Investigating *Taqpep* Expression in Tabby Cats

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May 2023

Summary

Coat color patterning in mammals is a fascinating developmental process whose molecular underpinnings are largely unexplored. Recently, mutations in *Transmembrane aminopeptidase Q* (*Taqpep*) were found in domestic cats (*Felis catus*) that change the regularly spaced dark stripes observed in Mackerel tabby cats to the whorled pattern observed in Blotched cats. With the knowledge that *Taqpep* acts during embryogenesis to shape the pigmentation pattern, we examined the temporal and spatial distribution of *Taqpep* expression in 18-25 day-post-conception (dpc) domestic cat embryos (Stage 12-15) using Advanced Cell Diagnostics' (ACD) RNAScope® technology and *Taqpep*-specific probes. Although we had originally hypothesized *Taqpep* expression to be concentrated in epidermal cells or in dermal cells that were close to the epidermis with pattern establishment believed to be happening in the epidermis, we found that *Taqpep* is to be first expressed at day 19 dpc (Stage 13) in deep dermal cells, a distance from the pattern-expressing cells in the epidermis that are marked by *Dickkopf 4* (*Dkk4*). Unpatterned *Taqpep* expression is maintained in the deep dermis at all embryonic stages examined. This study is the first of its kind to visualize *Taqpep* expression in cat embryos, and expands our understanding of the cell populations that contribute to mammalian patterning and highlights that communication among cell populations is essential in shaping the pattern.

Introduction

Periodic patterns and their development have been a subject of great interest to genetic and evolutionary biologists for several decades. From digit formation to the creation of intricate coat patterns, the mechanism of action for such patterning remains largely unknown (Miura et al., 2006, Sick et al., 2006). Some of the most prominent and visible examples of such patterning are found in various species of felids and equids, such as cheetahs and zebras, which creates ethical

and accessibility complications as models for biological investigation (Kaelin et al., 2021, Kaelin et al., 2013, Kaelin et al., 2010). Such insight from felids in particular is very pertinent to biologists as it has the potential to explain certain mechanisms at play during human development as well (Economou et al., 2012).

Domestic cats, however, present us with a unique opportunity for research as a model organism. Trap-neuter-release veterinary clinics across the country work to spay and neuter feral cats, whose populations have been on the rise and have become pervasive and harmful to humans (Wallace et al., 2006). After treatment and vaccination, cats are released back into their environment. These clinics manage the feral cat population without having to resort to euthanasia (Wallace et al., 2006). During spaying, embryos from incidentally pregnant feral cats are typically discarded. During the Spring (February to May), the percentage of incidentally pregnant feral cats can be upwards of 30% (Kaelin et al., 2021). By working in partnership with these clinics, this otherwise discarded tissue can be processed and preserved for the purposes of scientific research.

Using this valuable resource, some research has already been done that has greatly advanced our understanding of patterning. Mammalian patterning occurs through three sequential stages: the *establishment* of a pre-pattern during development, the *implementation* of this pattern in individual hair follicles, and the *maintenance* of this pattern in the adult animal (Kaelin et al., 2021). Much of the work discussed in Kaelin et al. concerns the first of these phases, pre-patterning. Pattern establishment occurs before the hair follicles have formed and before pigment cells (melanocytes) migrate to the skin, establishing a "molecular" pre-pattern. This molecular pre-pattern cannot be visualized without techniques for detecting gene expression (Kaelin et al., 2021).

Previous work has identified *Transmembrane aminopeptidase Q* (*Taqpep*), formerly known as *laeverin* or *aminopeptidase Q*, as a gene involved in patterning in domestic cats (*Felis catus*) (Kaelin et al., 2012). This was determined as mutant cats with a homozygous recessive *Taqpep* mutation (Ta^b/Ta^b) show the Blotched patterning of whorls of black and yellow coat color while the wildtype with at least one dominant *Taqpep* allele (Ta^M/Ta^-) displayed the typical regular black and yellow striping characteristic of the Mackerel phenotype (see Fig. 1.a) (Kaelin et al., 2021). Taqpep, was originally identified in the human placenta in human extravillous trophoblasts (EVTs) which play an important role in embryo implantation (Fujiwara et al., 2003). Although it had been suggested that Taqpep might be involved in preeclampsia (PE), a dangerous gestational hypertension disorder, because of its role in EVT invasion (Horie et al., 2012), other studies have found that Taqpep levels showed no correlation with PE in the first trimester (Pihl et al., 2018). Furthermore, *Taqpep* knockout in mice showed no significant changes in embryo implantation and as a single factor are not responsible for PE in mice (Tobita et al., 2019). Taqpep is a transmembrane aminopeptidase (Fujiwara et al., 2003) but the exact

functioning and its particular mechanism of action in patterning is still unknown. Some insight came from recent work on embryonic cats which showed that *Taqpep* mutations change the molecular pre-pattern from regularly spaced stripes to a blotched and whorled pattern that is similar to the Mackerel and Blotched phenotype that develops for adult cats, respectively (Fig. 1.a) (Kaelin et al., 2021). This study also identified epidermal *Dickkopf 4* (*Dkk4*) mRNA expression as a marker of the pre-pattern: *Dkk4* is differentially expressed in the two epidermal cell populations that give rise to the molecular pre-pattern. (Fig.1.b) (Kaelin et al., 2021). This previous work suggests that *Taqpep* acts during embryonic development around Stage 15 (see Table 1), which is roughly 23-25 dpc (Kaelin et al., 2021).

