (Gizmo), which is listed with a coloration of black, smoke and white, were also heterozygous for the p.Ala18Val allele and thus should be ticked. A black smoke cat suggests a solid-colored cat with at least one copy of the *Inhibitor* allele (*I*). The *ASIP* data suggested that this cat was not solid-colored, and perhaps the amount of white on it obscured the tabby markings. Eleven cats were homozygous for the p.Ala18Val allele, which were all Burmese from Australia, but as these cats were solid, the ticked phenotype could not be determined.

The breeding colony that produced the reference cat, Cinnamon, was established in Sweden (~1983) and several cats were moved to the University of Missouri – Columbia in 2001 to re-establish the colony (Narfstrom 1983; Menotti-Raymond *et al.* 2010). Outcrossed, natural matings with European short-haired cats were performed to increase the heterozygosity in the colony, and 55 backcrossed cats were generated as part of the project to map the *Ticked* and *Tabby* loci. However, overall, Cinnamon was selected as the reference cat from over 1000 genotyped cats because she

was highly inbred, leaning towards having minimal allelic variation (Pontius *et al.* 2007). Alternatively, Abyssinians and other cats may have more than one *Ticked* allele; thus, even if they are highly inbred, they could be segregating for different alleles that produce the same phenotype. In addition, many cat breeds with temperature-sensitive, as well as solid but dilute, colorations, such as Siamese, Burmese and blue cats (korats, chartreux and Russian blue), and cats with *Orange* coloration, may also be *Ticked* because 'ghost patterning' can bleed through in these coat colorations when the cat is young or as it ages, which is not ideal for many cat breed competitions. Thus, breeders may select for the *Ticked* allele in these breeds, even though the cats are solid in coloration and patterning is not of interest. The allele count suggested that several Burmese cats in the 99 Lives dataset do not have the p.Cys63Tyr allele but instead the p.Ala18Val *Ticked* allelic variant.

DKK4 is a member of the dickkopf (Dkk) family of cysteine-rich secretory proteins that are antagonists of Wnt signaling pathways, involved in antero-posterior axial patterning, limb development, somitogenesis and eye formation (Niehrs 2006). Four Dkk proteins exist in humans (Dkk1-4) and all contain two CRDs, each of which contains five disulfide bonds. Dkk1, Dkk2, and Dkk4 inhibit Wnt signaling by binding to the Wnt co-receptors LRP5/6 via the CRD2 domain. In humans, *DKK4* is localized to 8p11.21, which is near the centromere but on the short arm of the chromosome, suggesting a re-positioning of the cat centromere. The Dkk4 p.Cys63Tyr and p.Ala18Val alleles are biologically plausible candidates for influencing the *Ticked* phenotype in domestic cats. The p.Ala18Val is predicted to interfere with correct signal peptide cleavage, which in turn could affect the efficient secretion of Dkk4. In contrast, p.Cys63Tyr disrupts a key disulfide bond responsible for stabilizing the tertiary structure of Dkk4. Interestingly, Dkk4 expression has also been previously implicated in hair follicle spacing and density (Sick *et al.* 2006), acting as an inhibitor of primary hair placode formation (Cui *et al.* 2010). Taken together, this provides a plausible biological link between *DKK4* dysfunction and the *Ticked* phenotype.

Supportive data has also been suggested in a detailed recent study by Kaelin *et al.* (2020). This study combines variant analyses from the published 99 Lives sequence data with an elegant investigation using single-cell gene expression analysis in fetal skin of domestic cats to define the location and timing of pattern development in felids. Association analysis of additional DNA samples across ($n = 115$) and within breeds ($n = 238$) provides further support that variation in *Dkk4* causes *Ticked*.

Phenotypic and genotypic data from the 99 Lives cat genome sequencing project has supported the identification of candidate variants for the *Ticked* phenotype in domestic cat breeds. Additional genotyping of the proposed variants, in a large cohort of phenotyped cats, as well as supportive functional data, would clarify the role of these variants in cat coat pattern development. The identified variants do not clarify the pathways leading to the production of the spotted coat phenotype in cats, suggesting that additional genes influence other tabby patterns in domestic cats. The allelic series for the *Ticked* locus is suggested as $Ti^A = Ti^{CK} > Ti^+$, where the Ti^A allele represents the p.Cys63Tyr variant and the Ti^{CK} allele represents the p.Ala18Val variant.

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Conflict of interest

The authors disclose there are no conflicts of interest. The funders had no role in study design, data collection, data analysis, interpretation of results or decision to publish.

Author contributions

Conceptualization, L.A.L.; methodology, R.J.H., L.A.L.; software, R.M.B.; validation, R.M.B., L.A.L.; formal analysis, R.J.H., L.A.L.; resources, L.A.L.; data curation, R.M.B., R.J.H., L.A.L.; writing – original draft preparation, L.A.L.; writing – review and editing; R.M.B., R.J.H., L.A.L.; 99 Lives Consortium; supervision, L.A.L.; project administration, L.A.L.; funding acquisition, L.A.L. All authors have read and agreed to publish this manuscript.

Data availability statement

Genome sequence and signalment for the 195 cats are available in the NCBI SRA (www.ncbi.nlm.nih.gov) and accession nos are provided in File S1. All supplementary files have been uploaded to a figshare data sharing site: https://doi.org/10.6084/m9.figshare.13611188.

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1 Appendix

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

File S1 99 Lives Cat Genome Sequencing dataset signalment, accessions and contacts.

File S2 (a) All variants in B1: 35–65 Mb for association with *Ticked*. (b) Filtering of variants in B1: 35–65 Mb for association with *Ticked* – strategy A.

File S3 (a) Filtering of variants in B1: 35–65 Mb for association with *Ticked* – strategy B. (b) All variants in B1: 35–65 Mb for association with *Ticked* – strategy B (filter 2 only).

File S4 Structural variants in B1: 35–65 Mb for association with *Ticked*.

File S5 *DKK4* variants in the 195 cat 99 Lives dataset.

Table S1 Cat assembly V9.0 locations of the previously linked STRs associated with the *Ticked* locus[1].