CHAPTER I

INTRODUCTION

1.1 Rationale of the study

Mutations of *TP63* genes have been known to be the causes of at least five different ectodermal dysplasia (ED) syndromes, including Ectrodactyly-Ectodermal dysplasia-Cleft lip/palate Syndrome (EEC3; MIM 604292) (Celli et al., 1999), Ankyloblepharon-Ectodermal defects-Cleft lip/palate Syndrome (AEC; MIM 106260) (McGrath et al., 2001), Rapp-Hodgkin Syndrome (RHS; MIM 129400) (Kantaputra et al., 2003), Limb-Mammary Syndrome (LMS; MIM 603543) (van Bokhoven et al., 2001), and Acro-Dermato-Ungual-Lacrimal-Tooth Syndrome (ADULT; MIM 103285) (Propping et al., 2000). The ectodermal defects found in these *TP63*-associated syndromes consist of skin defects, missing or abnormally-shaped (small or conical) teeth, sparse hair, eyebrows, and eyelashes, and breast abnormalities such as aplastic or hypoplastic mammary glands or nipples, other exocrine glands such as sweat glands, salivary glands (McGrath et al., 2001; Rinne et al., 2007; van Bokhoven et al., 1999; van Bokhoven et al., 2001; van Bokhoven and Brunner, 2002). Furthermore, the additional anomalies in EEC syndrome are lacrimal duct abnormalities, conductive hearing loss, facial dysmorphism, chronic respiratory infections, urinary tract problems and developmental delay (Celli et al., 1999; Wessagowit et al., 2000).

In addition to ectodermal dysplasia syndromes, isolated or non-syndromic split hand-split foot malformation (SHFM4, MIM 605289) (Ianakiev et al., 2000) and non-syndromic cleft lip (Leoyklang et al., 2006) have been described as being caused by mutations in *TP63*. It was hypothesized that besides non-syndromic cleft lip/palate, *TP63* might be responsible for non-syndromic hypodontia, non-syndromic orofacial clefts and syndromic hypodontia with/without orofacial clefts.

This study examined *TP63* mutations in patients with non-syndromic hypodontia and non-syndromic orofacial clefts and syndromic hypodontia with/without orofacial clefts. The methods were composed of case selection, genomic DNA preparation, polymerase chain reaction (PCR) and direct gene sequencing, and data analysis. The results of the mutations and of single nucleotide polymorphisms (SNPs) of *TP63* were collected.

1.2 Literature review

1.2.1 *p63* and its structure and functions

p63 is a homologue of *p73* and of the prototypic tumor suppressor gene *p53* (Yang et al., 1998). *p63* and *p73* have much greater molecular complexity than does *p53* because they are expressed both as multiple, alternatively-spliced, C-terminal isoforms, and as N-terminally-deleted, dominant-negative proteins that have reciprocal functional regulation (Melino et al., 2003). *TP63* is located on human chromosome 3q27-29 (Yang et al., 1998), consisting of 16 exons with approximately 220 kb, and introns approximately 100 kb in length (Barbieri and Pietenpol, 2006). *TP63* was previously thought to have 16 exons (Yang et al., 1998). There are significant differences between *p63* and *p53*. *p63* expresses 10 known mRNA isoforms (Mangiulli et al., 2009). Each isoform expresses its protein by encoding a DNA-binding domain (DBD) and an oligomerization (tetramerization) domain (OD). The DBD of each isoform in human TAp63 is located at amino acids 142-321 and is approximately 60% identical, at the amino acid level, to that of p53. The OD is located at amino acids 353-397 and is about 37% identical to that of p53. The DNA-binding domains of p53 and p63 are highly homologous; all the amino acid residues in the p53 DNA-binding domain that directly contact DNA, or coordinate with a zinc ion necessary for DNA binding activity, are 100% conserved in the p63 DNA-binding domain. *p53* has only one promoter, but *p63* has at least two separate promoters directing the expression of two different classes of proteins that either contain or lack an N-terminal transactivation (TA) domain. Isoforms that occupy an N-terminal acidic TA domain with low homology to the transactivation domain of p53 (22%

identical) are known as TA isoforms, whereas isoforms that lack a TA domain are known as ΔN isoforms (Barbieri and Pietenpol, 2006). TAp63 isoforms are generated by the activity of a promoter upstream of exon 1, whereas ΔNp63 isoforms are generated by an alternative promoter located in intron 3. Each TA- and ΔN-transcript is alternatively spliced to produce five different isoforms, truncating at the C-termini, denoted α, β, γ, δ, and ε. Altogether, the *TP63* gene expresses at least ten mRNA variants, which encode for ten different p63 protein isoforms: TAp63α, TAp63β, TAp63γ, TAp63δ, TAp63ε, ΔNp63α, ΔNp63β, ΔNp63γ, ΔNp63δ, and ΔNp63ε **(Figure 1.1)**. Each p63 isoform has a DBD and an OD (Mangiulli et al., 2009).

