Linking new genes to an odontogenesis *TP63*-mediated gene regulatory network that is peripheral to jaw morphogenesis

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ABSTRACT

Despite that teeth and jawbones can develop and evolve independently of each other, the genetic processes that independently regulate cranial and dental morphogenesis remain unclear. Previous microarray screens indicated that a *TP63*-mediated gene regulatory network is integral to odontogenesis but peripheral to jaw morphogenesis.

Here, I characterized the expression of four genes flagged by our lab's previous microarray screens, comparing embryonic wild-type and *TP63*-null (*TP63*$^{-/-}$) mutant mouse mandible prominences. I hypothesized that in the normal dental epithelium, *TP63* up-regulates *Fermt1* and *Pltp* and down-regulates *Cbln1* and *Krt8*.

I validated the expression domains of these genes in *TP63*$^{-/-}$ mutant and wild-type mice using RNA *in-situ* hybridization on paraffin tissue sections at two stages, E11.5, just after odontogenesis begins, and at E13.5, just after odontogenesis arrests in the *TP63*$^{-/-}$ mutant. My *in situ* results validated our lab's previous microarray screens. My work revealed that *Fermt1* is expressed in wild-type oral epithelium and dental epithelium, and that *Pltp* is expressed in dental epithelium. Compared to wild-type littermates, in *TP63*$^{-/-}$ mice, *Fermt1* and *Pltp* expression intensity in the dental epithelium was decreased. *Cbln1* was not expressed in wild-type dental epithelium, although it expressed intensely in the dental epithelium of *TP63*$^{-/-}$ littermates. *Krt8* was expressed in the dental epithelium of both wild-type and *TP63*$^{-/-}$ embryos, with increased intensity in the *TP63*$^{-/-}$ mice. In sum, my results largely supported my hypothesis.

As such, I propose that *Fermt1, Pltp, Cbln1*, and *Krt8* belong to a *TP63*-mediated gene regulatory network and co-regulate odontogenesis by mediating cell adhesion, cell signaling, and epithelial-mesenchymal interactions at early stages of tooth development.

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LIST OF ABBREVIATIONS

AP	Alkaline Phosphatase
BMP	Bone morphogenic protein
BM	Basement membrane
Bmp4	Bone morphogenic protein 4
bp	Base pairs
Brdm2	Homozygote mutant of TP63
C	Celcius
Cbln1	Cerebellin 1 Precursor
cDNA	complementary DNA
ddH2O	double-distilled water
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
E	Embryonic day
E.coli	Escherichia coli
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
Fermt1	Fermitin Family Member 1
FGF	Fibroblast growth factor
Fgf8	Fibroblast growth factor 8
GRN	Gene regulatory network
H2O	Water
HCl	Hydrogen chloride
HDL	High-density lipoprotein
ISH	In situ hybridization
Krt8	Keratin 8
LDL	Low-density lipoprotein
LiCl	Lithium chloride
MAB	Maleic acid buffer
MABT	Maleic acid buffer containing Tween 20
MdP	Mandibular prominence
MgCl2	Magnesium chloride
mRNA	Messenger ribonucleic acid
Msx1	Msh homeobox 1
NaCl	Sodium chloride

NCBI	National Center for Biotechnology Information
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
Pltp	Phospholipid Transfer Protein
RNA	Ribonucleic acid
RT	Room temperature
Shh	Sonic hedgehog
SOC	Super optimised broth with the addition of glucose
TAE	Tris-acetate-EDTA
TBE	Tris/Borate/EDTA
TNF	Tumor necrosis factor
TP53	Tumor protein 53
TP63	Tumor protein 63
$TP63^{-/+}$	Heterozygote
$TP63^{+/+}$	Wild-type
TP73	Tumor protein 73
Tris	Trisaminomethane
UTR	Untranslated region
UV	Ultra violet
WT	Wild-type
ΔN-p63	TP63 isoform missing N-terminal transactivating domain

CHAPTER 1 - INTRODUCTION

1.1 Molecular biology of odontogenesis exclusive of jaw morphogenesis

In vertebrates, diverse shapes and sizes of teeth and jaws fit and function together; this successful adaption guarantees an individual's feeding and consequently a species' survival (McCollum and Sharpe 2001). Teeth are developmentally and evolutionarily integrated with the jaw structures; yet teeth and jawbones can develop and evolve independently of each other (Rücklin et al. 2012; Paradis et al. 2013). This independence suggests that even though dentitions and jaw skeletons are strongly integrated, there is a genetic process that is vital to odontogenesis but peripheral to jaw morphogenesis. Thus, some genes may be crucial to tooth but not jaw development, and vice-versa.

The genetics of odontogenesis are complex. A large number of genes are responsible for coding growth factors (e.g., Fibroblast growth factor (*FGF*) and Bone morphogenic protein (*BMP*)), signaling molecules (e.g., Sonic hedgehog (*Shh*) and Tumor necrosis factor (*TNF*)), and transcription factors (e.g., Msh homeobox 1 *(Msx1*)) that regulate multiple downstream target genes (Thesleff 2006; Frase et al. 2009). These many genes, as well as their complex interactions, make it challenging to define the molecules and pathways regulating tooth formation, particularly independent of jaw development. Our knowledge of the molecular biology of odontogenesis is based on the targeted mutation of genes such as *Fgf8, Bmp4, Shh,* and *Msx1* that are critical for both odontogenesis and jaw development. Thus experimental mutation of these genes perturbs both the dentition and the jaw skeleton (Cobourne and Sharpe 2003), making it technically complex to study the genetic processes and pathways that drive odontogenesis distinct from jaw development (Raj and Bougher 2016).

Nonetheless, understanding the conserved gene regulatory network(s) (GRN) that regulates teeth exclusive of jaws is crucial to clarify the factors that synchronize dental and craniofacial development (Paradis et al. 2013). Moreover, this knowledge is important to understand the putative ability for each of these structures - dentition and jaw - to change relative to one another during evolution (Boughner and Halgrimmson 2008).

1.2 The developmental stages of tooth morphogenesis in mammals

Odontogenesis is complex due in part to the highly coordinated epithelial-mesenchymal genetic interactions required to form a tooth (Chatterjee and Boaz 2011). In fact, odontogenesis relies on uninterrupted mesenchymal-epithelial signaling (Thesleff et al. 1991).

The basement membrane (BM) is a thin layer of extracellular matrix (ECM) between dental epithelium and underlying neural crest-derived mesenchyme cells that plays an important role in regulating epithelial-mesenchymal interactions (Thesleff et al. 1991). Also, the extracellular matrix (ECM) between epithelial cells regulates cell interactions during tooth morphogenesis (Fukumoto and Yamada 2005).

More than 100 years ago, the basic stages of tooth morphogenesis were first described (e.g., Owen 1840-1845). From that time and based on all vertebrates studied since, these four distinct stages are similar and consist of: 1) dental placode formation, followed by; 2) bud, then; 3) cap, and finally; 4) bell stage (Fig. 1) (Jernvall and Thesleff 2012). Among the vertebrate model systems that have been used to study the discrete stages and structures of odontogenesis, the mouse is a well-established classical model system. Because of the conserved processes of odontogenesis, data from mouse odontogenesis can be translated to that of other mammalian if not also other vertebrate groups (Thesleff and Ekblom 1984; Chai et al. 1998).

In mice, the first morphological sign of odontogenesis is the thickening of the oral epithelium that happens at embryonic day (E)10.5 (Fig. 1A). At E11.5, dental placodes develop and then forms the primary dental lamina, where the future tooth will form (Thesleff and Tummers 2009; Lumsden 1988). Which they share morphological as well as molecular features with placodes of other ectodermal derivatives, such as hair and nails (Pispa and Thesleff 2003). As the earliest, primary signaling center of the tooth, the dental placode emits signals that help govern tooth bud formation via the localized proliferation of dental lamina cells by E13.5 (Fig. 1B) (Thesleff 2003). One of these important signals is Sonic hedgehog (*Shh*), which is essential to vertebrate tooth development and a highly-conserved marker for odontogenesis (Hardcastle et al. 1998). *Shh* signaling begins very early in tooth development and specifies the odontogenic epithelium in the oral cavity during the initiation of the dental lamina formation (Hu et al. 2013). Later, *Shh* is important for epithelial differentiation and morphogenesis, and promotes epithelial cell movement in the early tooth bud (Li et al. 2016). For the first molar tooth, during the bud stage (E13.5), the dental epithelium invaginates the underlying dental mesenchyme and these epithelium cells divide into two lineages: 1) a single layer of peripheral basal epithelial cells contacting BM, and 2) the stellate reticulum loosely arranged located in the center. (Pansky 1982; Thesleff and Tummers 2009). During the cap stage starting at E14.5 (Fig. 1C), the dental epithelium is in turn invaginated by a group of condensed mesenchymal cells, called the dental papilla, that later gives rise to dentine and dental pulp. The dental epithelium folds and forms a cap-shaped structure called the enamel organ, which covers the dental papilla. The primary enamel knot, another epithelium-derived signaling center, also appears at this stage (Pansky 1982; Thesleff and Tummers 2009). The bell stage starts at E18.5; during this stage and the cap stage (Fig. 1C-D), enamel and dentin are secreted to create the tooth crown (Pansky 1982).

Enamel and dentin are the hard tissues of the tooth. Enamel is secreted by ameloblasts, which are epithelial derivatives, while dentin is secreted by odontoblasts, which are mesenchymal derivatives. Tooth crown size and shape are regulated by the enamel knots, which control where cusps form (Mitsiadis et al. 2010). After crown formation is complete, root formation starts as still-secreting odontoblasts and ameloblasts migrate away from the occlusal surface of the crown. By the time the tooth emerges into the oral cavity, the roots have reached their full length (Pansky 1982; Thesleff and Tummers 2009).

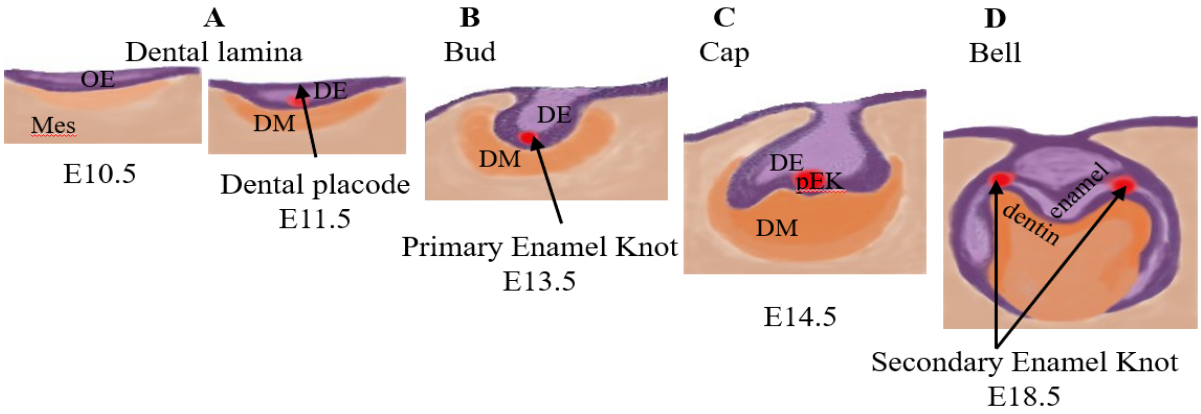

Figure 1. The four distinct stages of odontogenesis: (**A**) dental lamina formation at E10.5 and dental placode formation at E11.5, (**B**) bud stage at E13.5, (**C**) cap stage at E14.5, and (**D**) bell stage at E18.5. Dental epithelium (DE) and oral epithelium (OE) is shown in purple, condense underlying neural crest-derived mesenchyme (DE) is in dark orange, while rest of the mesenchyme cells (Mes) around the tooth formation area is shown in light orange. Signaling centers (i.e., dental placode at E11.5; primary enamel knot at (pEK) E13.5 & 14.5 and secondary enamel knot at E18.5) active at each stage are shown as red dots.

1.3 The integral role of *TP63* in initiating odontogenesis

One of the major transcription factors that required for epithelial cell layer development during odontogenesis is tumor protein 63 (*TP63*) (Yang et al. 2002; Belyi and Levine 2009; Joergegr et al. 2009). To date, it is commonly accepted that, compared to its homologues *TP53* and *TP73*, *TP63* most resembles the ancestral gene of the *TP53* family (Belyi and Levine 2009; Joergegr et al. 2009) and gene duplication events gave rise to the two additional homologs, *TP53*

and *TP73* (Belyi et al. 2010). The main function of the *TP53* family of genes is to protect the germline from DNA damage. In response to DNA damage caused by mutations, these proteins prevent the gene repair process and initiate apoptosis so that the mutation is not passed on in the germ-line (Belyi et al. 2010).

The *TP63* gene has two variants, one that contains an N-terminal transactivation domain (*TA-p63*), and another that does not (*ΔN-p63*) (Yang et al. 1998). Each of the two variants gives rise to three different isoforms: alpha (α), beta (β), and gamma (γ), due to alternative splicing at the variants' C terminus (Yang et al. 1998; Mills *et al*. 1999). *TP63* is required to initiate and regulate the development of epithelial cell layers regardless of their ectodermal or endodermal origin (Koster et al. 2004; Yoh and Prywes 2015; Kouwenhoven et al. 2015). Targeted disruption of *TP63* (e.g., the Brdm2 mouse mutant generated by Mills et al. in 1999 (Fig. 2)) causes limb and skin defects, among other pathological phenotypes. This Brdm2 mouse model was valuable because it revealed the main functions of *TP63* in the proliferation and differentiation of epithelial cells (Mills *et al*. 1999). Studies using this *TP63* mutant showed that *TA-p6*3 isoforms are expressed in epithelial cells prior to *ΔN-p63* isoforms during embryogenesis. Also, these studies revealed that *TA-p6*3 isoforms are counterbalanced by *ΔN-p63* isoforms. This counterbalance means that *TA-p6*3 isoforms' expression shifts to *ΔN-p63* isoforms' expression to allow epithelial cells to respond to signals required for their maturation. Signals such as *Bmp4* and *Fgfs* that come from underlying neural crest-derived mesenchymal cells (Yang et al. 1999; Mills *et al*. 1999; Koster et al. 2004). Further studies revealed, odontogenesis relies on activity of *ΔN-p63* isoforms (Rufini et al. 2006). During epithelial and tooth development *TP63* alpha and beta isoforms but not gamma isoform are predominantly expressed (Laurikkala et al. 2006). Also, other studies on this mutant showed that *TP63* regulates epithelial cell adhesion, integrity,

and homeostasis. In particular, *TP63* mediation of extracellular matrix adhesion molecules plays a critical role in normal epithelial-mesenchymal interactions (Carroll et al. 2007).

In Brdm2 mutant mice, in which both *TP63* alleles are mutated ($TP63^{-/-}$), all epithelial derivatives are malformed if they are not actually missing (Fig. 2A) (Mills *et al*. 1999). This severe $TP63^{-/-}$ phenotype indicates that *TP63* is crucial during the development of structures with epithelial origin such as limbs, skin, hair follicles, and teeth (Laurikkala et al. 2006), that also all require uninterrupted epithelial-mesenchymal crosstalk (Yang et al. 1999; Kouwenhoven et al. 2015). However, while the *TP63*-null mutant fails to form teeth because odontogenesis arrests shortly after it begins, the lower jaw (mandible) develops normally (Fig. 2D) (Paradis et al. 2013). It is salient to note that the mammalian mandible is composed of only the dentary bone, which has an exclusively mesenchymal origin. Therefore, *TP63* has an integral role in initiating odontogenesis and dental epithelium proliferation and differentiation that is exclusive of mandible morphogenesis (Rufini et al. 2006; Matsuura et al. 2012; Raj and Boughner 2016).