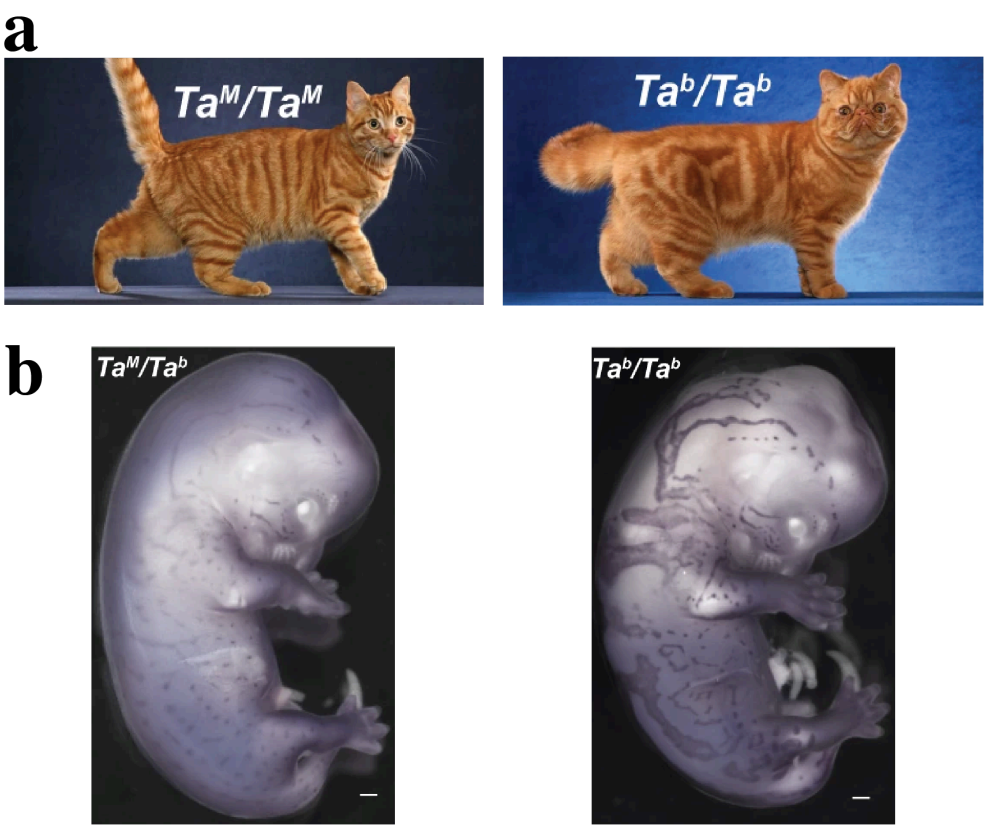
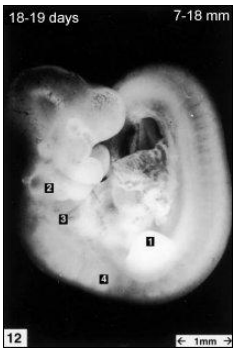
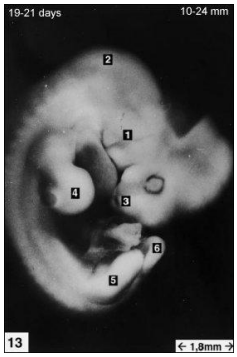
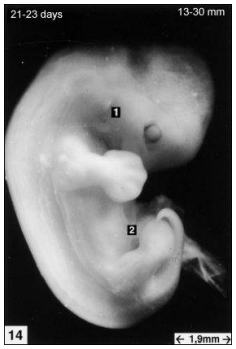
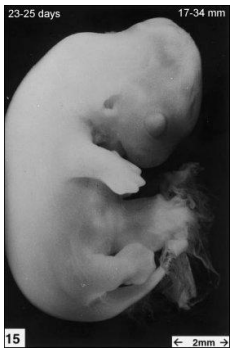


Figure 1: Images of color patterning and *Dkk4* expression for Ta^M/Ta^- and Ta^b/Ta^b cats. *(a) Representative image of coat markings for Mackerel (Ta^M/Ta^M) and Blotched (Ta^b/Ta^b) adult domestic cats; (b) Dkk4, a marker of the embryonic pre-pattern, expression (purple) in Stage 16 Mackerel (Ta^M/Ta^b) and Blotched (Ta^b/Ta^b) whole embryos (Kaelin et al., 2021).*

To explore how *Taqpep* influences the embryonic pre-pattern and the eventual pigmentation pattern we performed *in situ* hybridization on tabby cat embryo samples ranging from Stage 12 to Stage 15 (see Table 1), the purpose being to better understand when and where *Taqpep* is expressed in these early stages of development. Understanding *Taqpep* expression qualitatively

may help shed light on the mechanism by which *Taqpep* influences pre-patterning as well as its potential involvement in other developmental processes.

Table 1: Stages of Domestic Cat Embryology during which *Taqpep* is Expressed

Stage #	Stage 12	Stage 13	Stage 14	Stage 15
Image				
Trimester	1st	1st	2nd	2nd
Days of Gestation	18-19	19-21	21-23	23-25
Crown-rump Length	7-18mm	10-24mm	13-20mm	17-34mm
Development	- forelegs and hind legs develop - cerebrum and brainstem in basic form	- front gill arch deepens - duct for olfactory organ deepens - cerebellum divided by a groove - forelegs divided -tail lengthens - veins differentiate - esophagus, stomach, intestines, pancreas, thyroid gland, kidney and liver develop - vertebrae formed	- upper lip, eyelids, earlobes, toes, and tongue form - genitalia further developed - skeleton and muscles differentiate further - thyroid gland, parathyroid gland, heart, and thymus develop	- toes of foreleg separate - kidneys, adrenal glands, and genitalia further differentiate - gray and white batter of of spinal cord separate - axons and ganglia differentiate - jaws, palate, and salivary glands develop - pituitary gland forms

(Knospe, 2002)

Results

Dermal *Taqpep* Staining of Embryos at Various Stages

To better understand when and where *Taqpep* is expressed during development, embryo samples from various developmental stages (12-15) were evaluated by *in situ* hybridization (Table 2) using ACD's RNAScope® technology. Samples at or before Stage 15 were evaluated as previous work showed a marked difference between the pre-pattern of *Taqpep* mutant and non-mutant embryos at this stage. (Kaelin et al., 2021). 100µm-serial sections were taken through the entire embryos at different developmental stages to provide a more complete understanding of the locations of *Taqpep* expression.

The earliest stage evaluated was Stage 12 (see Table 1) but no staining was observed throughout the sample.

The earliest stage in which *Taqpep*-positive cells were observed was Stage 13. In the 1.0 cm sample analyzed (So19F103F3), *Taqpep* appeared in low quantities in cells (within nuclei) beneath in the lower dermis (Fig. 2).

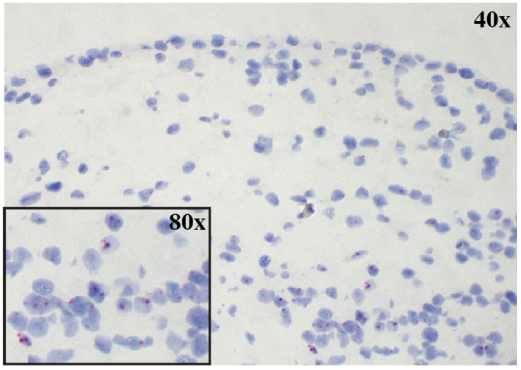


Figure 2: Dermal Staining of Stage 13, 1.0 cm embryo (So19F103F3). *Representative image of dermal staining observed across multiple sections of sample. Low levels of Taqpep expression (red) observed in the deep dermis. Inset is a high magnification image showing Taqpep-positive dermal cells (80x).*

Taqpep expression did not appear more prominently until embryos reached Stage 14. In addition to higher levels of *Taqpep* staining, the *Taqpep*-positive cells also appeared closer to (but still under) the upper dermal layer as seen in Figure 3.

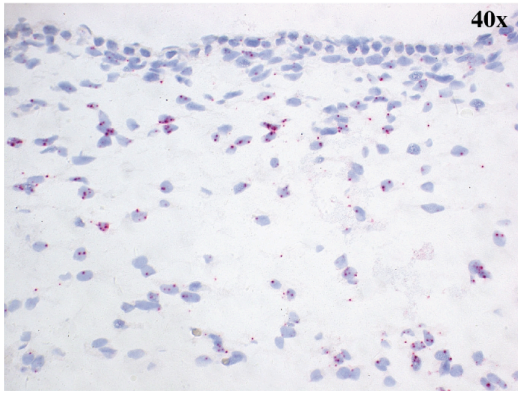


Figure 3: Dermal Staining of Stage 14, 1.4 cm (So19C105F2). *Representative image of dermal staining observed across multiple sections of sample. Higher levels of Taqpep expression (red) observed below the upper dermal layer but closer than in Stage 13.*

By Stage 15, there was a considerable increase in cell density generally and a prominent layer of subcutaneous muscle (Fig. 4.a). There was also more *Taqpep* staining than at Stage 14, and the clustering of staining was noticeably deeper in the dermis than it was at Stage 14 (Fig. 4.a, b).

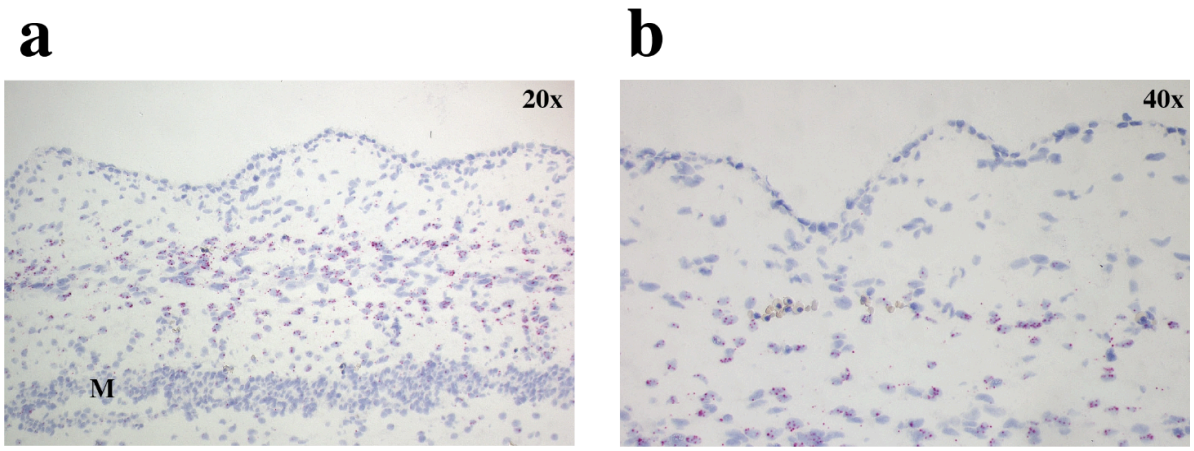


Figure 4: Dermal Staining of Stage 15, 1.6 cm embryo (So19C16F4). *Representative image of dermal staining observed across multiple sections of sample; higher levels of Taqpep expression (red) observed below the upper dermal layer, further down than Stage 14; (a) magnification of 20x, the M indicates the developing muscle layer; (b) 40x image .*

Double Stain of *Taqpep* and Pattern Element (*Dkk4*)

To examine whether *Taqpep* expression is patterned, we performed a double staining for *Taqpep* and *Dickkopf 4* (*Dkk4*) using ACD's Duplex RNAScope® in situ hybridization technology. We chose *Dkk4* as a marker of the epidermal pre-pattern (Kaelin, 2021). Observationally, there was no difference in *Taqpep* expression below *Dkk4*-positive and *Dkk4*-negative epidermal regions (Fig. 5.a, b, c). To determine if *Taqpep* expression was patterned (relative to the epidermal pre-pattern), we counted the number of *Taqpep*-positive cells below *Dkk4*-positive and adjacent *Dkk4*-negative epidermal regions from eight unique anatomic locations around the Stage 15 embryo (Fig. 5b, c). While the number of *Taqpep*-positive cells varied across the different anatomic locations, we did not observe a difference in the number of *Taqpep*-positive cells below *Dkk4*-positive regions and adjacent *Dkk4*-negative regions (Fig. 5d, paired Student t-test, $p=0.32309$, $n=8$ anatomic locations from one Stage 15, 1.8cm embryo). In fact, the number of *Taqpep*-positive cells below *Dkk4*-positive and *Dkk4*-negative regions from a single anatomic location was highly correlated (Fig, 5d, $R^2=0.9579$).