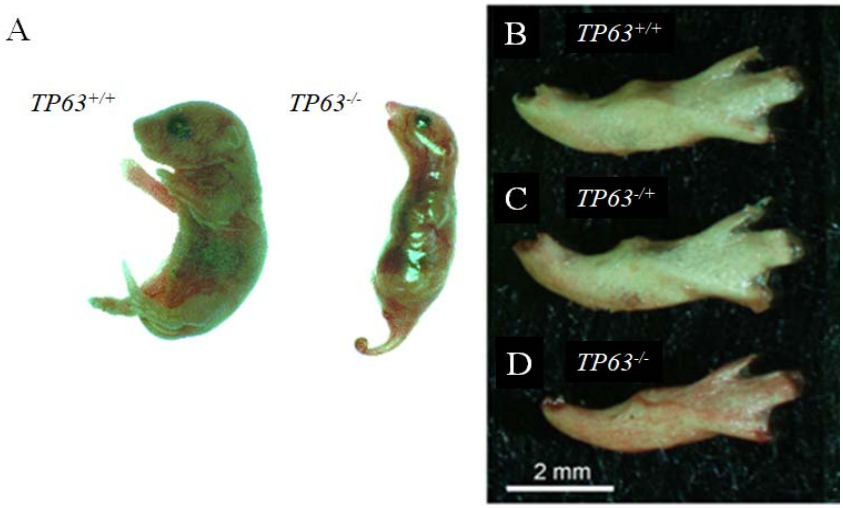

Figure 2. (**A**) Neonatal Brdm2 mouse (right) showing the two *TP63* mutated alleles ($TP63^{-/-}$) phenotype that lacks epithelial derivatives, in comparison to neonatal wild-type mouse (left, $TP63^{+/+}$) (Adapted from Mills et al. 1999). (**B-D**) Comparing mandible (dentary bone) shapes among (**B**) *TP63* wild-type ($TP63^{+/+}$), (**C**) *TP63* heterozygote ($TP63^{-/+}$) and (**D**) *TP63*-null fetuses (E18) ($TP63^{-/-}$) shows that the dentary bone develops normally but with a 12-24 hours developmental delay in the toothless mouse (Adapted from Paradis et al. 2013).

1.4 Thesis research questions and objectives

1.4.1 Adding new genes to a *TP63*-mediated gene regulatory network active during mouse embryonic development

In *TP63*$^{-/-}$ mice, tooth development arrests at E11.5, shortly after odontogenesis has initiated; consequently, no tooth buds ever form (Laurikkala et al. 2006). To better understand the genes that regulate odontogenesis exclusive of jaw development, a recent publication by Raj and Boughner (2016) used high-throughput gene expression microarray assays validated by reverse-transcription quantitative PCR (RT-QPCR) to contrast gene transcripts between the RNA extracted from surgically excised mandibular prominences in toothless *TP63*$^{-/-}$ and toothed wild-type mouse embryos. This microarray screen identified a set of genes with significantly increased or decreased levels of expression in *TP63*-null mice compared to wild-type littermates at embryonic days (E) 10-13, four ages that encompass the earliest stages of tooth formation (Table 1). Among this set of genes, some had never before been connected to odontogenesis or to the *TP63* pathway. To more precisely understand the roles of these newly flagged genes in odontogenesis, the exact expression domains of these genes during the early stages of tooth development needed to be probed. Also, to connect these genes to a *TP63*-mediated gene regulatory network (GRN), changes in the expression of these genes relative to functional *TP63* presence or absence needed to be studied. In Raj and Boughner's (2016) study, several genes had significantly different fold changes between mutant and toothed strains that were either increased (positive fold changes) or decreased (negative fold changes). Also, comparison of the fold changes among different stages (E10-13) showed the changes in some of these genes were not only limited to one stage, but were consistent across all the stages (E10-13). The genes with the largest differences in expression levels (highest/lowest fold changes) which had not been previously connected to the odontogenesis were the ideal candidates for this thesis research.

Table 1. Results of microarray analysis showing the differences in gene expression caused by the null mutation of TP63 during odontogenesis. Fold changes of the genes that I studied here are: *Fermt1 (-2.9, -2.1), Pltp (-2.2, -1.6), Cbln1 (2.2,2.3*), and *Krt8 (2.7,4.5)* at early stages of tooth development (E11,13). These genes showed constant decrease (*Fermt1* and *Pltp*) or increase (*Cbln1* and *Krt8*) in their fold changes. For example: *Fermt1* has significant fold changes (lower expression in *TP63*$^{-/-}$ in comparison to wild type) in both E11 and E13. (Adapted from Raj & Boughner 2016).

	Fold Change				Gene associated with:	
Gene	**E10**	**E11**	**E12**	**E13**	**odontogenesis**	***p63***
TP63	-2.7	-2.1	-1.9	-2	Laurikkala et al., 2006	Mills et al., 1999
Fermt1	-2.7	-2.9	-2.3	-2.1	-	-
Pltp	-2.3	-2.2	-1.8	-1.6	-	-
Cbln1	1.8	2.2	2.6	2.3	-	-
Krt8	2.4	2.7	4.1	4.5	-	De Rosa et al., 2009

Therefore, my aim in this project was to add and connect four of these genes, Fermitin1 (*Fermt1*), Phospholipid Transfer Protein (*Pltp*), Cerebellin-1 (*Cbln1*), and Keratin 8 (*Krt8*), to a novel *TP63*-controlled GRN that is specific to odontogenesis but that does not influence jaw development. In the next section I briefly explain the known roles of these four genes.

Fermt1 is one of the Fermitin family homologues and is also known as *Kindlin 1*. This gene family encodes a group of highly conserved proteins called Fermitins. All the members of this family contain a FERM domain, the name coming from the first proteins that identified in this family: Four-point-one, Ezrin, Radixin, and Moesin (Pearson et al. 2000). Fermitins mediate the integrin activation and initiation intracellular signals called integrin inside-out signaling (Malinin et al. 2010). The gene *Fermt1* generally expresses in epithelium and encodes a cell structural protein linking actin to the extracellular matrix (ECM) (Jobard et al. 2003; Siegel et al. 2003). Next, Phospholipid Transfer Protein (*Pltp*) gene encodes the PLTP protein along with three other proteins that are all members of the lipid transfer/lipopolysaccharide binding protein (LT/LBP) gene family (Albers et al. 1996). The main established role for PLTP is the metabolism of lipoproteins. The PLTP protein also has the ability to bind to lipids and transfer

lipids between plasma lipoproteins t(Lagrost et al. 1998). Third, Cerebellin-1 (*Cbln1*) encodes CBLN1 which is a secreted glycoprotein. This protein is critical for synaptic integrity and function in neurons of brain cerebellar granule (Hirai et al. 2005). CBLN1 belongs to a protein family (CBLN1-CBLN4) which all share the C1q domain at their C termini (Urade et al. 1991). Lastly, Keratin 8 (*Krt8*) encodes KRT8, one of the many Keratin proteins. These proteins are the major component of intermediate filament proteins and predominantly expressed in epithelial cells (Makino et al. 2009). Keratin proteins are divided into two types based on their molecular weights: type I (K9-22), and type II (KRT1–KRT8) (Hesse et al. 2001). KRTs are essential cytoskeletal components important for cell morphology, mitosis, differentiation, and apoptosis (Tan et al. 2017). KRT8 expresses in tumors and has been associated with tumor progressions, notably cell migration, adhesion, and drug resistance (Fang et al. 2017).

1.4.2 Mapping the expression of four new genes at the beginning of tooth initiation and at the later bud stage of tooth development

In summary, none of these four genes had previously been linked to tooth development and despite the results of the microarray screen, it is not known exactly where in the developing tooth each gene may be expressed. Here I hypothesized that in normal dental epithelial cells, *TP63* up-regulates *Fermt1* and *Pltp*, and down-regulates *Cbln1* and *Krt8*. To test this hypothesis, I used *digoxigenin-labeled* RNA probe *in situ* hybridization to localize and map the expression of these four genes in dental and oral epithelium, as well as in underlying mesenchyme, and compare the expression of these genes in *TP63*-null mice with wild-type littermates. The expression domain and intensity of these genes were studied at two different time points of tooth development: E13.5, when the tooth bud formed in WT, and E11.5, when dental placode is fully form and starts signaling. Both of these stages overlap with two of the four stages studied by Raj and Boughner's (2016) microarray work.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Study methodology: Overview

My data were collected from RNA *in situ* hybridization (ISH) experiments performed on sections of paraffin-embedded embryonic mouse heads. Mice were phenotyped and genotyped to identify Brdm2 toothless mutants (*TP63*$^{-/-}$) and toothed wild-type (*TP63*$^{+/+}$) embryos. Next, wild-type and mutant mice aged embryonic day (*E*) E13.5 and E11.5 were collected for RNA ISH. Embryos were fixed in paraformaldehyde (PFA), dehydrated in graded ethanol washes, embedded in paraffin, then sectioned and slide-mounted. I probed these tissue sections for four genes: *Fermt1, Pltp, Cbln1,* and *Krt8*. As positive controls, I used Sonic Hedgehog (*Shh)* for both toothless and toothed mice, since *Shh* is expressed in dental epithelium where future teeth will form (Koyama et al. 1996). I used *TP63* as another positive control for toothed mice since, similar to *Shh*, *TP63* expresses intensely in oral and dental epithelium but is missing in the toothless mice. Sense RNA probes were used as negative controls. Both anti-Sense and Sense RNA probes were designed and synthesized by me. At least two different embryonic specimens were assayed per gene, for each time point.

2.2 Study materials

2.2.1 Mice

All the following studies were performed using pregnant and prenatal mice derived from the B6.129S7-*TP63tm2Brd/J* strain (Jackson Laboratory, Bar Harbor, U.S.A.) and *C57BL/6J* (stock # 003568 and # 000664, respectively). All mice were cared for by animal technicians in the Vivarium in the Health Sciences Building at the University of Saskatchewan. This care included breeding heterozygote adult mice and watching for vaginal plugs, which indicate

pregnant females. After 11 or 13 days post-coitum, pregnant dams were sacrificed by cervical dislocation in accordance with University of Saskatchewan animal use protocol # 20110008. Immediately after sacrifice, dissection forceps were used to extract all embryos from the uterus, which was immersed in 10X Phosphate Buffered Saline (PBS, Molecular Biology grade), under a dissecting microscope (Olympus, model SZX2ILLK).

2.2.2 Phenotyping

Brdm2 toothless mutants (*TP63*$^{-/-}$) were clearly distinguished from phenotypically normal wild-type (*TP63*$^{+/+}$) and heterozygote (*TP63*$^{-/+}$) embryos. From at least E9.5, homozygote mice have conspicuous morphological deformities, most notably their highly atrophied or absent limb buds (Mills *et al*. 1999). As such, these *TP63*$^{-/-}$embryos did not need to be genotyped; however, homozygote tail clips were collected and used as positive controls for the polymerase chain reaction (PCR) work. In contrast, because heterozygote and wild-type mice are morphologically indistinguishable from each other, PCR was performed on these embryonic tissues using primers complementary to the *TP63* insertion mutation (Jackson Laboratory, Bar Harbor, U.S.A.) (Table 2).

2.3 DNA extraction and genotyping

Each embryonic tail clip was collected in its own sterile (DNase/RNase-free) microcentrifuge tube and immediately snap-frozen dry at -20° Celcius (C) for subsequent DNA extraction and PCR work. All DNA extraction was done using a Phire Tissue Direct PCR master mix (Thermo Scientific, Waltham, U.S.A.). First, DNA release buffer (0.5µl) and dilution buffer (20µl) were added to each tissue sample, and then incubated at room temperature (RT) for 5 min. Next, samples were heated at 98°C for 2 min. After this step, the supernatant was collected and used immediately for PCR work or stored at -20°C until the next day, when the samples were

thawed, and used for PCR work. Extracted DNA was tested for quantity and quality using a NanoDrop Lite Spectrophotometer (Thermo Scientific). PCR was performed using the primers listed in Table 2. The presence of the *TP63* insertion mutation was detected with its specific primers (Table 2, top two rows). If the insertion mutation was present in the DNA sample, these primers amplified an insertion mutation that yields a sequence that is 410 base pairs (bp) in length. As an internal positive control, the genomic sequence coding for *T cell receptor* was amplified, with a product length of 210 bp *(*Table 2*)*. A wild-type embryo would show a band for only this 210 bp band.

Table 2. List of PCR primers that were designed and validated by Jackson Laboratories and purchased from Sigma. Please refer to the following link for more primer and *PCR* protocol information: http://jaxmice.jax.org/protocolsdb/f?p=116:2:753578398088607::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:5248,003568.

TP63 Insertion mutation	Forward (oIMR1029) 5' GTGTTGGCAAGGATTCTGAGACC 3'
	Reverse (oIMR1030) 5' GGAAGACAATAGCAGGCATGCTG 3'
T cell receptor	Forward (oIMR8744) 5' CAAATGTTGCTTGTCTGGTG 3'
	Reverse (oIMR8745) 5' GTCAGTCGAGTGCACAGTTT 3'

All PCR reaction ingredients listed below were stored at -20°C and thawed on ice before they were mixed to prepare the master reaction mixture *(*Table 3*)*.

Table 3. Each PCR reaction mixture consisted of the following reagents:

Ingredient	**Volume (µl)**
Forward Primer (20 µM)	0.375
Reverse Primer (20 µM)	0.375
10x Buffer - $MgCl_2$ (Invitrogen)	1.5
dNTP 10 µM (Invitrogen)	3.0
50x $MgCl_2$ (Invitrogen)	0.75
Ultra pure H_2O (Gibco)	7.85
DNA (100-200 ng)	0.5-1.0
Total volume	**14.35-14.85**

After heating the thermocycler (Bio-Rad, Hercules, U.S.A.) to the temperature noted in Step 1, Table 4*,* the program was paused. While the sample tubes were still on ice, 0.150 µl of *TAQ* polymerase (Taq DNA Polymerase, recombinant CAT# 10342020,500 *units*) was added to each reaction mixture. Then all of the tubes were placed into the thermocycler, and the program was resumed and allowed to run to completion.

Table 4. Thermocycler program:

Step 1. 94°C for 5min
Step 2. 94°C for 30sec
Step 3. 55°C for 30sec
Step 4. 72°C for 30sec
Step 5. Back to step 2 and repeat 32x
Step 6. 72°C for 5min
Step 7. 4°C hold

Once the PCR was finished, all the tubes were removed from the thermocycler and placed on ice. Next, 2µl of 5x *BlueJuice* gel loading buffer (Life Technologies, Carlsbad, U.S.A.) was added to each tube of PCR product, which was then loaded into a 1.5% agarose gel (Invitrogen UltraPure Agarose, Life Technologies, Carlsbad, U.S.A.). The electrophoresis gel was made in 1x TAE (2M tris acetate, 0.05M EDTA, pH 8.3. Life Technologies) with 4µl *GelRed* Nucleic Acid added (Gel Stain, 10,000x in water, Biotium, U.S.A.). The gel was then run for approximately 40 minutes at 120 Volts. Next, the gel was imaged using a *UVP* BioDoc-It imaging system (UVP, Upland, CA, U.S.A.), and photographed with *UVP*-software (VisionWorksLS) (Fig. 3).

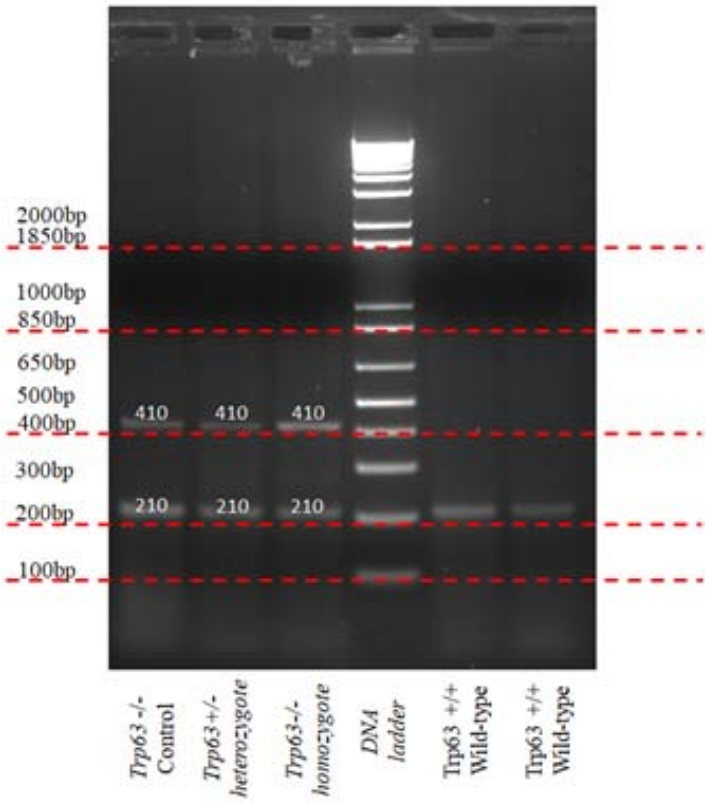

Figure 3. UV light view of a typical PCR agarose gel. Showing internal control band (210 bp) and mutated allele in *TP63*$^{-/+}$ (heterozygote) or *TP63* $^{-/-}$ (homozygote) via amplification of a 410 bp band. The absence of a 410 bp band indicates that both alleles are normal, and that the embryo is *TP63*$^{+/+}$ (wild-type), as shown below the gel image.