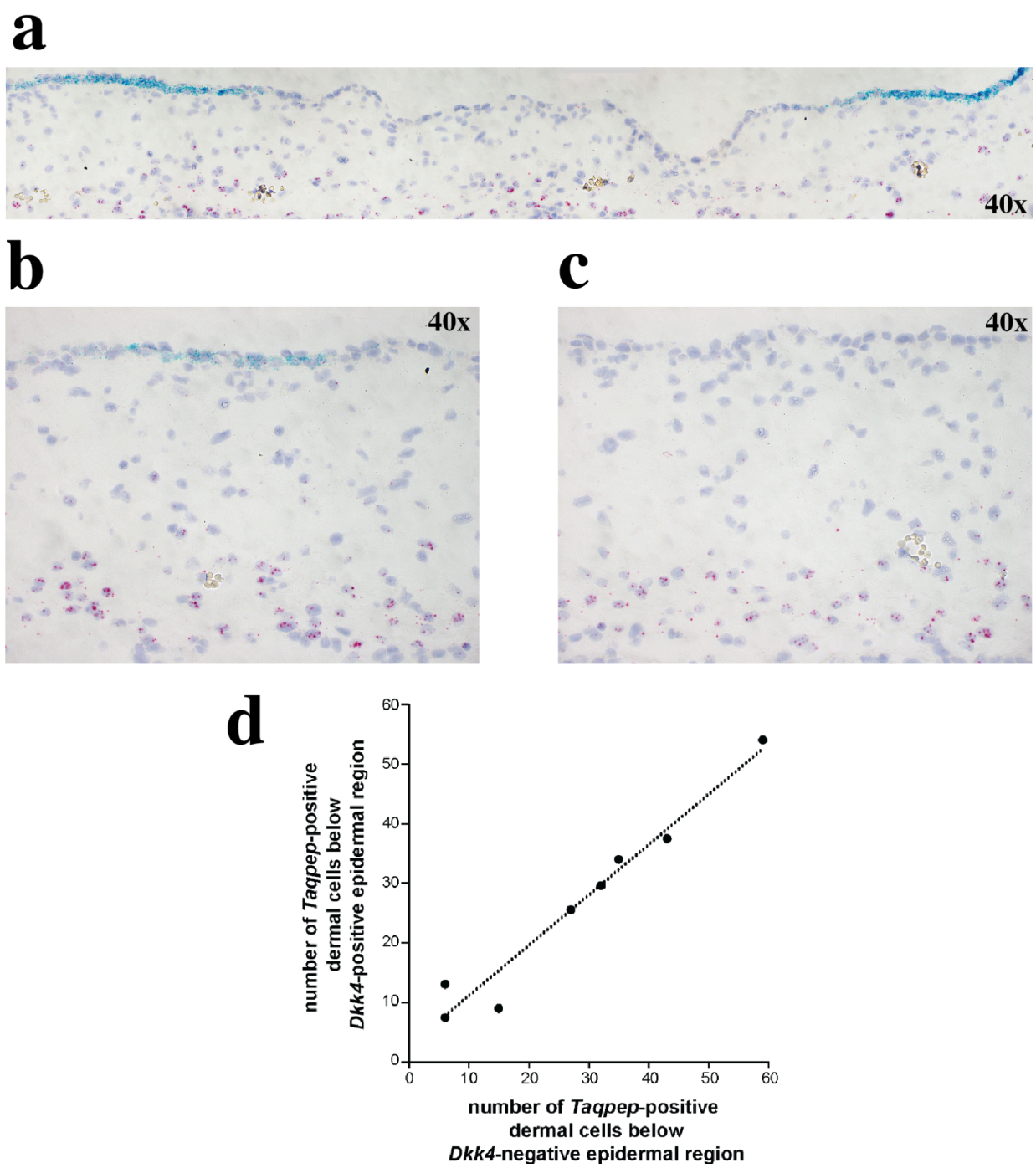


Figure 5: Double Staining of Stage 15, 1.8 cm embryo (So21C4F2). *(a) Tiled image of adjacent Dkk4-positive (blue) and negative epidermal regions showing Taqpep expression (red) in the dermal layer; (b) sample Dkk4-positive region used for quantitative analysis; (c) sample Dkk4-negative region used for quantitative analysis, adjacent to Dkk4-positive region (b); (d) the number of Taqpep-positive dermal cells does not differ between Dkk4-positive and Dkk4-negative epidermal regions (R^2=0.9579, paired Student t-test, p=0.32309, N=8 anatomic locations).*

Extracutaneous Staining

Our experiments identified *Taqpep* expression in a number of extracutaneous locations. At Stage 15, we noted *Taqpep* expression around the developing neural tube (Fig. 6.a). Similarly at an earlier point in Stage 13, *Taqpep* was observed in neural crest cells (Fig. 6.b). All the samples analyzed (except for Stage 12), staining was found in the mesonephros, developmental areas that give rise to the embryonic kidney (Fig. 6.c and Fig. 6.d.). Another region where *Taqpep* was found was the intercostal tissue surrounding the developing ribs (Fig 6.e).

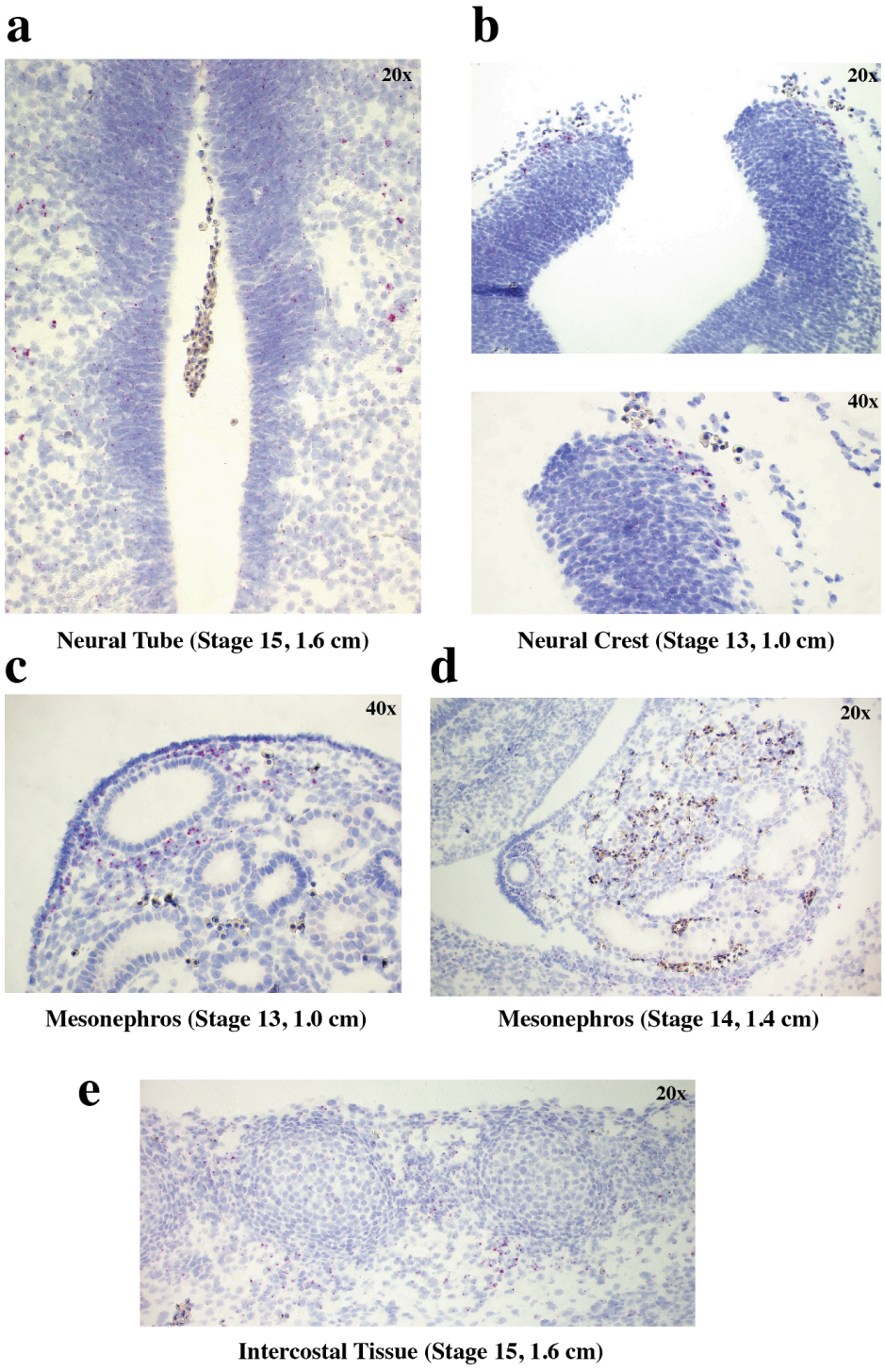


Figure 6: Images of extracutaneous staining. *Observed areas of additional Taqpep expression besides dermal tissue; (a) some Taqpep staining in cells surrounding neural tube of Stage 15, 1.6 cm embryo (So19C16F4); (b) staining of neural crest cells of Stage 13, 1.0 cm embryo (So19F103F3) at magnifications of 20x and 40x; staining around the mesonephric duct; (c) Stage 13, 1.0 cm embryo (So19F103F3) and (d) Stage 14, 1.4 cm (So19C105F2); (e) staining of intercostal tissue of Stage 15, 1.6 cm embryo (So19C16F4).*

Discussion

In this study, we sought to better understand where and when *Taqpep* is expressed in cat embryonic development and to examine how this expression might point towards the mechanism by which *Taqpep* influences coat color patterns in domestic cats.. To do this, we performed *in situ* hybridization on five different embryos ranging from Stage 12 to Stage 15 (see Table 2) using ACD's proprietary RNAScope® technology. Our results found that *Taqpep* was generally expressed in the deep dermis–a distance from the patterned epidermis–starting at Stage 13 (Fig. 2) but becoming more prominent and slightly closer to the surface by Stage 14 (Fig. 3). Stage 15 showed even greater levels of *Taqpep* expression but lower in the dermis and above the developing muscle layer at this stage (Fig. 4.a, b). Our double staining of *Taqpep* with *Dkk4*, a known marker of the embryonic pre-pattern, further suggested that *Taqpep* expression is not patterned in the same way that *Dkk4* is. Additionally, *Taqpep* was found in extracutaneous locations, including the neural tube, neural crest cells, mesonephros, and interstitial tissue (Fig. 5.a, b, c, d, e). The compilation of these findings advances our understanding of *Taqpep* expression and builds upon the previous work done by Kaelin et al. that had identified mutations in the *Taqpep* gene as factor responsible for changing the molecular pre-pattern from regularly spaced stripes of the Mackerel phenotype to the whorled pattern (Fig. 1.a, b).

The observation that *Taqpep* is expressed in the deep dermis is surprising. We expected *Taqpep* expression to be located close to the epidermis, the location of the *Dkk4*-expressing cells and the pre-pattern (Fig. 6.a). Deep dermal *Taqpep* expression underscores that communication between cutaneous cell populations is important in pattern establishment and brings forward new hypotheses to explain *Taqpep*'s influence on pattern formation. First, as a transmembrane aminopeptidase, Taqpep may be cleaved and diffuse to the epidermis, where it acts on the molecular pre-pattern (Fig. 6b). To assay if Taqpep is cleaved *in vivo*, we could use a Taqpep antibody and Western blotting on skin extracts to assay the size of Taqpep isoforms in the skin. In parallel, immunostaining with a Taqpep antibody would tell us if the protein diffuses away from the deep dermal cells, if it is located near the epidermal pre-pattern, and if its expression is patterned. Second, Taqpep may act on other dermal proteins that diffuse to the epidermis (Fig. 6c). Mass spectrometry and protein on skin from wild-type and *Taqpep*-mutant embryonic cat skin samples would give us a list of candidate proteins for further investigation.