2.4 Embryo processing and sectioning

To perform the RNA probe *in sit*u hybridization on paraffin tissue sections, first, dissected embryos were fixed overnight in 4% paraformaldehyde (PFA). (Note: fixative volume should be 20 times that of tissue on a weight per volume ratio; therefore, the whole embryo was immersed in 2 ml of 4% PFA.) The next day, tissues were washed twice in 1X PBS (in DEPC water) for 10 min at RT. Then, tissues were dehydrated with graded ethanol washes based on the following Table 5. After step 3, tissues were stored in fresh 70% EtOH/1X PBS at 4ºC.

Table 5. Tissue dehydration steps. Ethanol washes used to dehydrate embryo samples.

Step 1. 1X wash in 25% EtOH/1X PBS for 15min at RT
Step 2. 1X wash in 50% EtOH/1X PBS for 15min at RT
Step 3. 1X wash in 70% EtOH/1X PBS for 15min at RT

Tissues were then processed through additional and graded washes of ethanol and xylene using a RVG/1 Vacuum Tissue Processor machine (Belair Instrument Company) located in the Core Histology Lab (Department of Anatomy & Cell Biology). Table 6 lists the processing protocol. Next, using a Tissue-Tek TEC 5 Tissue Embedding Console System (Model # 5100) processed tissues were embedded in paraffin blocks with heads in the coronal orientation. (See Table 6, following page.)

Table 6. Tissue processor progeam. Solutions and wash times used to process embryos:

Solution	**Time**	**Temperature**
EtOH 70%	1 hour	RT
EtOH 80%	1 hour	RT
EtOH 95%	35 minutes	RT
EtOH 95%	35 minutes	RT
EtOH 100%	50 minutes	RT
EtOH 100%	35 minutes	RT
EtOH 100%	35 minutes	RT
Xylene - 100% EtOH	45 minutes	RT
Xylene	40 minutes	RT
Xylene	40 minutes	RT
Paraffin	1hour	60^{o}C
Paraffin	35 minutes	60^{o}C
Paraffin	35 minutes	60^{o}C
Paraffin	35 minutes	60^{o}C

Once the paraffin blocks were allowed to solidify (overnight, at 4^{o}C), each block was placed in a rotary microtome and oriented so that the blade cut straight across the paraffin block. Sections were cut at 8 µm thickness. Section thickness is important: sections must be thin enough for the probe to penetrate and thick enough to have enough tissue to detect RNA expression of the gene. Sections were picked up using tissue forceps and floated on a bath of 40-45^{o}C DEPC-treated water in an autoclaved glass trough. After the sections were allowed to flatten out on the warm water, they were picked out of the water using Superfrost Plus microscope slides (U.S.A of Swiss Glass, CAT#12-550-15), placed on a slide warmer set at 37°C and left to bake overnight.

2.5 Hematoxylin and Eosin staining

Solutions:

Modified Harris Hematoxylin was used because it requires a less toxic chemical (sodium iodate instead of mercuric oxide), and the solution preparation is simpler and faster (no boiling of the solution is required). Staining results are comparable to the original solution.

Modified Harris Hematoxylin Working Solution:

100 g	Aluminum Potassium Sulfate (Al $K(SO_4)_2.12H_2O$) Or
	Aluminum Ammonium Sulfate (Al $NH_4(SO_4)_2.12H_2O$)
1000 mL	Distilled Water
5 g	Hematoxylin (*C.I. No. 75290*)
50 mL	Absolute Alcohol (i.e. 100% Ethanol)
0.5 g	Sodium Iodate
30 mL	Glacial Acetic Acid

Eosin Stock Solution: 1.25% Eosin Y (*C.I. No. 45380*) in 70% Ethanol
(Add 1 mL of Glacial Acetic Acid to every 100 mL).
Eosin Working Solution: 1-part Stock Solution to 4 parts 70% Ethanol

Table 7. H & E staining protocol

3 minutes each	2 changes of Xylene
1 minute	Xylene / Absolute Alcohol
1 minute each	2 changes of Absolute Alcohol
1 minute	95 % Ethanol
1 minute	Tap Water
Rinse	Distilled Water
3 - 5 minutes	Harris Hematoxylin
Wash	Tap Water (several changes)
2 dips	Acid Alcohol (0.5% HCl in 95% Ethanol)
Wash	Tap Water (several changes)
5 seconds	Saturated Aqueous Lithium Carbonate
3 minutes	Running Tap Water
Rinse	Distilled Water
1 minute	Eosin
1 minute	95 % Ethanol
1 minute	Absolute Alcohol 1
1 minute	Absolute Alcohol 2
1 minute	Absolute Alcohol / Xylene
1 minute each	3 changes of Xylene
Leave slides in xylene until ready to coverslip; **don't let them dry out!** Coverslip (LOT#17907) slides in a permanent mounting medium.	

2.6 RNA *in situ* hybridization

I designed each RNA probe sequence based on that gene's cDNA sequence and sent these sequences to Bio Basic Company (Ontario, Canada) to be cloned. Then I synthesized the *Digoxigenin-labeled* RNA probe from the DNA plasmid returned to me by the company.

2.6.1 Designing RNA probes

To design the RNA probe, based on published mouse cDNA sequences I chose a specific fragment of the 3' *UTR* region of each gene of interest specifically since these 3' regions are unique for each gene. The length of each chosen fragments was between 500-900 bases. Also, gene protein sequences were BLASTed with other genes in the same family to exclude homology domains (using the multiple sequence alignment software T-COFFEE: http://tcoffee.crg.cat/) and protein conserved domains (using NCBI Conserved Domains software: https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The T3 and T7 promoters were added specifically to the beginning and end of the RNA probe sequence. The final designed probe sequences were sent to Bio Basic Company (Ontario, Canada) to be cloned and inserted into pUC57 plasmids. Specifically, pUC57 is 2710 bp in length and is a high expression vector that is regularly used as a cloning vector in *E. coli*. This vector contains a *bla* gene for ampicillin resistance. Bio Basic returned by mail the lyophilized plasmids that I then transformed. First, before opening the container, I centrifuged the lyophilized DNA, then added TE buffer (10 mM, PH 8.5) and dissolved the DNA in TE buffer.

To transfer the plasmid into the bacteria, the appropriate *E. coli* competent cells were used (NEB 5-alpha competent *E. coli*, High Efficiency, CAT# C2987H). 100 ng of original plasmid DNA were added into *E. coli* competent cells and the mixture was incubated on ice for 30 minutes. Then, the mixture was heated at 42°C for 30 seconds and then immediately incubated on ice for 5 minutes. 950 µl *SOC* media were added to the mixture and incubated at

37°C for 60 minutes. This yielded my bacterial stock. 2µl of the bacterial stock was incubated on LB agar plates at 37°C overnight. The next day, a single colony that was well separated from other colonies was collected and inoculated in LB medium with Ampicillin, the antibiotic against which my plasmids are resistant, for large scale culture. The next day, DNA from the large culture was purified using a QIAprep Spin Miniprep Kit (CAT# 27104).

2.6.2 Probe synthesis

Making linearized template

1. I used a restriction enzyme that cuts a single site at the 5' end of the cDNA insert and linearized the plasmid equivalent to 10µg of DNA.

2. Mix:

10µg DNA
5µg of 10x buffer
4µg of restriction enzyme

3. Add Ultrapure distilled water (CAT#10977015) to a final volume of 50µl.

4. Incubate at 37°C for 3 hrs to overnight.

5. Transfer 2µl of DNA to a new 25 µl tube, mix with 7µl DEPC water and 1-2µl of loading dye, and run a 0.8% gel to check whether the DNA is linearized. (Also, run 1µl of uncut mini prep DNA as a control.)

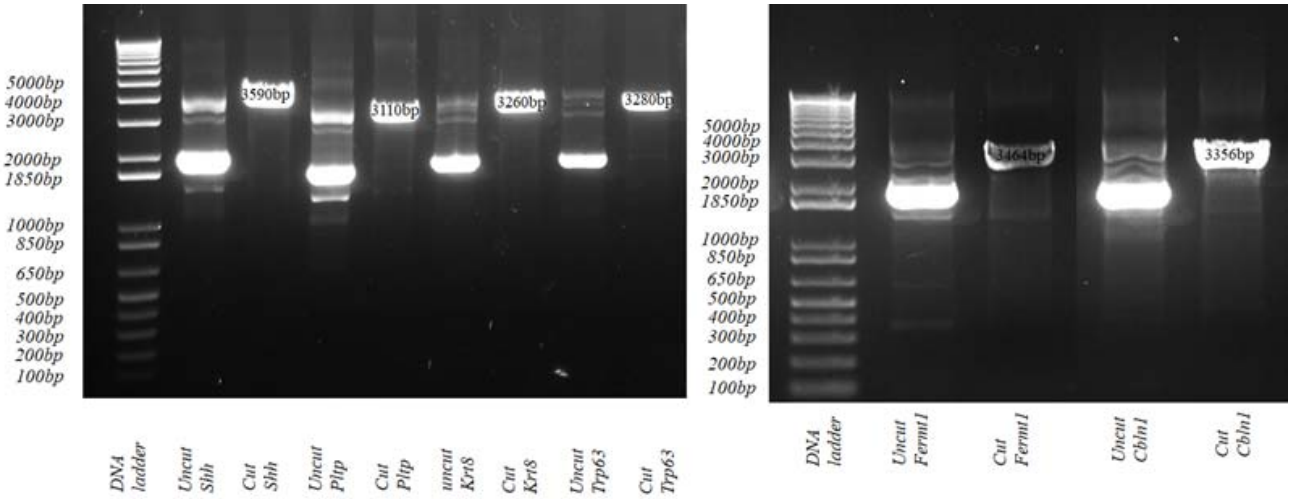

Figure 4. UV light view of agarose gel of all the genes of interest after DNA plasmids linearization, cut products along with their specific uncut stock DNA.

If the DNA wasn't cut properly then I repeated the linearization again.

If the DNA was cut properly then I proceeded to the following steps:

6. Add 100µl of ultrapure distilled water and 150µlof phenol/chloroform/IAA, mix, and spin at 10,000-15,000 rpm for 5min. Transfer only the top layer to a new tube.

7. Repeat step 6 (phenol/chloroform/IAA extraction).

8. Transfer the top layer to a new tube, extract with 150µl chloroform, and spin for 5 min.

9. Add 1/10 Vol of 3M sodium acetate and 2.5 volumes of ethanol, store at -70°C for 30 min.

10. Spin down at 10,000-15,000 rpm for 15 min at 4°*C*.

11. Carefully pipet off and discard the liquid. Wash the precipitate with 200µl of 80% ethanol, spin for 5 min, then pipet off and discard this liquid. Do a final quick spin and pipet with a sharp tip carefully to get rid of the last drops of EtOH.

12. Dissolve the precipitate in 40µl of ultrapure distilled water, and measure the concentration using a NanoDrop Lite Spectrophotometer (Thermo Scientific).

(Note: one could purify DNA using DNA clean up kit, instead of phenol/chloroform for purification. DNA clean up kit (for 5µg DNA): DNA clean and concentrator ZYMO CAT#D4003).

13. Transfer 1µl of the DNA to a new tube, mix with 7µl *DEPC* water and 1-2µl of loading dye, and run a gel to check whether you have gotten the DNA back.

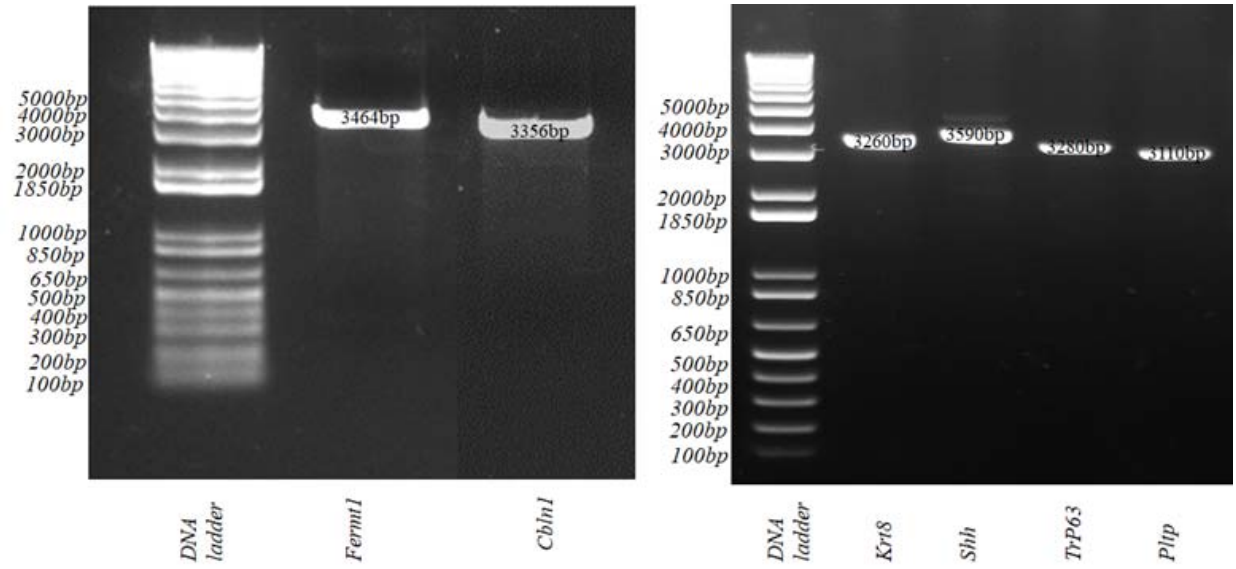

Figure 5. UV light view of an agarose gel showing all four of the genes of interest after DNA plasmid purification.

Making the RNA Probe

14. Mix together the 1X transcription reaction combine in a 1.5 ml tube:

1µg of linearized and purified plasmid (calculate volume based upon concentration)

2µl of 10X Transcription buffer (or 4µlof 5X buffer)

2µl of 10X nucleotide mix (with digoxigenin-UTP or fluorescein-UTP)

1µl of RNase inhibitor (40 units)

1µl of the appropriate T7, T3 (40u/µl) polymerase (20units/µl)

Ultrapure distilled water to give a final reaction volume of 20 µl

15. Incubate the mixture for 2hr at 37°*C*.

16. Add 4µl (40 units) of DNase 1 for 1X reaction. Incubate at 37°C for 15 min to remove the plasmid DNA.

17. Stop the reaction by adding 0.8 µl of 500mM EDTA pH 8.0.

18. Precipitate the RNA with 2.5 µl 4M LiCl, and 75 µl prechilled (-20°*C*) ethanol, keep at -70°C for 30min, if you can keep it longer you get better result.

19. Spin down the pellet at 10,000-15,000 rpm for 15min and discard the liquid.

20. Wash with 200µl of 80% EtOH then spin for 5min and discard the liquid. Next, do one quick spin for 10 seconds, and pipet with a sharp tip carefully to get rid of the last drop of ETOH. (Note: one could use RNA clean up kit instead of ETOH precipitation. RNA cleans up kit: ZYMO CAT#R1015.)