Another possibility is that the *Taqpep* is involved in other time periods and anatomic locations that were not assayed in this study. Similarly, the cells currently expressing *Taqpep* at the stages analyzed may migrate to the epidermis at a later stage. This study has conveyed a great deal of new information regarding *Taqpep* and its location in tabby cat embryos from Stage 12 to Stage 15, and although there are many aspects still unknown, it has allowed us to begin to ask more directed questions to continue to learn more about this molecule and its action.

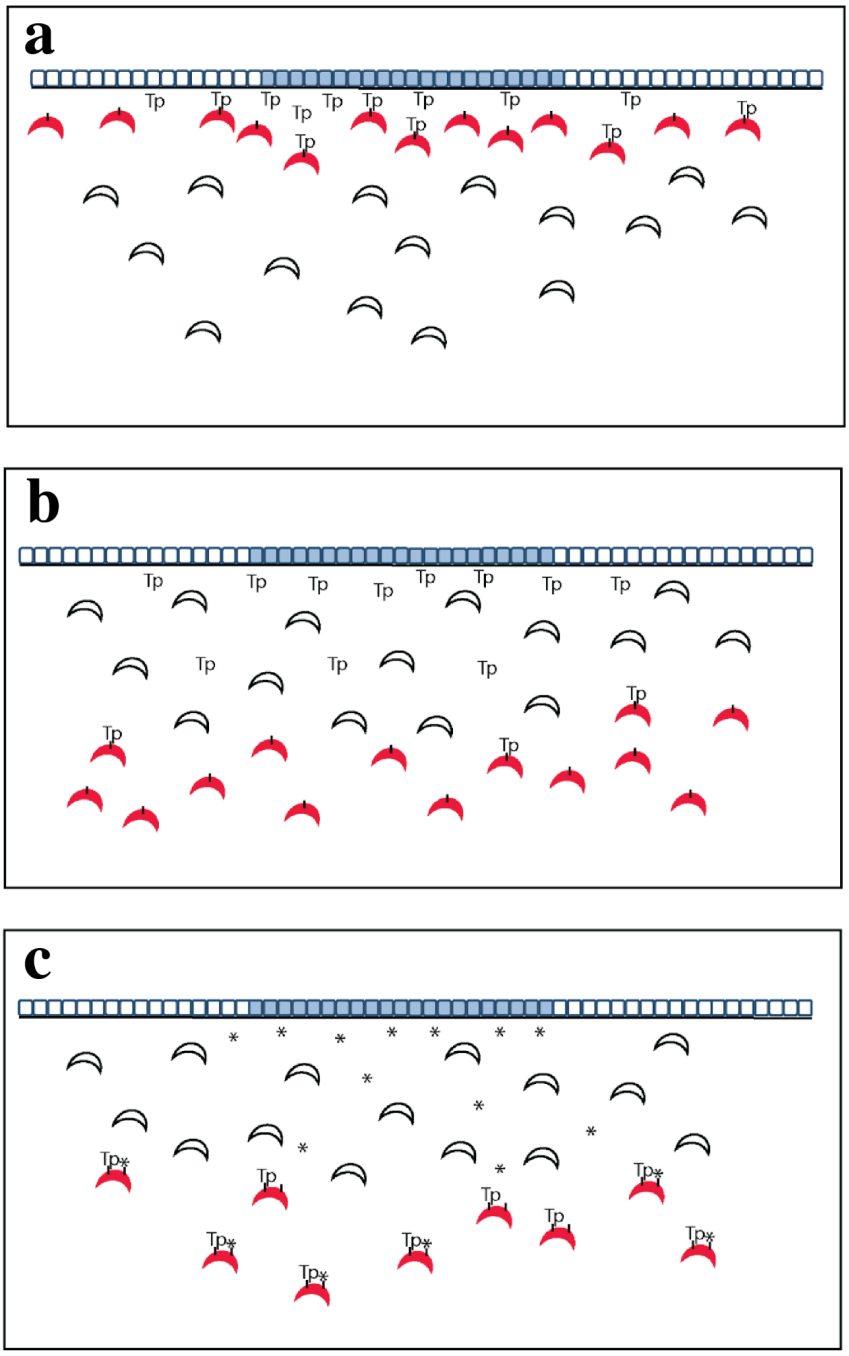


Figure 6: Possible Hypotheses for Taqpep's role in Patterning. *(a) Hypothesis prior to this study: Taqpep expression located in dermal cells adjacent to the epidermis; (b) alternative new hypothesis: Taqpep expression located deeper in the dermis, cleaved Taqpep proteins diffuse to epidermis; (c) alternative new hypothesis: Taqpep expression located deeper in the dermis, Taqpep proteins act on other dermal proteins that diffuse to epidermis. Dkk4-positive epidermal cells (blue squares), Dkk4-negative epidermal cells (white squares), Taqpep-positive dermal cells (red crescents), Taqpep-negative dermal cells (white crescents), Taqpep protein (Tp), dermal protein modified by Taqpep (*).*

Another notable finding from our study is that unlike *Dkk4*, *Taqpep* does not appear to be patterned which leads us to wonder how it might influence a pattern without being patterned itself. Additional analyses would confirm our observation. First, we could use a larger sample size of sets of *Dkk4*-positive and *Dkk4*-negative regions. Additionally, we could perform an analysis on the average amount of *Taqpep* expressed (red dots) per cell or on the total amount of *Taqpep* expressed (red dots) in a field of view (independent of the number of cells). We could also measure the distance between *Taqpep* expressing cells and the patterned epidermis. Finally, and most importantly, immunostaining with a Taqpep antibody could be used to explore Taqpep protein expression patterns.

A major strength of this study was its effective use of ACD's proprietary RNAScope® technology. In comparison to other traditional methods of *in situ* hybridization with radio- or DIG-labeled probes, ACD's method provides a much more sensitive and specific probe. This sensitivity, largely due to the multi-step system of amplifiers, is especially important for this experiment as *Taqpep* is generally expressed in low levels in the skin, which would make traditional in situ hybridizations difficult to visualize. ACD's version also has a great degree of specificity because of their proprietary system of two adjacent Z probes that must hybridize in tandem for amplification to occur. This specificity is also very important for *Taqpep* hybridization since *Taqpep* has many related genes that we would want to reduce non specific binding to. All that being said, the ACD's RNAScope® technology does have limitations. For our purposes, their method has not been applied to whole mount embryos, so our analysis with this technology is limited to an analysis of serial sections.

Although we were able to observe *Taqpep* expression for samples at Stage 13, 14, and 15, we failed to detect it at Stage 12 (So19C113F6, Table 2). This may have occurred for one of several reasons. One possibility is that there is no expression at Stage 12 which would be consistent with the low expression observed at Stage 13 (Fig. 2). But due to the fact that we only evaluated one sample at this stage, it may also be that the embryo used had not been fixed and processed properly. To further confirm our findings for this stage, the experiment would need to be repeated for other Stage 12 cat embryos.

This study has advanced our current understanding of *Taqpep* and has provided us with valuable new insight into the mechanism by which *Taqpep* influences the coat color patterns in felids. We found it to be located deeper in the dermis than had originally been expected, leading to a number of new hypotheses to how Taqpep might act. This work has provided us with some novel insight from which future studies can continue to build off of to better understand the genetic underpinnings of coat patternings and, more broadly, the mechanism by which developmental patterns emerge in different mammals. This work is an example of the potential for non-model organisms to elucidate insight into developmental and cellular processes, and highlights that collaboration with community organizations is an essential component of many scientific studies.

This partnership with the trap-neuter-release clinic, Forgotten Felines of Sonoma County, reminds us of the importance of tying scientific research to and effectively communicating what we learn to interested communities and the general public.

Experimental Procedures

Processing and Embedding Samples

For the purposes of this study, samples from Stages 12-15 were evaluated, as previous work has indicated that *Taqpep* is most likely expressed at or before Stage 16 since the pre-pattern of a wild type differs from that of a mutant *Taqpep* cat at Stage 15/16 (Kaelin et al., 2021). The stage number and specifications of the embryos evaluated in this experiment are listed in Table 2. Samples were collected from the Forgotten Felines of Sonoma County trap-neuter-release (TNR) clinic located in Santa Rosa, CA. Samples were dissected within 12 hours of the spaying procedure and a small piece of tail skin was used for *Taqpep* genotyping. All embryos were Ta^{M}/Ta^{-}. Embryos were fixed in 4% paraformaldehyde in PBS at 4℃ overnight. Serial coronal sections were taken from embedded embryos such that pattern elements–*Dkk4*-positive regions that will become black stripes in the adult–and background–*Dkk4*-negative regions that will become yellow background in the adult–could be viewed in the same section (Table 2).The Stage 12 embryo, 1.0 cm (So19C113F6), was too small, having curled up due to dehydration, so it was embedded laterally. On the following day, the samples were washed with PBS for 15 minutes, 3 times at room temperature. Samples were dehydrated in an ethanol series (70% ethanol for 1 hour, 95% ethanol for 1 hour, and 100% ethanol for 1 hour, twice). Samples were cleared using xylene in the fume hood for 30 minutes (two washes), washed in paraffin wax for 15 minutes and then incubated in paraffin overnight at 65℃. Tissues were embedded in paraffin in tissue molds. The molds were then stored between 2-8℃ until sectioning.

Sample Sectioning

A microtome supplied by Leica Biosystems was used to take 5-micron sections of the samples. Sections were taken every 100 microns through the entire embryo, moving from the dorsum to the ventrum. The section was floated in a Precision™ water bath set to 37℃ and then mounted onto Superfrost® Plus Slides. The slides were then dried overnight at room temperature.

RNAScope® *In Situ* Hybridization

To analyze the sections prepared as described above, Advanced Cell Diagnostics' (ACD) proprietary *in situ* hybridization technology was used. For this assay, we used the RNAscope 2.5

HD Reagent Kit–RED assay combined with *Taqpep*-specific probes custom developed by ACD by sending them the *Taqpep* sequence. The following methodology sections detail an adapted version of the ACD User Manuals Formalin-Fixed Paraffin-Embedded (FFPE) Sample Preparation and Pretreatment and RNAscope® 2.5 HD Detection Reagent - RED specific to our experiment.

Deparaffinization of Slides

The slides were baked in a dry oven at 60℃ for 1 hour. They were then placed in a Tissue-Tek® Slide Rack and submerged into a xylene-containing dish (in the fume hood) and incubated for 5 minutes with occasional agitation of the rack. This xylene-bath was repeated again with a fresh container of xylene. The same washing was performed with two 100% ethanol baths but for 1 minute intervals. The slides were removed from the rack and placed on absorbent paper, section side up, and set to dry for 5 minutes.

Equipment Preparation

Before beginning the sample pretreatment, the HybEZ™ oven which will be used to incubate the slides must be equilibrated. To do this, it was turned on and set to 40℃. A sheet of humidifying paper was placed in the humidity control tray and wetted completely with distilled water. Then the tray was covered and placed into the oven to be warmed for 30 minutes at 40℃.

Sample Pretreatment

Drops of RNAscope® Hydrogen Peroxide were applied using a micropipette onto the slides to cover the entire section. The slides were then incubated with the hydrogen peroxide applied for 10 minutes at room temperature. After this period, excess solution was flicked off and the slides were inserted into a Tissue-Tek® Slide Rack submerged in distilled water. The slides were washed by agitating the rack up and down and then transferred into 700 ml of boiling RNAscope® Target Retrieval Reagent (prepared by combining 630mL of distilled water with 70mL of 10x RNAscope® Target Retrieval Reagents) for 3 minutes. After being removed from boiling reagent, the slides were rinsed in a bath of distilled water and then transferred into an 100% ethanol bath for 3 minutes and placed on absorbent paper. Once the slides had dried, the Immedge™ hydrophobic barrier pen was used to create a barrier outlining each section. The barrier was set to be dried completely (approximately 1 minute), and then the slides were transferred onto the HybEZ™ Slide Rack. RNAscope® Protease Plus was applied using a micropipette to cover each section within the Immedge™ barrier. The HybEZ™ Humidifying Control Tray (prepared as stated in **Equipment Preparation** section) was removed from the oven and the slide rack was inserted in the tray. The tray was then inserted back into the

HybEZ™ oven to incubate at 40℃ for 30 minutes. After the incubation period, any excess reagent was flicked off and the slides were inserted into a Tissue-Tek® Slide Rack submerged in distilled water and washed up and down.