21. Dissolve probe in 100 µl of RNase-free water containing (ultrapure distilled water) 1µl (40 units) of RNase inhibitor, and measure concentration using NanoDrop Lite Spectrophotometer (Thermo Scientific).

22. Check the probe's quality by running 2.5 µl of the stock volume on a 0.8% agarose, 1X TBE or TAE minigel. Wash the apparatus thoroughly before preparing the gel and run the samples quickly to avoid problems with RNase.

2.6.3 *in situ* hybridization

DAY 1

Deparaffinization:

1. Rehydration (in fume hood)
   2x xylene, 5 minutes each
   2x 100% EtOH, 1 minute each

1x 70% EtOH, 1 minute
1x 1X PBS, 5 minutes

2. Fix in 4% PFA-DEPC for 20 min.

3. Rinse 1X in 1X PBS for 5 min.

4. Incubate slides in 0.2N HCl-DEPC at RT for 10 min (use slide mailers).

5. Rinse 2X in 1X PBS-DEPC for 5 min each.

6. Proteinase K (3 µg/ml 1X PBS-DEPC) @37*℃* for approximately 10 -15 min.

7. Rinse 1X in 1X PBS -DEPC.

8. Post -fix in 4% PFA -DEPC for 15 min.

9. Pre -hybridize sections in hybridization buffer for 3 hours at 58*℃*.

10. Prepare the probe in 100 µl of hybridization solution at a final concentration of 0.2 -0.4 ng/µl. Heat at 70*℃* for 5 min, then put on ice. Load the probe onto the tissue sections and coverslip.

11. Incubate slides O/N at 58*℃*.

DAY 2

12. Transfer the slides to a slide Mailer immersed in washing solution (enough to cover the slides) at 58ºC.
Wash 1X 15 min at 58*℃* to allow coverslips to fall off.
Wash 2X 30 min at 58*℃* in washing solution.

13. Wash 2X 30 min in 1X MABT.

Blocking and antibody staining:

14. Block at least 1 hour (2 -3 hours is desirable) in 1X MABT + 2% Blocking reagent + 20% heat inactivated sheep serum at RT (no coverslips).

15. Incubate O/N at 4*℃* with fresh 1X MABT + 2% Blocking reagent +20% sheep serum containing a 1 -1000 dilution of anti -DIG Alkaline Phosphatase antibody in a humidified chamber (with 1X PBS -DEPC). Use 100 µl per section and coverslip.

DAY 3

Post -antibody washes:

16. Transfer slides to a slide mailer immersed in 1X MABT (enough to cover the slides) Wash 4x 30 min each in 1X MABT.

17. Wash 2X, 10 min each in AP staining buffer. Use a shaker at RT for this step.

Staining reaction:

18. Add 1ml per slide of BM purple AP substrate and keep the slides in humidifier chamber

protected from light, for 1 -24 hours at RT to reveal the expression.

19. Wash 3X, 5 min each in PBS.

20. Dehydrate with EtOH:
    Wash slides 1X for 5 min in 70% EtOH.
    Wash slides 1X for 5 min in 95% EtOH.
    Wash slides 2X, for 5 min each in 100% EtOH.

21. Mount slides in permount for photography.

SOLUTIONS (all solutions are prepared with DEPC water)

4% PFA in PBS - mix 4g paraformaldehyde (PFA) with 100ml 1X PBS.
Place on a heating block until solution becomes clear, immediately filter into a bottle placed on ice aliquot and freeze. Aliquots can only be thawed once

10X Salt - 2M NACl, 100 mM Tris -HCl pH 7.5, 100mM phosphate buffer pH 7.4, 50 mM EDTA pH8 100 ml 5M NaCl 25 ml 1M Tris -HCl pH7.5 25 ml 0.5M EDTA 19.35 ml 1M Na2HPO4 5.65 ml 1M NaH2PO4 H2O QSP 250 ml

50X Denhardt's - 100 ml stored aliquoted at -20°C
1gm bovine serum albumin (1% W/V)
1gm Ficoll™ (1%W/V)
1gm polyvinylpyrrolidone (Mol.Wt. 360,000)
Make up to 100 ml with ddH_2O

Hybridization buffer - stored aliquots at -20°C

	10ml	**20ml**	**50ml**	**100ml**	**Final Conc**
1X salt	1ml	2ml	5ml	10ml	1X
Deionized formamide	5ml	10ml	25ml	50ml	50%
dextran sulphate	1gm	2gm	5gm	10gm	10%
Torula RNA 1 mg/ml	200µl	400µl	1ml	2ml	1mg/ml
50X Denhardt's	200µl	400µl	1ml	2ml	1X
ddH_2O	3.6ml	7.2ml	18ml	36ml	

Washing solution - 1X SSC, 50% formamide, 0.1% Tween20
1X MABT - 100 mM maleic acid, 150mM NaCl, 0.1% Tween20, pH 7.5

5X MAB - 21.91g NaCl 29.02g Maleic Acid pH to 7.5 with NaOH - first add 18g NaOH pellets, then adjust pH with concentrated NaOH solution H2O QSP 500ml

Blocking reagent - Roche CAT# 11 096 176 001. Make 10% stocks in 1X MAB (no tween20) and store at -20°C

Alkaline phosphatase (AP) buffer
**Prepare freshly just before use from stock solutions.

Stock Soln.	100ml	Final Conc.
5M NaCl	2 ml	100 mM
1M MgCl2	5 ml	50 mM
1M Tris pH 9.5	10 ml	100 mM
20% Tween 20	0.5 ml	0.1%
ddH2O	82.5 ml	- - - - -

2.7 Gene expression documentation

Gene expression domain and intensity were visually mapped and analyzed based on the pattern of the BM purple dye location. To document the gene expression domains and intensities, slides of the assayed tissue sections were photographed using a digital camera (Olympus, model: DP21, D21-CU) and dissection microscope (Olympus, model SZX2ILLK).

CHAPTER 3 - RESULTS

Recent work by Raj and Boughner (2016) flagged a new set of the genes that showed significant increases or decreases in their expression levels in the mandibular prominences (MdPs) of *TP63*-null mice (*TP63*$^{-/-}$) compared to wild-type littermates at embryonic days (E) 10-13. In this MSc thesis project, I characterized the expression domain and intensity of four of these genes - *Fermt1*, *Pltp, Cbln1, Krt8* - in mouse tooth organ using Antisense *Digoxigenin-labeled* RNA probe *in situ* hybridization. I hypothesized that *TP63* up-regulates *Fermt1* and *Pltp*, and down-regulates *Cbln1* and *Krt8*, in dental epithelial cells.

3.1 Digoxigenin-labeled RNA probe synthesis results

In this project, all the RNA probes were designed by me and this was the first time that these probes were used in embryonic mouse dental and jaw tissues. Therefore, after my antisense and sense *Digoxigenin-labeled* RNA probes were synthesized, to confirm the size of the probes I ran all of them on a 0.8% agarose gel. Figures 6 and 7 show the UV light view of these agarose gels. RNA probes usually appear as a smear instead of a single band. I used a DNA ladder (CAT# 10787018) as a guide for the RNA probe sizes. Smears located at about the size that was expected for each probe is evidence that the probe was successfully synthesized (Fig. 6, 7). Also, as another check, probes were sent for sequencing to Eurofins Genomics company, and sequencing results were blasted against published sequences of mRNA. This confirmed that my antisense probes were the reverse complements to published mRNA sequences (Appendix A). In contrast, sense probes (my negative controls) were same sequences as for published mRNA.

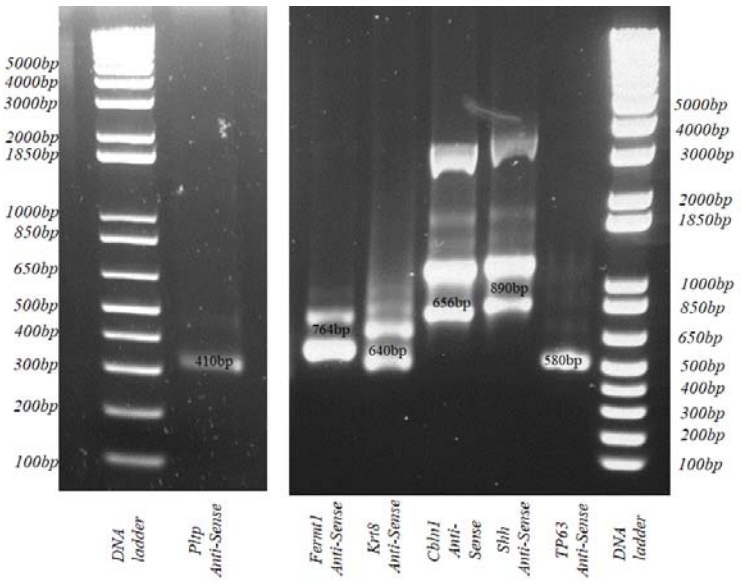

Figure 6. UV light view of an agarose gel showing antisense probes for six genes and smears approximately around the size that was expected.

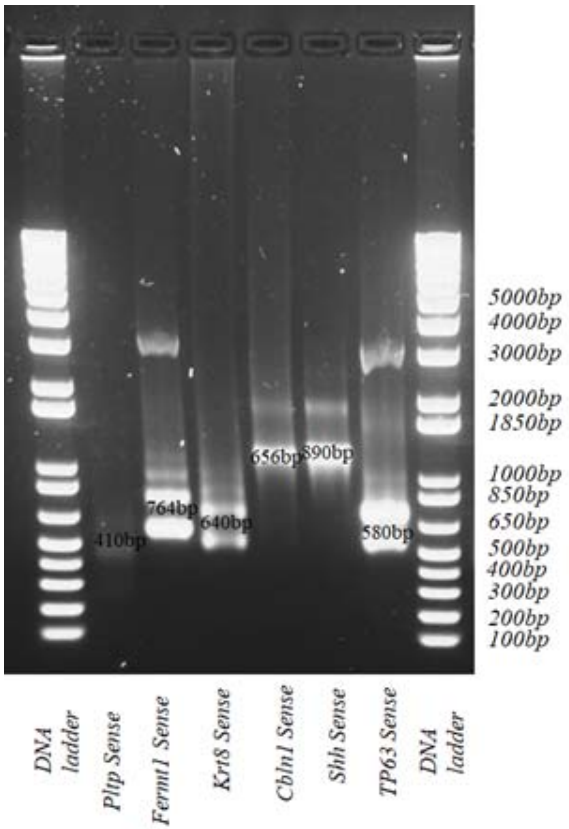

Figure 7. UV light view of an agarose gel showing sense probes for six genes and smears approximately around the size that was expected. The expected size for sense probe is same as antisense probe for the same gene.

In situ hybridization (ISH) assays probed coronal tissue sections of wild-type (*WT*) and *TP63*-null (*TP63*$^{-/-}$) embryos. Expression of *Fermt1, Pltp, Cbln1* and *Krt8* were observed in the tooth organs of both the upper and lower jaw primordia. Two different stages of tooth development - placode and bud stages - were assayed to compare differences and changes in expression level of the same four genes, in addition to control genes *TP63* and *Shh*. The latter gene is a well-known marker for early tooth formation (Seppala et al. 2017). Embryonic day (E) 13.5 is the stage when the tooth bud forms in wild-type mice, and E11.5 is about the time when dental placode formation is arrested in the *TP63*-null mice. In this study, genes of interest and control genes ISH were performed on adjacent sections of each individual.

3.2 The expression of *TP63*, *Shh*, and four novel genes (*Fermt1*, *Pltp, Cbln1,* and *Krt8*) in tooth organ and jaw at E13.5

I used H & E staining to help view and identify the tooth organ morphology (Fig. 8A). Hatched lines represent the dental epithelium in the tooth bud. My ISH results showed that, at bud stage, *TP63* expression in mice was restricted to oral and dental epithelium (Fig. 8B). *Shh* expression was restricted to the specific area of dental epithelium in both wild-type and *TP63*$^{-/-}$ mice which were used as a marker to specify the dental epithelium in both models (Fig 9A-B,10A-B). *Fermt1* expression in wild-type mouse was more intense in oral epithelium and dental epithelium than in dental mesenchyme (Fig. 9E). *Pltp* expression in the same wild-type individual was intense and more restricted to the dental epithelium than to the oral epithelium or dental mesenchyme (Fig. 9G). *TP63*, *Fermt1*, and *Pltp* expression domains in dental epithelium overlapped with each other in the wild-type embryos (Fig. 9A-C-E-G). Compared to their wild-type littermates, in the *TP63*$^{-/-}$ mice *Fermt1* was expressed at very low intensity in the oral and dental epithelium (Fig 9F). In the same *TP63*$^{-/-}$ mouse, *Pltp* expression was seen in the dental

epithelium but, compared to the littermate wild-type, the intensity of expression was decreased, notably in the dental epithelium (Fig. 9H). Decreased expression of *Fermt1* and *Pltp* in *TP63*$^{-/-}$ embryos were mostly in the same area, which according to *Shh* expression was marked as dental epithelium (Fig. 9B-D-F-H). Conversely, *Cbln1* did not show any expression in the dental epithelium, oral epithelium, and dental mesenchyme in the wild-type mice (Fig. 10E), although *Cbln1* was expressed intensely in the dental epithelium of *TP63*$^{-/-}$ littermates (Fig. 10F). *Krt8* expression was seen in both wild-type and *TP63*$^{-/-}$ littermatesand was restricted to the dental and oral epithelium in both mouse strains. Comparing *Krt8* expression intensity between wild-type and *TP63*$^{-/-}$ littermates showed higher expression in *TP63*$^{-/-}$ dental epithelium (Fig.10G&H). Changes in the level of expression of *Cbln1* and *Krt8* in, are mostly in the same area that *Shh* expression flagged as the dental epithelium and that overlaps with differences in *TP63* expression.

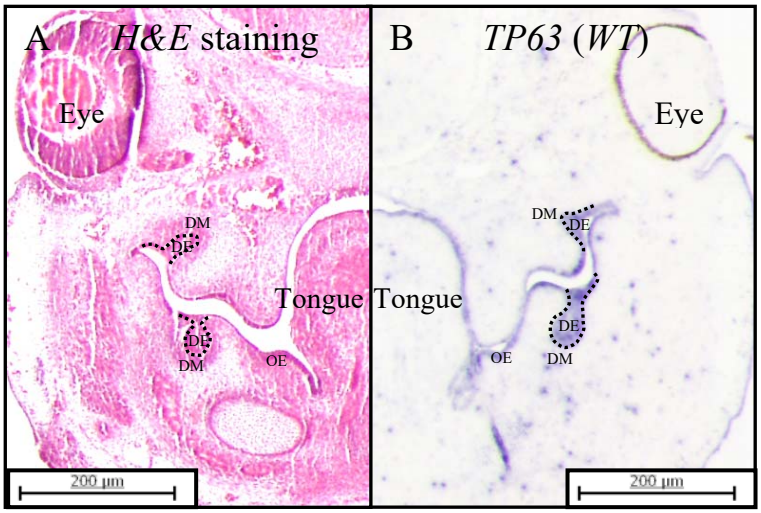

Figure 8. Coronal view of mouse head (**A**), showing tooth organ at bud stage (hatched lines are defining dental epithelium in tooth organ) and **(B)** *TP63* expression (purple) at E13.5 which is restricted to oral epithelium and dental organ. Oral Epithelium (OE), Dental Epithelium (DE), and Dental Mesenchyme (DM).