RNAscope® 2.5 Assay Using *Taqpep*-specific Probes

Before conducting the RNAscope® 2.5 Assay, the detection reagents (AMP 1-AMP 6) should be removed from the fridge and placed at room temperature. Likewise, the *Taqpep*-specific probes should be warmed in the HybEZ™ oven at 40℃ for at least 10 minutes. After completing the sample pretreatment, the slides were placed in the HybEZ™ Slide Rack and *Taqpep*-specific probes designed by ACD were pipetted on to cover each sample. The rack was inserted into the HybEZ™ Humidifying Control Tray and incubated in the oven at 40℃ for 2 hours. The tray was removed, excess liquid was flicked off, and the rack was washed in a series of 2 baths of 1X Wash Buffer, rocking back and forth for 2 minutes each. In total, 3L of 1X Wash Buffer should be prepared by combining 2.94 L of distilled water with the 60mL bottle of RNAscope® Wash Buffer (50X). The same process of applying reagent, incubating in the HybEZ™ oven at 40℃, and performing the 2 washes was then performed for the detection reagents AMP 1-AMP 4 but instead incubating for alternating periods of 30 minutes (AMP 1, AMP 3) and 15 minutes (AMP 2, AMP 4). AMP 5 and AMP 6 underwent the same application and wash processes but their incubation occurred at RT for 30 minutes and 15 minutes respectively. After the amplification reagents, the next step was signal detection. The signal detection solution was prepared by mixing Fast RED-B reagent with Fast RED-A reagent in a 1:60 ratio (eg. mix 7µL of Fast RED-B with 413µL of Fast RED-A). The solution was pipetted up and down to mix and then applied onto the samples. The RED solution was set to incubate for 10 min in the HybEZ™ Humidifying Control Tray at RT. After incubation, excess liquid was gently flicked off and the slides were placed in a Tissue-Tek® Slide Rack submerged in distilled water. The rack was transferred into a second fresh bath of distilled water and transported to the fume hood for the counterstaining (staining the cell nuclei blue). The slide rack was transferred into a dish containing 50% Hematoxylin staining solution. This 50% solution was made by adding 100mL of Gill's Hematoxylin to 100mL of distilled water. The rack was then washed through a series of 3-5 fresh distilled water baths until the water remained clear. The rack was placed into a 0.02% ammonia bath. This solution was prepared by adding 167 µL of 30% ammonium hydroxide to 250mL of distilled water. After the ammonia bath, they are placed in another water bath and dried in a 60℃ dry oven for 15 minutes. Then each slide was mounted using Fisher Scientific coverslips. To do so, each slide was dipped in fresh xylene (in the fume hood) and a drop of EcoMount by Biocare was applied. The 24mm x 50mm coverslip was then carefully placed over the section, avoiding trapping air bubbles. This was repeated for each slide, and all the slides were set to dry overnight in the hood.

Sample evaluation

Each prepared slide was analyzed within a week of the assay using the Leica DMRXA2 microscope and its accessories with a magnification of 10-40x. Combined with a Dell computer, images were captured of notable regions observed under the microscope in various regions of the sample. The location on each embryo where each image was taken was carefully noted down to ensure the images could later be interpreted properly and in the context of their location on the section.

RNAScope® Double *In Situ* Hybridization

To perform a double staining of *Taqpep* and *Dickkopf 4* (*Dkk4*), a previously identified marker of the pre-pattern, Advanced Cell Diagnostics' (ACD) proprietary double in situ hybridization technology was used. For this assay, we used the RNAscope 2.5 HD Duplex Assay Reagent Kit with Channel 1 *Dkk4*-specific probes (HRP-based Green) and *Taqpep*-specific probes (AP-based Fast Red), both custom developed by ACD by sending their respective gene sequences. The procedure for this hybridization matched those described above for the single assay, except for the added set of probes and amplifying reagents which are detailed in the ACD User Manual RNAscope® 2.5 HD Duplex Assay.

Quantitative Analysis

To perform a quantitative analysis of the sections processed with the Duplex Assay, photomicrographs at 40x were taken of *Dkk4*-positive regions and the two *Dkk4*-negative regions flanking it. This was done for eight unique anatomic locations across the various sections evaluated of the 1.8cm embryo. The number of *Taqpep*-positive cells (a blue nucleus with two or more red dots) was counted in each image. The number of *Taqpep*-positive cells below the two *Dkk4*-negative regions was averaged, and a graph comparing the number of *Taqpep*-positive cells below *Dkk4*-positive and *Dkk4*-negative epidermis was generated. A paired Student's t-test was performed to determine whether there was a statistically significant difference in the mean of *Taqpep*-positive cell counts of the *Dkk4*-positive and -negative regions.

Samples Analyzed

All the cat embryo samples analyzed were from the trap-neuter-release (TNR) clinic Forgotten Felines of Sonoma County. Their developmental stage was evaluated based on previous work on cats and mice (Kaelin et al., 2021). We evaluated serial sections through one embryo at each developmental time point.

Table 2: Details on Samples Evaluated

Name (location_year_cat #_fetus#)	Year Collected	Stage	Crown-rump Length	Orientation	RNAScope® Assay Performed
So19C16F4	2019	15	1.6-1.7 cm	dorsal down	*Taqpep*
So19C105F2	2019	14	1.3-1.4 cm	dorsal down	*Taqpep*
So19F103F3	2019	13	1.0-1.1 cm	dorsal down	*Taqpep*
So19C113F6	2019	12	1.0 cm	lateral	*Taqpep*
So21C4F2	2021	15	1.8 cm	dorsal down	*Taqpep* + *Dkk4*

Acknowledgements

I would like to thank Dr. Kelly McGowan for her guidance throughout this project. I cannot express how grateful I am for her mentorship and support. I would also like to thank our partners at Forgotten Felines of Sonoma County who generously donated their time and resources to our work. Lastly, I would like to acknowledge and thank Mr. Dietrich Schuhl for his support and understanding through each step of this project.

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