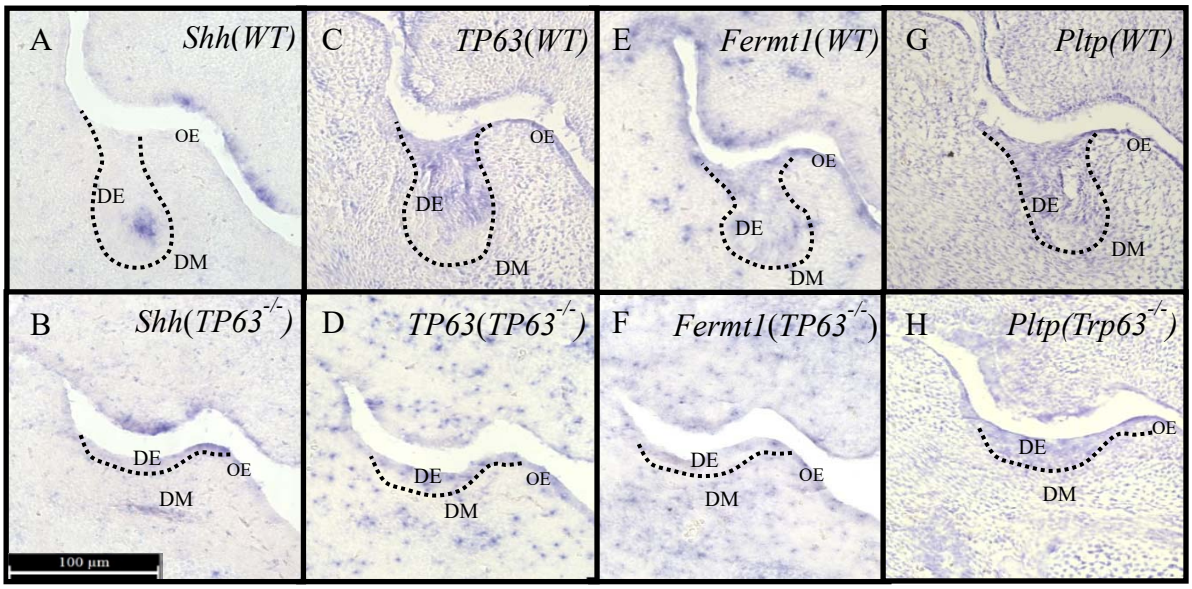

Figure 9. RNA *in situ* hybridization on coronal sections of tooth organ at E13.5 with expression of (**A, B**) *Shh* in dental placode used as positive controls in both *WT* (toothed) mice (top row) and *TP63*$^{-/-}$ (toothless) mice (bottom row) and as guides to show where teeth normally form. Hatched lines are schematic representation of histology of tooth bud morphology and in *TP63*$^{-/-}$ show the small thickening in oral epithelium, (**C, D**) Presence of *TP63* expression in dental epithelium and tooth organ in *WT* and absence of *TP63* expression in *TP63*$^{-/-}$ mouse was confirmed, (**E, F**) expression level of *Fermt1* in dental epithelium in *WT* and *TP63*$^{-/-}$ mice, (**G, H**) *Pltp* level of expression in dental epithelium in *WT* and *TP63*$^{-/-}$ mice. *Fermt1* and *Pltp* express in dental epithelium and expression intensity for these two genes in *TP63*$^{-/-}$ mice is lower than *WT*. Oral Epithelium (OE), Dental Epithelium (DE), and Dental Mesenchyme (DM)

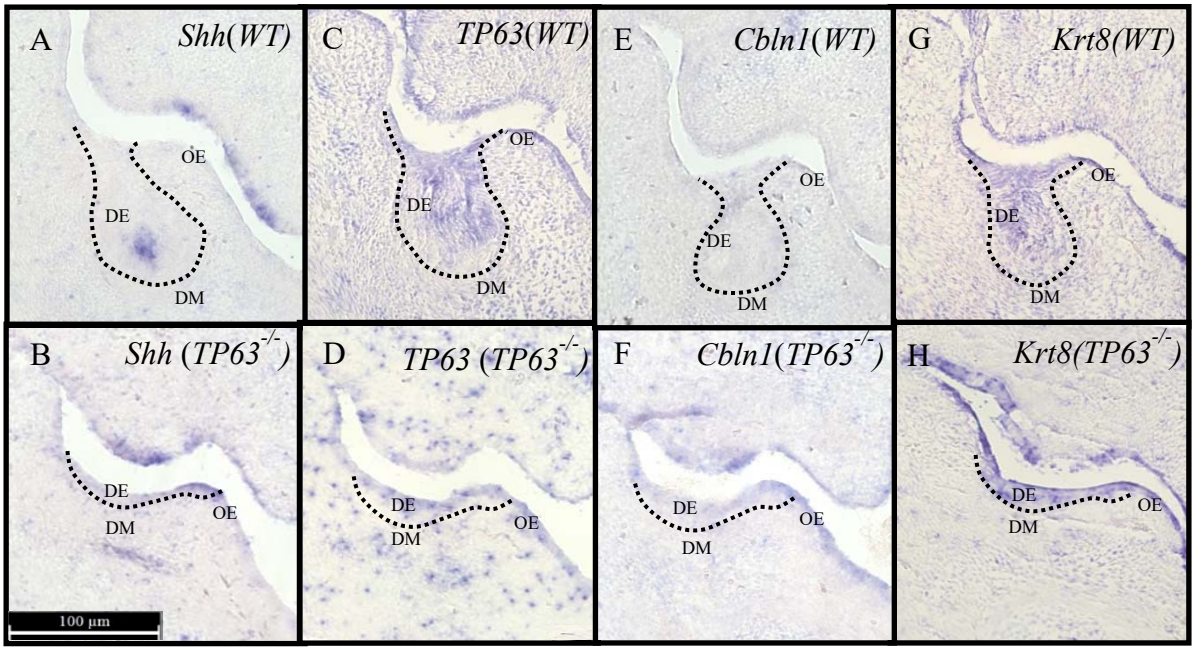

Figure 10. RNA *in situ* hybridization on coronal sections of tooth organ at E13.5 with expression of (**A, B**) *Shh* in dental placode that used as positive controls in both *WT* (toothed) mice (top row) and *TP63*$^{-/-}$ (toothless) mice (bottom row) and as guides to show where teeth normally form. Hatched lines are schematic representation of histology of tooth bud morphology and in *TP63*$^{-/-}$ show the small thickening in oral epithelium, (**C, D**) Presence of *TP63* expression in dental epithelium and tooth organ in *WT* and absence of *TP63* expression in *TP63*$^{-/-}$ mice was confirmed, (**E, F**) *Cbln1* level of expression in dental epithelium in *WT* and *TP63*$^{-/-}$ mice, (**G, H**) expression level of *Krt8* in dental epithelium in *WT* and *TP63*$^{-/-}$ mice. *Cbln1* and *Krt8* express in dental epithelium and expression intensity of these two genes in *TP63*$^{-/-}$ mice is higher than *WT*. Oral Epithelium (OE), Dental Epithelium (DE), and Dental Mesenchyme (DM).

.

3.3 The expression of *TP63, Shh*, and four novel genes (*Fermt1*, *Pltp, Cbln1,* and *Krt8*) in tooth organ and jaw at E11.5

After visually confirming the differences in expression level and domain of my four genes of interest at E13.5, I mapped the expression of these same four genes at E11.5. My *in situ* results at E11.5 align with my findings at E13.5, showing that these four genes expression domains are similar at early and later stages of tooth formation. Also, in the absence of functional *TP63*, *Fermt1* and *Pltp* expression decreased (Fig. 11), and *Cbln1* and *Krt8* expression increased (Fig. 12) in the dental epithelium at E11.5. These findings confirmed the previous microarray data regarding decrease and increase in the level of expression of these four genes at E11.5 and revealed that these changes happen in the same tooth organ domain as the later stage (E13.5).

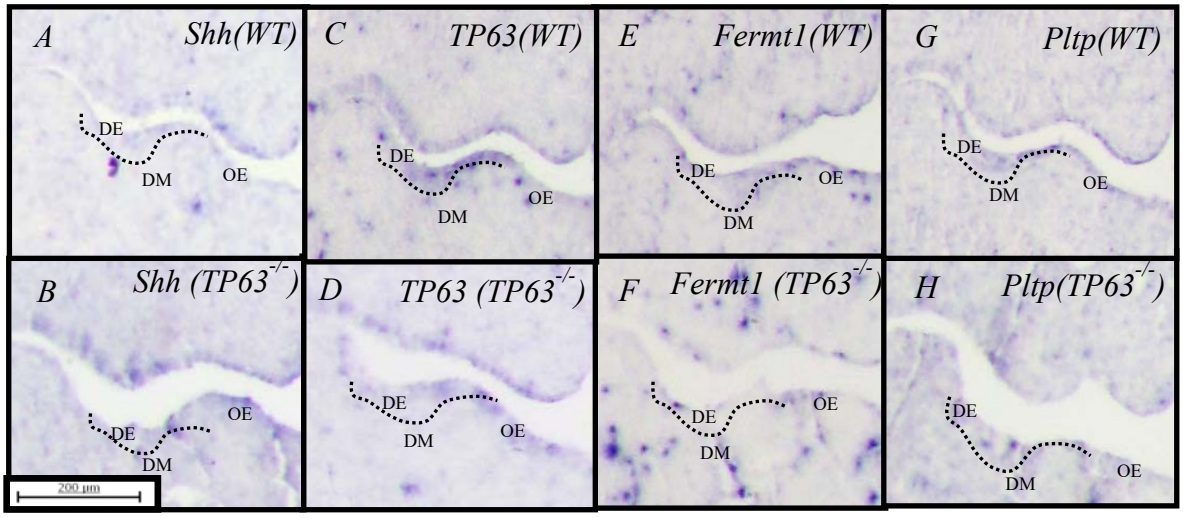

Figure 11. RNA *in situ* hybridization on coronal sections of tooth organ at E11.5 with expression of (**A, B**) *Shh* in dental placode that used as positive controls in both *WT* (toothed) mice (top row) and *TP63*$^{-/-}$ (toothless) mice (bottom row) and as guides to show where teeth normally form. Hatched line showing typical dental epithelium thickening morphology, (**C, D**) Presence of *TP63* expression in dental epithelium and tooth organ in *WT* and absence of *TP63* expression in *TP63*$^{-/-}$ mice was confirmed, (**E, F**) expression level of *Fermt1* in dental epithelium in *WT* and *TP63*$^{-/-}$ mice, (**G, H**) *Pltp* level of expression in dental epithelium in *WT* and *TP63*$^{-/-}$ mice. *Fermt1* and *Pltp* express in dental epithelium and expression intensity in *TP63*$^{-/-}$ mice is lower than *WT*. Oral Epithelium (OE), Dental Epithelium (DE), and Dental Mesenchyme (DM).

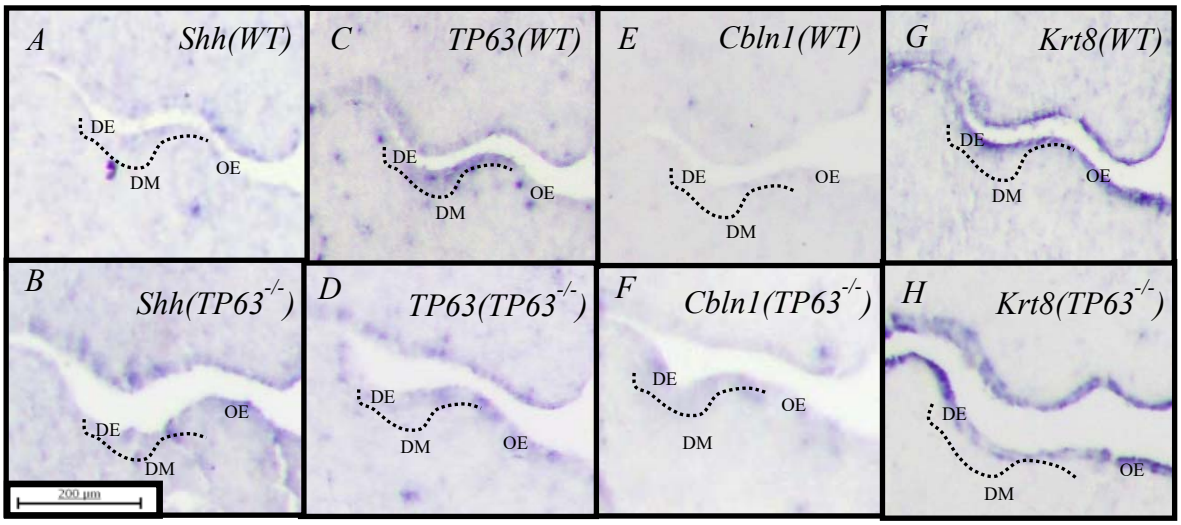

Figure 12. RNA *in situ* hybridization on coronal sections of tooth organ at E11.5 with expression of (**A, B**) *Shh* in dental placode that used as positive controls in both *WT* (toothed)mice (top row) and *TP63*$^{-/-}$ (toothless) mice (bottom row) and as guides to show where teeth normally form. Hatched line showing typical dental epithelium morphology, (**C, D**) Presence of *TP63* expression in dental epithelium and tooth organ in *WT* and absence of *TP63* expression in *TP63*$^{-/-}$ mice was confirmed, (**E, F**) *Cbln1* level of expression in dental epithelium in *WT* and *TP63*$^{-/-}$ mice. (**G, H**) expression level of *Krt8* in dental epithelium in *WT* and *TP63*$^{-/-}$ mice. *Cbln1* and *Krt8* express in dental epithelium and expression intensity in *TP63*$^{-/-}$ mice is higher than *WT.* Oral Epithelium (OE), Dental Epithelium (DE), and Dental Mesenchyme (DM).

3.4 Negative controls using sense probes

Specific sense probes were used as a negative control for each specific gene, to confirm the accuracy of antisense probes expressions (Fig. 13).

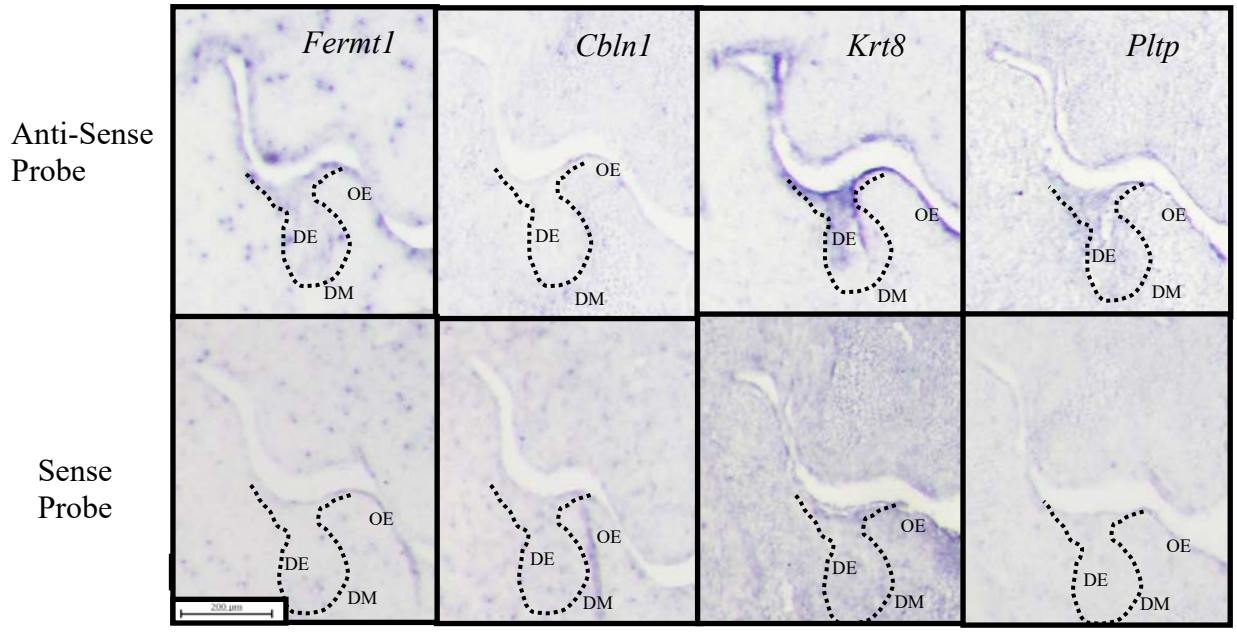

Figure 13. RNA *in situ* hybridization on coronal sections of tooth organ at E13.5*,* top row use of *antisense* RNA probes for *Fermt1, Pltp, Cbln1, and Krt 8* in *WT* mice, bottom row use of *sense* RNA probes for *Fermt1, Pltp, Cbln1, and Krt 8* in *WT* mice as a negative control for each probe.Oral Epithelium (OE), Dental Epithelium (DE), and Dental Mesenchyme (DM).

In summary, RNA *in situ* hybridization experiments revealed the expression domains of *Fermt1*, *Pltp*, *Cbln1*, and *Krt8* for the first time in mouse tooth organ. Also, these experiments visualized the correlation between the presence and absence of *TP63* with all four genes. Lastly, studying the expression domains and intensities of all four genes at two different time points during the earliest stages of odontogenesis suggested that each of these genes is targeted by *TP63* during early tooth formation. The expression of these four genes appears to be simultaneously affected by TP63 expression.

CHAPTER 4 - DISCUSSION AND CONCLUSIONS

4.1 The importance of the *TP63* transcription factor and its gene regulatory network in the oral dentition in mammals

TP63 is one of the main transcription factors required for onset of odontogenesis (Laurikkala et al. 2006). My RNA *in situ* hybridization experiments using E11.5 and E13.5 aged *TP63*$^{-/-}$ and wild-type mice visualized the expression domains of four genes of interest (*Fermt1, Pltp, Cbln1,* and *Krt8)* in the tooth organ. This thesis research also showed a strong correlation between the expression of *TP63* and that of the four other genes, *Fermt1, Pltp, Cbln1,* and *Krt8*, in embryonic tooth tissues. My work revealed that *Fermt1* and *Pltp* expression intensities decreased specifically in the dental epithelium in the absence of *TP63*. In contrast, *Cbln1*and *Krt8* expression intensities increased, also in the dental epithelium, in the absence of *TP63*. These results suggest that *TP63* up-regulates *Fermt1* and *Pltp*, and down-regulates *Cbln1* and *Krt8* in dental epithelial cells at early stages of tooth development.

Also, the expression domains and intensities of all four of these genes of interest in dental epithelium (which belongs to the first molar) are comparable between the lower and upper jaw primordia. This result is somewhat surprising because: 1) the molecular profiles that give rise to these two anatomically different facial structures differ (Lee et al. 2004), and; 2) even in the *TP63*$^{-/-}$ mouse, the lower jaw forms normally while the upper jaw is typically deformed (Paradis et al. 2013), including presenting with midfacial clefting (Thomason et al. 2010). These results suggest that the role that *Fermt1, Pltp, Cbln1,* and *Krt8* play is important and equivalent during the early stage of tooth development in both the upper and lower dentitions. In *TP63*$^{-/-}$ embryos the teeth but not jaws fail to form: comparing the level of expression of these four genes in dental

epithelium between toothed wild-type and toothless *TP63*$^{-/-}$ embryos suggests that *Fermt1, Pltp, Cbln1,* and *Krt8* belong to a *TP63*-controlled gene regulatory network that drives odontogenesis with neither input from, nor influence on, jaw morphogenesis (Fig. 14).

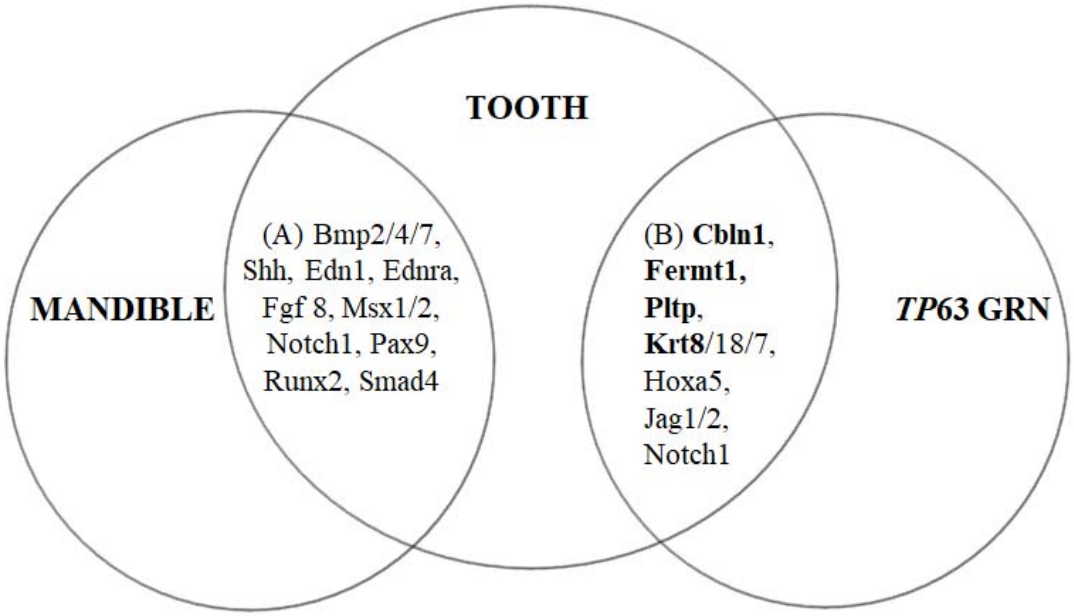

Figure 14. Venn diagram based on my recent ISH results, past microarray screens results and MGI/NCBI databases showing: **(A)** genes known to be important for mandible and tooth development that I posit do not act within a *TP63* odontogenic *GRN*; versus **(B)** candidate members of a *TP63* odontogenic *GRN* that have redundant/null roles for mandible morphogenesis. The four bold-font gene names (***Fermt1, Pltp, Cbln1, and Krt8***) are those genes presented here (Adapted from Raj & Boughner 2016).

These results also suggest that as early as E11.5 *TP63* plays a role in regulating the expression of all four genes of interest. E11.5 is an important stage of tooth development: first, the dental placode, which is the first signaling center, forms, and then regulates tooth bud formation. Second, in the *TP63*$^{-/-}$ mouse, tooth formation is arrested, and the dental placode fails to form the tooth bud, due to absent *TP63* and perturbed signaling pathways that are important for tooth formation. Indirectly, my results suggest that four genes of interest are important if not indispensable to early tooth morphogenesis.

The *TP63* up-regulation effect on *Fermt1* and *Pltp,* and its down-regulation effect on *Cbln1* and *Krt8* in dental epithelium in two important time points of tooth formation suggests that each of these new genes (*Fermt1, Pltp, Cbln1, and Krt8*) that I connected to the dental epithelium, and to the *TP63* gene regulatory network more broadly, are distinct downstream targets of *TP63*. As next steps to build on my thesis work, protein expression profiling, study of later stages of tooth development, and functional study of these four genes are needed to understand how the expression of these genes might change relatively to each other and to help test the reliability of my hypothesis that the four genes play a key role in earliest odontogenesis. In the next section I focus on the putative roles that each of the four genes might play in the developing tooth.

4.2 Putative roles of these four new candidate genes in a tooth-specific *TP63* gene regulatory network

Fermt1 has been studied intensively, with a focus on its function in keratinocytes and skin. Herz et al., (2006) showed that FERMT1 protein is expressed in epidermal keratinocytes, which are located near the skin basement membrane. This study also revealed that, due to FERMT1 deficiency in skin cells, the proliferation of keratinocytes was significantly reduced. Also, *in vitro* experiments confirmed that *Fermt1* deficiency in cultured keratinocytes caused the same disease phenotype seen in human skin (Herz et al. 2006). This phenotype, called *Kindler* syndrome, is a rare autosomal recessive genodermatosis caused by a mutation in *Fermt1*. Fragile and blistering skin are the main characteristics of this syndrome (Has et al. 2011). Also, work on *Fermt1* deficiency in keratinocytes showed that this gene encodes a protein with an important role in cell adhesion to the extracellular matrix, cell migration, and proliferation (Herz et al. 2006). Based on previous studies of FERMT1, this protein seems necessary for keratinocyte

homeostasis, binds to and activates integrin, and is involved in epithelial cell adhesion to the extracellular matrix (Calderwood et al. 2013).

Although in some databases (e.g., genecards.org), *Fermt1* expression in dental epithelium has been reported, I couldn't find a specific publication that reported the results and the exact expression domains or role in tooth organ for this gene. The novel ISH results that I presented in this thesis define the wild-type expression domain of *Fermt1* in dental epithelium during early odontogenesis, both placode and bud stages, and show decreased *Fermt1* expression in the dental epithelium of same-aged *TP63*-null mice. Since this gene is required for epithelial cell adhesion to the extracellular matrix, cell migration, my results along with those from previous studies suggest that *Fermt1* helps normal dental epithelium cell shape and polarity. I also suggest that *Fermt1* helps to maintain the bond between dental epithelium cells and their extracellular matrix and, consequently, the underlying mesenchyme. This physical bond helps the epithelium and mesenchyme to signal to each other, which is vital for dental epithelium maturation and proliferation, as well as for mesenchymal cell proliferation at later stages.

Next, *Pltp* has primarily been studied relative to lipid metabolism (Albers et al. 1995). In general, the majority of published studies on PLTP function focus on how this protein relates to high-density lipoprotein (*HDL*) and low-density lipoprotein (*LDL*) metabolism, and suggest that PLTP has a key role in lipoprotein metabolism and lipid transport (Albers et al. 2012). PLTP is secreted by a variety of tissues. 2003, O'Brien *et al*.'s study on human atherosclerosis (in which plaque accumulates inside arteries) suggested that PLTP accumulates on the extracellular matrix and acts as a connecting molecule to mediate the *HDL* binding to ECM. Many cellular processes during tooth development such as epithelium cells migration, proliferation, and differentiation affect cell adhesion to other cells and the ECM. Lipids in the epithelial cell membrane are crucial

for the maintenance of cell adhesion structures. These structures along with lipids in the cell membrane enable epithelial cells to attach to ECM (Márquez et al. 2008). Also Márquez et al. in 2012 revealed lipid composition in the epithelial cell membrane has a key role in maintaining cell adhesion to ECM. In 2012, Su et al. found that *TA-p63*$^{-/-}$ mice develop obesity: excess lipid accumulates in different tissues of *TA-p63*$^{-/-}$ mice. These results revealed that *TA-p63* isoforms are crucial for regulating lipid metabolism (Su et al. 2012). Other studies showed that *Pltp* has roles besides lipid metabolism, such as the transfer of alpha-tocopherol (the most common form of *Vitamin E*) between different cellular compartments, which needs the presence of PLTP (Kostner et al. 1995). A study by Vuletic et al. (2003) showed that PLTP is expressed in neurons in the human brain but, unfortunately, they did not clarify the functions of PLTP protein.

Reviewing previous studies on *Pltp* shows that no role has been reported for this gene in any developmental processes. For the first time, Raj and Boughner's (2016) associated *Pltp* expression with odontogenesis and *TP63*, and my results support this finding by showing that *Pltp* is expressed in early stages of tooth development in the dental epithelium. More broadly, and perhaps more closely related to development than lipid metabolism, many studies explain the role of lipids in cell-cell adhesion (Sumigray and Lechler 2015), mediating epithelial cells interactions with each other, or other cell types, and mediating cell signaling (Colgan 2002; Serhan et al. 1996), and finally, in cell structure and differentiation (Wertz 1992). This knowledge suggests a possible link between *Pltp* function and *TP63* regulatory effect on *Pltp*. More to the point, this knowledge suggests that the expression of *Pltp* in dental epithelium is important for transferring lipids or other essential components. Also, this past work suggests that *Pltp* is essential for regulating lipids on the dental epithelium membrane and, consequently, epithelial adhesion to the ECM. Thus *Pltp* may mediate the physical bond between dental

epithelium and underlying mesenchyme so they can interact and signal to each other and support the normal differentiation of the dental epithelium.

Cbln1 is well known for its role in the early stages of brain development (Miura et al. 2006) and this gene is widely expressed in the cerebellum (Slemmon et al. 1984, 1988). A study by Hirai *et al.* (2005) on a mouse mutant lacking *Cbln1* reported that this gene is expressed in the post or presynaptic neurons of Purkinje cells and is important for the integrity and normal arrangement of cerebellar Purkinje cells (Hirai et al. 2005). Other studies revealed a potential role for *Cbln1* in a signaling pathway that is essential for synapse plasticity and integrity, and found that CBLN1 is a secreted glycoprotein that acts as a signaling molecule in neurons (Miura et al. 2006). Related to my thesis work, perhaps *Cbln1* is among the genes that, according to molecular and immunohistochemical analyses of the first molar in mice, helps regulate development of the first trigeminal dental nerve. This nerve reaches the dental epithelium at E10, and axons start growing toward the tooth at the early bud stage (Lumsden 1988; Kettunen et al. 2005). The dental nerve also innervates the developing tooth dental mesenchyme (Luukko et al. 2005). Epithelial-mesenchymal interactions along with different signaling molecules appear to regulate local innervation of the tooth. At early stages of tooth development, signals from dental epithelium predominantly mediate tooth innervation (Kettunen et al. 2005; Moe et al. 2012). Since my work showed that *Cbln1* is expressed in epithelium (and at higher levels in the *TP63* mutant), perhaps *Cbln1* helps regulate these earliest stages of tooth innervation.

My ISH results during placode and bud stages of tooth development showed for the first time that *Cbln1* was significantly expressed in dental epithelium in the absence of *TP63*. Therefore, I propose that *Cbln1* expression has a signaling role in mesenchymal-epithelial interactions. I suggest that the balance in the level of this gene is important for tooth

development and more specifically tooth innervation, and that in the absence of *TP63*, *Cbln1* over-expression perturbs signaling between epithelium and underlying mesenchyme in *TP63*-null mice and, consequently, perturbs nerve growth in dental mesenchyme.

Krt8 is expressed in simple or single layered internal epithelial cells and is expressed extensively in carcinomas. In contrast to my other three genes of interest, *Krt8* has been associated with *TP63*, where *TP63* repressed the expression of *Krt8* and *Krt18* (De Rosa et al. 2009). Related to this finding, a study on KRT8 levels in epidermal tissues (Truong et al. 2006) reported that down-regulation of *ΔN-p63* resulted in the induction of KRT8. In addition, *Krt8* expression has been indirectly associated with *tenascin*, which is an extracellular matrix protein (Aufderheide and Ekblom in 1988). *Tenascin* is expressed in the mesenchyme around growing epithelia in the embryo and, more to the point here, regulates tooth development (Aufderheide and Ekblom 1988; Vainio et al. 1989). Also, *tenascin* is important for cell adhesion and cell interactions (Oberhauser et al. 1998). KRT8 has been associated with tumor progressions like cell adhesion and migration (Galarneau et al. 2007; Fortier et al. 2013). On the other hand, for the first time, my ISH results showed that *Krt8* expresses extensively in dental epithelium at early stages of tooth development in the *TP63*-null mouse. *TP63* is a tumor-suppressor and *Krt8* is involved in cancerous growth, which suggests that (as shown by past studies, noted above) *TP63* would logically suppress *Krt8*. Therefore, perhaps *Krt8* mediates dental epithelium cell adhesion and consequent interactions between epithelial cells and the underlying mesenchyme. In addition, possible functions for *Krt8* in apoptotic signaling were also suggested by Oshima in 2002. Programmed apoptosis is an important mechanism in bud formation and morphogenesis and shutting off the signaling centers during the tooth development. Also, a balance between cell division and apoptosis is essential for controlling the tooth shape and formation of the

appropriate tooth bud. Moreover, during the cell differentiation in an organ, apoptosis is the mechanism that controls the numbers cells (Matalova et al. 2004). From the above, another possibility is that *Krt8* regulates the apoptotic signals in dental epithelium cells that are going through differentiation.

In conclusion, based on the newly characterized *Fermt1, Pltp, Cbln1,* and *Krt8* expression domains - including the up-regulation effect of *TP63* on *Fermt1* and *Pltp*, and down-regulation effect of *TP63* on *Cbln1* and *Krt8* - during early stages of normal tooth development, I suggest that these four genes support the structural integrity, cell-cell adhesion, signaling and cross-talk between epithelial and underlying neural crest-derived mesenchymal cells. These four genes of interest along with *TP63* help mediate the epithelial-mesenchymal interactions that are required for dental epithelial cell maturation and proliferation during dental morphogenesis.

To explain the putative role for each of the genes of interest and the complicated mechanisms that they may help regulate during odontogenesis, further experiments are, of course, required. Although for some of these genes mutant organisms have been generated (Jiang et al. 1999; Ussar et al. 2008; Hirai et al. 2005), phenotype changes of teeth or during tooth development haven't been studied yet. Post translation and protein expression level profiling might be a good approach to test whether *TP63* interacts with any protein product of four new genes that I connected to *TP63*-mediated GRN (*Fermt1, Pltp, Cbln1,* and *Krt8*). Using techniques such as Chromatin Immunoprecipitation (ChIP) assay might be helpful to test weather *TP63* transcription factor directly regulates the expression of these genes (*Fermt1, Pltp, Cbln1,* and *Krt8*). Also, gene manipulation studies can help to understand the mechanism of function for each gene in odontogenesis. Functional experiments and study the phenotype changes of mutant organisms for each gene can lead us to more confident speculation about the function of these

genes. Other *in vitro* functional experiments and tissue engineering studies can help us to explain the role of these genes in epithelial structure, maturation and/or cell-cell cross-signaling.

Finally, since *TP63* is one of the major transcription factors required for initiating and regulating the development of epithelial cell layers, not only in mammals but also in other vertebrates, the proposed *TP63*-controlled gene regulatory network might provide broader insight about the evolution of the dentate jaw in vertebrates. However, so far, no published study has related my four candidate genes to odontogenesis in other vertebrates, although unpublished data suggests that at least some of these genes (*Pltp* and *Cbln1*) are expressed in shark (small-spotted catshark) tooth organ tissues (Gareth Fraser, Univ. Sheffield, pers. comm.). In the future, performing gene expression studies on other dentate vertebrates, such as evolutionarily ancient animals like garfish, might help link a tooth specific *TP63*-controlled gene regulatory network to earlier dentate ancestors to test whether the role of a *TP63* GRN in odontogenesis is deeply conserved.

CHAPTER 5 - REFERENCES

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Appendix A

Sequencing results from Eurofins Genomics company blasted against published sequences of mRNA in Basic Local Alignment Search Tool (Standard Nucleotide BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome)

Sonic hedgehog (*Shh*) synthesized anti-Sense probe with T7 polymerase:

Mus musculus sonic hedgehog (Shh), mRNA

Sequence ID: NM_009170.3 **Length:** 2727 **Number of Matches:** 1

Range 1: 339 to 1156 GenBank Graphics ▼ Next Match ▲ Previous I

Score	Expect	Identities	Gaps	Strand
1506 bits(815)	0.0	818/819(99%)	1/819(0%)	Plus/Minus

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Query  25    AGCAGGTGCGCGGCGGTGAGCAGCAGGCGCTCGCGCGGCTCCAGCGTCTCGATCACGTAG  84
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1156  AGCAGGTGCGCGGCGGTGAGCAGCAGGCGCTCGCGCGGCTCCAGCGTCTCGATCACGTAG  1097

Query  85    AAGACCTTCTTGGCGCCTTCGTCGCGGTCCAGGAAGGTGAGGAAGTCGCTGTACAGCAGC  144
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1096  AAGACCTTCTTGGCGCCTTCGTCGCGGTCCAGGAAGGTGAGGAAGTCGCTGTACAGCAGC  1037

Query  145   CGGCCCTGGTCGTCAGCCGCCAGCACGCGGTCTCCGGGACGTAAGTCCTTCACCAGCTTG  204
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1036  CGGCCCTGGTCGTCAGCCGCCAGCACGCGGTCTCCGGGACGTAAGTCCTTCACCAGCTTG  977

Query  205   GTGCCGCCCTGCTCCAGGTGCACGGTGGCGGATCCCGGGAAACAGCCGCCGGATTTGGCC  264
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Sbjct  976   GTGCCGCCCTGCTCCAGGTGCACGGTGGCGGATCCCGGGAAACAGCCGCCGGATTTGGCC  917

Query  265   GCCACGGAGTTCTCTGCTTTCACAGAACAGTGGATGTGAGCTTTGGATTCATAGTAGACC  324
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Sbjct  916   GCCACGGAGTTCTCTGCTTTCACAGAACAGTGGATGTGAGCTTTGGATTCATAGTAGACC  857

Query  325   CAGTCGAAACCTGCTTCCACAGCCAGGCGAGCCAGCATGCCGTACTTGCTGCGGTCCCGG  384
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Sbjct  856   CAGTCGAAACCTGCTTCCACAGCCAGGCGAGCCAGCATGCCGTACTTGCTGCGGTCCCGG  797

Query  385   TCGGACGTGGTGATGTCCACTGCTCGACCCTCATAGTGTAGAGACTCCTCTGAATGATGG  444
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Sbjct  796   TCGGACGTGGTGATGTCCACTGCTCGACCCTCATAGTGTAGAGACTCCTCTGAATGATGG  737

Query  445   CCGTCCTCATCCCAGCCCTCGGTCACTCGCAGCTTCACTCCAGGCCACTGGTTCATCACA  504
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Sbjct  736   CCGTCCTCATCCCAGCCCTCGGTCACTCGCAGCTTCACTCCAGGCCACTGGTTCATCACA  677

Query  505   GAGATGGCCAAGGCATTTAACTTGTCTTTGCACCTCTGAGTCATCAGCCGGTCTGCTCCC  564
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Sbjct  676   GAGATGGCCAAGGCATTTAACTTGTCTTTGCACCTCTGAGTCATCAGCCGGTCTGCTCCC  617

Query  565   GTGTTTTCCTCATCCTTAAATATGATGTCGGGGTTGTAATTGGGGGGTGAGTTCCTTAAA  624
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  616   GTGTTTTCCTCATCCTTAAATATGATGTCGGGGTTGTAATTGGGGG-TGAGTTCCTTAAA  558

Query  625   TCGTTCGGAGTTTCTTGTGATCTTCCCTTCATATCTGCCGCTGGCCCCTAGGGTCTTCTC  684
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Sbjct  557   TCGTTCGGAGTTTCTTGTGATCTTCCCTTCATATCTGCCGCTGGCCCCTAGGGTCTTCTC  498

Query  685   GGCTACGTTGGGAATAAACTGCTTGTAGGCTAAAGGGGTCAGCTTTTTGGGGTGCCGCCT  744
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Sbjct  497   GGCTACGTTGGGAATAAACTGCTTGTAGGCTAAAGGGGTCAGCTTTTTGGGGTGCCGCCT  438

Query  745   CTTTCCAAACCCCCTGCCGGGCCCACAGGCCAGCCCGGGGCACACCAGCAGCGAGGAAGC  804
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Sbjct  437   CTTTCCAAACCCCCTGCCGGGCCCACAGGCCAGCCCGGGGCACACCAGCAGCGAGGAAGC  378

Query  805   AAGGATCACCAGAAAACATCTGGCCAGCAGCAGCAGCAT  843
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Sbjct  377   AAGGATCACCAGAAAACATCTGGCCAGCAGCAGCAGCAT  339
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Sonic hedgehog (*Shh*) synthesized Sense probe with T3 polymerase:

Mus musculus sonic hedgehog (Shh), mRNA

Sequence ID: NM_009170.3 **Length:** 2727 **Number of Matches:** 1

Range 1: 358 to 1187 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1533 bits(830)	0.0	830/830(100%)	0/830(0%)	Plus/Plus

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Query  14    GATGTTTTCTGGTGATCCTTGCTTCCTCGCTGCTGGTGTGCCCCGGGCTGGCCTGTGGGC  73
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Sbjct  358   GATGTTTTCTGGTGATCCTTGCTTCCTCGCTGCTGGTGTGCCCCGGGCTGGCCTGTGGGC  417

Query  74    CCGGCAGGGGGTTTGGAAAGAGGCGGCACCCCAAAAAGCTGACCCCTTTAGCCTACAAGC  133
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Sbjct  418   CCGGCAGGGGGTTTGGAAAGAGGCGGCACCCCAAAAAGCTGACCCCTTTAGCCTACAAGC  477

Query  134   AGTTTATTCCCAACGTAGCCGAGAAGACCCTAGGGGCCAGCGGCAGATATGAAGGGAAGA  193
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Sbjct  478   AGTTTATTCCCAACGTAGCCGAGAAGACCCTAGGGGCCAGCGGCAGATATGAAGGGAAGA  537

Query  194   TCACAAGAAACTCCGAACGATTTAAGGAACTCACCCCCAATTACAACCCCGACATCATAT  253
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Sbjct  538   TCACAAGAAACTCCGAACGATTTAAGGAACTCACCCCCAATTACAACCCCGACATCATAT  597

Query  254   TTAAGGATGAGGAAAACACGGGAGCAGACCGGCTGATGACTCAGAGGTGCAAAGACAAGT  313
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Sbjct  598   TTAAGGATGAGGAAAACACGGGAGCAGACCGGCTGATGACTCAGAGGTGCAAAGACAAGT  657

Query  314   TAAATGCCTTGGCCATCTCTGTGATGAACCAGTGGCCTGGAGTGAAGCTGCGAGTGACCG  373
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Sbjct  658   TAAATGCCTTGGCCATCTCTGTGATGAACCAGTGGCCTGGAGTGAAGCTGCGAGTGACCG  717

Query  374   AGGGCTGGGATGAGGACGGCCATCATTCAGAGGAGTCTCTACACTATGAGGGTCGAGCAG  433
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Sbjct  718   AGGGCTGGGATGAGGACGGCCATCATTCAGAGGAGTCTCTACACTATGAGGGTCGAGCAG  777

Query  434   TGGACATCACCACGTCCGACCGGGACCGCAGCAAGTACGGCATGCTGGCTCGCCTGGCTG  493
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  778   TGGACATCACCACGTCCGACCGGGACCGCAGCAAGTACGGCATGCTGGCTCGCCTGGCTG  837

Query  494   TGGAAGCAGGTTTCGACTGGGTCTACTATGAATCCAAAGCTCACATCCACTGTTCTGTGA  553
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  838   TGGAAGCAGGTTTCGACTGGGTCTACTATGAATCCAAAGCTCACATCCACTGTTCTGTGA  897

Query  554   AAGCAGAGAACTCCGTGGCGGCCAAATCCGGCGGCTGTTTCCCGGGATCCGCCACCGTGC  613
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Sbjct  898   AAGCAGAGAACTCCGTGGCGGCCAAATCCGGCGGCTGTTTCCCGGGATCCGCCACCGTGC  957

Query  614   ACCTGGAGCAGGGCGGCACCAAGCTGGTGAAGGACTTACGTCCCGGAGACCGCGTGCTGG  673
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Sbjct  958   ACCTGGAGCAGGGCGGCACCAAGCTGGTGAAGGACTTACGTCCCGGAGACCGCGTGCTGG  1017

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Query  734   AAGGCGCCAAGAAGGTCTTCTACGTGATCGAGACGCTGGAGCCGCGCGAGCGCCTGCTGC  793
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Sbjct  1078  AAGGCGCCAAGAAGGTCTTCTACGTGATCGAGACGCTGGAGCCGCGCGAGCGCCTGCTGC  1137

Query  794   TCACCGCCGCGCACCTGCTCTTCGTGGCGCCGCACAACGACTCGGGGCCC  843
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Sbjct  1138  TCACCGCCGCGCACCTGCTCTTCGTGGCGCCGCACAACGACTCGGGGCCC  1187
```

Tumor Protein 63 (*TP63*) synthesized anti-Sense probe with T7 polymerase:

Mus musculus transformation related protein 63 (Trp63), transcript variant 5, mRNA

Sequence ID: NM_001127264.1 **Length:** 4707 **Number of Matches:** 1

Range 1: 3927 to 4417 GenBank Graphics ▼ Next Match ▲ Previous M

Score	Expect	Identities	Gaps	Strand
893 bits(483)	0.0	487/491(99%)	0/491(0%)	Plus/Minus

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Sbjct  4417  GTATTCAGGGGGGATATCAAGAAAACAAAAGCTAACTGGATTAACAAAGAGAATCCTGCT  4358

Query  80    TTCTATCCTATCTGCTTCACCACCAAGTGAAGGAATCCCATTCTCCACTGAGAGGCATCC  139
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Query  140   AGCAGATGTCTGTTCTCTGTGTTCTTCCCCACAGCTCTGGCTATTNANACACTAAACACT  199
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Sbjct  4297  AGCAGATGTCTGTTCTCTGTGTTCTTCCCCACAGCTCTGGCTATTCACACACTAAACACT  4238

Query  200   GGTGTGAGGAGACAAACTGTGCCTACCTATGGCTAATGCCAAAATCATCAGATACAGTAC  259
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Sbjct  4237  GGTGTGAGGAGACAAACTGTGCCTACCTATGGCTAATGCCAAAATCATCAGATACAGTAC  4178

Query  260   AGGAATTTCANNGTCCCTAGTGCCACCTAAATAGTGCTGAAATGCCACAGAGTCCCCTGT  319
             |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4177  AGGAATTTCAGAGTCCCTAGTGCCACCTAAATAGTGCTGAAATGCCACAGAGTCCCCTGT  4118

Query  320   GCCACACCAACACTGCTGAAACAGCCCAAATACTTACAACACACAGGAAGCCATTTGTCT  379
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Sbjct  4117  GCCACACCAACACTGCTGAAACAGCCCAAATACTTACAACACACAGGAAGCCATTTGTCT  4058

Query  380   TCCTAACATGGCTAAGACACAGCTTTCTTCTCCACCCATTGAATTTGTAAAGCTGTGAGT  439
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Sbjct  4057  TCCTAACATGGCTAAGACACAGCTTTCTTCTCCACCCATTGAATTTGTAAAGCTGTGAGT  3998

Query  440   TTGTTACAGAAGTTAATGATGCTTATCATTATTTAAACACAACAAAATGTTGAAATTAAG  499
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Sbjct  3997  TTGTTACAGAAGTTAATGATGCTTATCATTATTTAAACACAACAAAATGTTGAAATTAAG  3938

Query  500   GACTCATTTGC  510
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Sbjct  3937  GACTCATTTGC  3927
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Tumor Protein 63 (*TP63*) synthesized Sense probe with T3 polymerase:

Mus musculus transformation related protein 63 (Trp63), transcript variant 5, mRNA

Sequence ID: NM_001127264.1 **Length:** 4707 **Number of Matches:** 1

Range 1: 3933 to 4446 GenBank Graphics Next Match Previous M

Score	Expect	Identities	Gaps	Strand
946 bits(512)	0.0	513/514(99%)	0/514(0%)	Plus/Plus

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Sbjct  3933  GAGTCCTTAATTTCAACATTTTGTTGTGTTTAAATAATGATAAGCATCATTAACTTCTGT  3992

Query  84    AACAAACTCACAGCTTTACAAATTCAATGGGTGGAGAAGAAAGCTGTGTCTTAGCCATGT  143
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3993  AACAAACTCACAGCTTTACAAATTCAATGGGTGGAGAAGAAAGCTGTGTCTTAGCCATGT  4052

Query  144   TAGGAAGACAAATGGCTTCCTGTGTGTTGTAAGTATTTGGGCTGTTTCAGCAGTGTTGGT  203
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Sbjct  4053  TAGGAAGACAAATGGCTTCCTGTGTGTTGTAAGTATTTGGGCTGTTTCAGCAGTGTTGGT  4112

Query  204   GTGGCACAGGGGACTCTGTGGCATTTCAGCACTATTTAGGTGGCACTAGGGACTCTGAAA  263
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Sbjct  4113  GTGGCACAGGGGACTCTGTGGCATTTCAGCACTATTTAGGTGGCACTAGGGACTCTGAAA  4172

Query  264   TTCCTGTACTGTATCTGATGATTTTGGCATTAGCCATAGGTAGGCACAGTTTGTCTCCTC  323
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Sbjct  4173  TTCCTGTACTGTATCTGATGATTTTGGCATTAGCCATAGGTAGGCACAGTTTGTCTCCTC  4232

Query  324   ACACCAGTGTTTAGTGTGTGAATAGCCAGAGCTGTGGGGAAGAACACAGAGAACAGACAT  383
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4233  ACACCAGTGTTTAGTGTGTGAATAGCCAGAGCTGTGGGGAAGAACACAGAGAACAGACAT  4292

Query  384   CTGCTGGATGCCTCTCAGTGGAGAATGGGATTCCTTCACTTGGTGGTGAAGCAGATAGGA  443
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Sbjct  4293  CTGCTGGATGCCTCTCAGTGGAGAATGGGATTCCTTCACTTGGTGGTGAAGCAGATAGGA  4352

Query  444   TAGAAAGCAGGATTCTCTTTGTTAATCCAGTTAGCTTTTGTTTTCTTGATATCCCCCCTG  503
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4353  TAGAAAGCAGGATTCTCTTTGTTAATCCAGTTAGCTTTTGTTTTCTTGATATCCCCCCTG  4412

Query  504   AATACGTTGAGTATGAGAGATATGTGGGTTTTTT  537
             ||||||||||||||||||||||||||||||||||
Sbjct  4413  AATACGTTGAGTATGAGAGATATGTGGGTTTTTT  4446
```

Fermitin 1 (*Fermt1*) synthesized anti-Sense probe with T7 polymerase:

PREDICTED: Mus musculus fermitin family member 1 (Fermt1), transcript variant X3, mRNA

Sequence ID: XM_017318210.1 **Length:** 4855 **Number of Matches:** 1

Range 1: 1639 to 2333 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1280 bits(693)	0.0	694/695(99%)	0/695(0%)	Plus/Minus

```
Query  22    AGGNCCTCATCGAGCGTTTCGTTTTGGTCCTTGGATCGGGTGGACAAGAAGATGTAGCCA  81
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2333  AGGTCCTCATCGAGCGTTTCGTTTTGGTCCTTGGATCGGGTGGACAAGAAGATGTAGCCA  2274

Query  82    CCAATGTACTCATGGACAATCTTGCAATCTGCACTTAGGCAGGTGAATGCGATGGAGACG  141
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2273  CCAATGTACTCATGGACAATCTTGCAATCTGCACTTAGGCAGGTGAATGCGATGGAGACG  2214

Query  142   TTTTGGTCAAACTCGATTGCCACCTGCCGGATTTCCCAGTTGACATTCCACTGCTTCATA  201
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2213  TTTTGGTCAAACTCGATTGCCACCTGCCGGATTTCCCAGTTGACATTCCACTGCTTCATA  2154

Query  202   TTGGCGAATCTCCACGTTGTAACTGGTATCCCAGTGACCGCATCGATTCTAATCAACCTG  261
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2153  TTGGCGAATCTCCACGTTGTAACTGGTATCCCAGTGACCGCATCGATTCTAATCAACCTG  2094

Query  262   TTGTATGCAACTCCCAGGATGTCATCTTTTTTGCTTCCTTTAAATCTGACAAGGTAGTAG  321
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2093  TTGTATGCAACTCCCAGGATGTCATCTTTTTTGCTTCCTTTAAATCTGACAAGGTAGTAG  2034

Query  322   GTGAGGCCGAACTCAGGTAAGGATTGCCAGGCCTGGATGAACTGCAGTTTGGCTTCGACC  381
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2033  GTGAGGCCGAACTCAGGTAAGGATTGCCAGGCCTGGATGAACTGCAGTTTGGCTTCGACC  1974

Query  382   AGAGGCATCTGGGCTACGTTATGGTGAGCTTCCAGGATCCGGGCCGCCAGCTGTTTAGAC  441
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1973  AGAGGCATCTGGGCTACGTTATGGTGAGCTTCCAGGATCCGGGCCGCCAGCTGTTTAGAC  1914

Query  442   TTGTGCTTTTTTGCACAGCAGGGCGATACTAAACATTCCGGGTTCATATCCATGTTTTCA  501
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1913  TTGTGCTTTTTTGCACAGCAGGGCGATACTAAACATTCCGGGTTCATATCCATGTTTTCA  1854

Query  502   AGACTGGAAGCCACCAGAGGTGATGAGTTCCGGTTTTTCATCTTGAGAAACGAAAGGATG  561
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1853  AGACTGGAAGCCACCAGAGGTGATGAGTTCCGGTTTTTCATCTTGAGAAACGAAAGGATG  1794

Query  562   CTGATGACCTCTGGCTGATAGGAGCTGTCTGCCATAGTTTTACCCTTCGATGCCAAGATG  621
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1793  CTGATGACCTCTGGCTGATAGGAGCTGTCTGCCATAGTTTTACCCTTCGATGCCAAGATG  1734

Query  622   CAGGCAGCCATCCATCGGGCATATTGATCCTCATGGTCACATCTCAAGTACACTTCATTC  681
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1733  CAGGCAGCCATCCATCGGGCATATTGATCCTCATGGTCACATCTCAAGTACACTTCATTC  1674

Query  682   ATACCATCAGCAACGGGGATTAGTAACTTGATTCC  716
             |||||||||||||||||||||||||||||||||
Sbjct  1673  ATACCATCAGCAACGGGGATTAGTAACTTGATTCC  1639
```

Fermitin 1 (*Fermt1*) synthesized Sense probe with T3 polymerase:

PREDICTED: Mus musculus fermitin family member 1 (Fermt1), transcript variant X3, mRNA

Sequence ID: XM_017318210.1 **Length:** 4855 **Number of Matches:** 1

Range 1: 1662 to 2364 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1299 bits(703)	0.0	703/703(100%)	0/703(0%)	Plus/Plus

```
Query  10    TGCTGATGGTATGAATGAAGTGTACTTGAGATGTGACCATGAGGATCAATATGCCCGATG  69
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1662  TGCTGATGGTATGAATGAAGTGTACTTGAGATGTGACCATGAGGATCAATATGCCCGATG  1721

Query  70    GATGGCTGCCTGCATCTTGGCATCGAAGGGTAAAACTATGGCAGACAGCTCCTATCAGCC  129
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1722  GATGGCTGCCTGCATCTTGGCATCGAAGGGTAAAACTATGGCAGACAGCTCCTATCAGCC  1781

Query  130   AGAGGTCATCAGCATCCTTTCGTTTCTCAAGATGAAAAACCGGAACTCATCACCTCTGGT  189
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1782  AGAGGTCATCAGCATCCTTTCGTTTCTCAAGATGAAAAACCGGAACTCATCACCTCTGGT  1841

Query  190   GGCTTCCAGTCTTGAAAACATGGATATGAACCCGGAATGTTTAGTATCGCCCTGCTGTGC  249
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1842  GGCTTCCAGTCTTGAAAACATGGATATGAACCCGGAATGTTTAGTATCGCCCTGCTGTGC  1901

Query  250   AAAAAAGCACAAGTCTAAACAGCTGGCGGCCCGGATCCTGGAAGCTCACCATAACGTAGC  309
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1902  AAAAAAGCACAAGTCTAAACAGCTGGCGGCCCGGATCCTGGAAGCTCACCATAACGTAGC  1961

Query  310   CCAGATGCCTCTGGTCGAAGCCAAACTGCAGTTCATCCAGGCCTGGCAATCCTTACCTGA  369
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1962  CCAGATGCCTCTGGTCGAAGCCAAACTGCAGTTCATCCAGGCCTGGCAATCCTTACCTGA  2021

Query  370   GTTCGGCCTCACCTACTACCTTGTCAGATTTAAAGGAAGCAAAAAAGATGACATCCTGGG  429
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2022  GTTCGGCCTCACCTACTACCTTGTCAGATTTAAAGGAAGCAAAAAAGATGACATCCTGGG  2081

Query  430   AGTTGCATACAACAGGTTGATTAGAATCGATGCGGTCACTGGGATACCAGTTACAACGTG  489
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2082  AGTTGCATACAACAGGTTGATTAGAATCGATGCGGTCACTGGGATACCAGTTACAACGTG  2141

Query  490   GAGATTCGCCAATATGAAGCAGTGGAATGTCAACTGGGAAATCCGGCAGGTGGCAATCGA  549
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2142  GAGATTCGCCAATATGAAGCAGTGGAATGTCAACTGGGAAATCCGGCAGGTGGCAATCGA  2201

Query  550   GTTTGACCAAAACGTCTCCATCGCATTCACCTGCCTAAGTGCAGATTGCAAGATTGTCCA  609
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2202  GTTTGACCAAAACGTCTCCATCGCATTCACCTGCCTAAGTGCAGATTGCAAGATTGTCCA  2261

Query  610   TGAGTACATTGGTGGCTACATCTTCTTGTCCACCCGATCCAAGGACCAAAACGAAACGCT  669
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2262  TGAGTACATTGGTGGCTACATCTTCTTGTCCACCCGATCCAAGGACCAAAACGAAACGCT  2321

Query  670   CGATGAGGACCTCTTCCACAAACTGACTGGCGGTCAGGACTGA  712
             |||||||||||||||||||||||||||||||||||||||||||
Sbjct  2322  CGATGAGGACCTCTTCCACAAACTGACTGGCGGTCAGGACTGA  2364
```

Cerebellin 1 (*Cbln1*) synthesized anti-Sense probe with T7 polymerase:

Mus musculus cerebellin 1 precursor protein (Cbln1), mRNA

Sequence ID: NM_019626.3 **Length:** 2345 **Number of Matches:** 1

Range 1: 325 to 920 GenBank Graphics ▼ Next Match ▲ Previous Ma

Score	Expect	Identities	Gaps	Strand
1062 bits(575)	0.0	587/596(98%)	1/596(0%)	Plus/Minus

```
Query  13   CGAGG-ANCCNGAGAAGGNTGAGTACTTCCAGCCCCCCATCAAGTTCCCCCGCTCCNGCT  71
            ||||| | || |||||||| ||||||||||||||||||||||||||||||||||| |||
Sbjct  920  CGAGGAATCCAGAGAAGGTTGAGTACTTCCAGCCCCCCATCAAGTTCCCCCGCTCCAGCT  861

Query  72   TGAGGTATGCTCGGTCGCCTTTCTCCATCTGGATGAGGACGCCGTTGCTGGCGGCCTNNN  131
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  860  TGAGGTATGCTCGGTCGCCTTTCTCCATCTGGATGAGGACGCCGTTGCTGGCGGCCTCGC  801

Query  132  GTGTCACGTCTTGGTCACCGGCGAAGGCTGAAATCACCGGCCACCCGTTCAACATGAGGC  191
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  800  GTGTCACGTCTTGGTCACCGGCGAAGGCTGAAATCACCGGCCACCCGTTCAACATGAGGC  741

Query  192  TCACCTGGATGGTCTGTCTGTTGTAGACTTTCACCACGTGGAAGTTAAAACTGTAGATGC  251
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  740  TCACCTGGATGGTCTGTCTGTTGTAGACTTTCACCACGTGGAAGTTAAAACTGTAGATGC  681

Query  252  CTTTGCGCGGGGCGATGAAAGTGCTGCGTTCTGAGTCAAAGTTGTTCCCGATGTTCACTA  311
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  680  CTTTGCGCGGGGCGATGAAAGTGCTGCGTTCTGAGTCAAAGTTGTTCCCGATGTTCACTA  621

Query  312  GTACCTGGTCGAAGTAGATGATCATGGTGCGATTACTCATCTCGGACGGCTCATGGTTGG  371
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  620  GTACCTGGTCGAAGTAGATGATCATGGTGCGATTACTCATCTCGGACGGCTCATGGTTGG  561

Query  372  TGCTCCTGATGGCAGAGAAAGCCACCTTGGCGCTGCCGGAGCGCACAGAGATGCCCAGAG  431
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  560  TGCTCCTGATGGCAGAGAAAGCCACCTTGGCGCTGCCGGAGCGCACAGAGATGCCCAGAG  501

Query  432  CAGTGCCCGTAGGGTCAGACGTGGGGTTGGAGTCACACACCACCAGGCACTTGCCCTCCA  491
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  500  CAGTGCCCGTAGGGTCAGACGTGGGGTTGGAGTCACACACCACCAGGCACTTGCCCTCCA  441

Query  492  GTACGATGGGCTCTGTCTCATTCTGCCCGCGGGCTGGGCCTGCCAGCCACGCAGTCCCCA  551
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  440  GTACGATGGGCTCTGTCTCATTCTGCCCGCGGGCTGGGCCTGCCAGCCACGCAGTCCCCA  381

Query  552  ACAGCAGCAGCTCCACGACGCCCAGCATCGCGCCTCCGGCGCCCACCCCGCCTCCC  607
            ||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  380  ACAGCAGCAGCTCCACGACGCCCAGCATCGCGCCTCCGGCGCCCACCCCGCCCCCC  325
```

Cerebellin 1 (*Cbln1*) synthesized Sense probe with T3 polymerase:

Mus musculus cerebellin 1 precursor protein (Cbln1), mRNA

Sequence ID: NM_019626.3 **Length:** 2345 **Number of Matches:** 1

Range 1: 355 to 943 GenBank Graphics ▼ Next Match ▲ Previou

Score	Expect	Identities	Gaps	Strand
1085 bits(587)	0.0	588/589(99%)	0/589(0%)	Plus/Plus

```
Query  26   GCTGGGCGTCGTGGAGCTGCTGCTGTTGGGGACTGCGTGGCTGGCAGGCCCNGCCCGCGG  85
            ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  355  GCTGGGCGTCGTGGAGCTGCTGCTGTTGGGGACTGCGTGGCTGGCAGGCCCAGCCCGCGG  414

Query  86   GCAGAATGAGACAGAGCCCATCGTACTGGAGGGCAAGTGCCTGGTGGTGTGTGACTCCAA  145
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  415  GCAGAATGAGACAGAGCCCATCGTACTGGAGGGCAAGTGCCTGGTGGTGTGTGACTCCAA  474

Query  146  CCCCACGTCTGACCCTACGGGCACTGCTCTGGGCATCTCTGTGCGCTCCGGCAGCGCCAA  205
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  475  CCCCACGTCTGACCCTACGGGCACTGCTCTGGGCATCTCTGTGCGCTCCGGCAGCGCCAA  534

Query  206  GGTGGCTTTCTCTGCCATCAGGAGCACCAACCATGAGCCGTCCGAGATGAGTAATCGCAC  265
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  535  GGTGGCTTTCTCTGCCATCAGGAGCACCAACCATGAGCCGTCCGAGATGAGTAATCGCAC  594

Query  266  CATGATCATCTACTTCGACCAGGTACTAGTGAACATCGGGAACAACTTTGACTCAGAACG  325
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  595  CATGATCATCTACTTCGACCAGGTACTAGTGAACATCGGGAACAACTTTGACTCAGAACG  654

Query  326  CAGCACTTTCATCGCCCCGCGCAAAGGCATCTACAGTTTTAACTTCCACGTGGTGAAAGT  385
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  655  CAGCACTTTCATCGCCCCGCGCAAAGGCATCTACAGTTTTAACTTCCACGTGGTGAAAGT  714

Query  386  CTACAACAGACAGACCATCCAGGTGAGCCTCATGTTGAACGGGTGGCCGGTGATTTCAGC  445
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  715  CTACAACAGACAGACCATCCAGGTGAGCCTCATGTTGAACGGGTGGCCGGTGATTTCAGC  774

Query  446  CTTCGCCGGTGACCAAGACGTGACACGCGAGGCCGCCAGCAACGGCGTCCTCATCCAGAT  505
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  775  CTTCGCCGGTGACCAAGACGTGACACGCGAGGCCGCCAGCAACGGCGTCCTCATCCAGAT  834

Query  506  GGAGAAAGGCGACCGAGCATACCTCAAGCTGGAGCGGGGGAACTTGATGGGGGGCTGGAA  565
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  835  GGAGAAAGGCGACCGAGCATACCTCAAGCTGGAGCGGGGGAACTTGATGGGGGGCTGGAA  894

Query  566  GTACTCAACCTTCTCTGGATTCCTCGTGTTTCCCCTCTGACTGGCACGT  614
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  895  GTACTCAACCTTCTCTGGATTCCTCGTGTTTCCCCTCTGACTGGCACGT  943
```