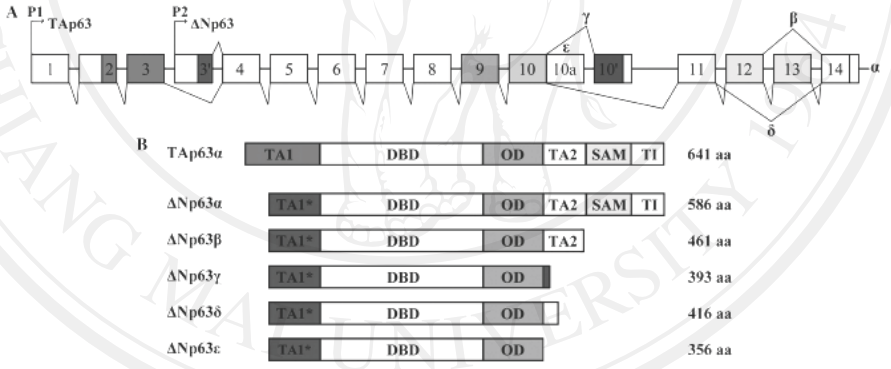


Figure 1.1 Structures of p63 proteins. The *TP63* gene is transcribed into ten different protein isoforms that originate from two distinct promoters, generating a transactivation domain (TA) or lack of it (ΔN). α isoforms have additional C-terminal domains, a sterile alpha motif (SAM) domain and a transactivation inhibitory domain (TID) (reproduced from Mangiulli et al., 2009).

The α isoform, the longest isoform, contains a sterile-α-motif (SAM) domain, located in human TAp63, at aminio acids 502–567, encoded primarily within exon 14 (Thanos and Bowie, 1999). In the proteins involved in the regulation of different developmental processes, SAM domains, which are highly conserved, are crucial for developmental processes (Schultz et al., 1997). The SAM domain is known to function as a protein-protein interaction via homodimerization, or via heterodimerization with other SAM domains. The SAM domain is also known to interact with RNA (Barbieri and Pietenpol, 2006; Radoja et al., 2007; Thanos and Bowie, 1999). Interestingly, the SAM domain of p63 is not associated with dimerization but is essential for transcriptional activity (Scoumanne et al., 2005). It has been hypothesized that the SAM domain may also be involved in apoptosis, transcriptional activation, focal adhesion, chromatin remodeling, receptor tyrosine kinase signaling, and SUMOylation (Westfall and Pietenpol, 2004). The SAM domain of p63 is important for regulatory transcription of Dlx3. Both Dlx3 and p63 are essential for epidermal morphogenesis. Therefore, the transcription of Dlx3 is abolished by mutations, in the SAM domain of *TP63,* that are associated with AEC syndrome. This may be the reason why dental anomalies, especially enamel hypoplasia, are more common in patients with Rapp-Hodgkin Syndrome or AEC syndrome than in patients with other *TP63*-associated syndromes, as the two former syndromes are caused by mutations in the SAM domain (Radoja et al., 2007).

The p63α isoforms contain a transactivation inhibitory (TI) domain that is able to physically bind and regulate the TA domain, creating an intramolecular regulatory mechanism (Ghioni et al., 2002; Serber et al., 2002). The splicing isoforms lacking the TI domain possess stronger transactivation activity. TAp63α, with no TI domain,

has the weakest transactivation activity. ΔNp63 isoforms are capable of forming complexes with TAp63 isoforms, increasing their stability, while keeping them inactive (Serber et al., 2002). Although only the TAp63 isoforms were initially considered as transactivating molecules, there is some evidence showing that ΔNp63 proteins can act both as transcriptional activators and repressors (Barbieri and Pietenpol, 2006; Ghioni et al., 2002; Mikkola, 2007; Westfall et al., 2003; Yang et al., 1998).

The TA isoforms can bind to DNA through a p53-responsive element (p53RE) and can activate transcription of target genes. This process may induce cell cycle arrest or apoptosis. However, the ΔNp63 isoforms are able to bind to DNA through p53RE and can exert dominant-negative effects over p53, p63, and p73 activities by either competing for DNA binding sites or by direct protein interaction (Benard et al., 2003). By expressing a variety of isoforms, p63 has the ability to regulate a great number of genes with great diversity of roles. The p63 isoforms possess opposing regulatory effects among themselves, the type of which depending on the type of isoform used. Each isoform has specific biological and biochemical activities. It has been demonstrated in mouse embryos that at Embryonic Day 7.5 (E7.5), TAp63 isoforms are the first to be detected in the single-layered surface ectoderm; subsequently the ectoderm starts epithelial stratification. Then, there is a transition from TAp63 to ΔNp63 isoforms. At approximately E9.5, ΔNp63 isoforms gradually replace TAp63 isoforms and are required for epidermal maturation (Koster and Roop, 2004a; b) **(Figure 1.2)**. In addition, in mature epidermis, ΔNp63α is required for the maintenance of basal keratinocytes to proliferate efficiently (King et al., 2003; Koster and Roop, 2004a; b; Parsa et al., 1999; Westfall et al., 2003).

TAp63 isoforms are also known to inhibit terminal differentiation and their functions are counterbalanced by ΔNp63 isoforms to allow cells to respond to signals required for the differentiation and maturation of embryonic epidermis (Koster and Roop, 2004a; b; Murray-Zmijewski et al., 2006). However, not all TAp63 isoforms can induce transactivation. Both TAp63β and TAp63γ are able to activate reporter genes containing consensus p53-binding sites, but TAp63α lacks of this ability (Yang et al., 1998). ΔNp63α, ΔNp63β and ΔNp63γ are capable of transactivating p53-responsive genes. ΔNp63 isoforms are capable of blocking the function of p53 and TAp63 isoforms. ΔNp63 isoforms not only function as dominant-negative repressors of transactivation but also have opposing effects compared with TAp63 isoforms (Bamshad, 2008). The SAM-containing TAp63α isoform is more stable than other isoforms because of its transactivation inhibitory domain, intramolecularly bound to the TA domain, subsequently interfering with p63α degradation. In general, TAp63 isoforms (half-life six minutes *in vitro*) are much less stable than ΔNp63 isoforms (half-life greater than five hours). The specific DNA-binding activity of TAp63 must be essential for its protein stability because the disease-related DNA-binding mutants of TAp63 are very stable (Moll and Slade, 2004).

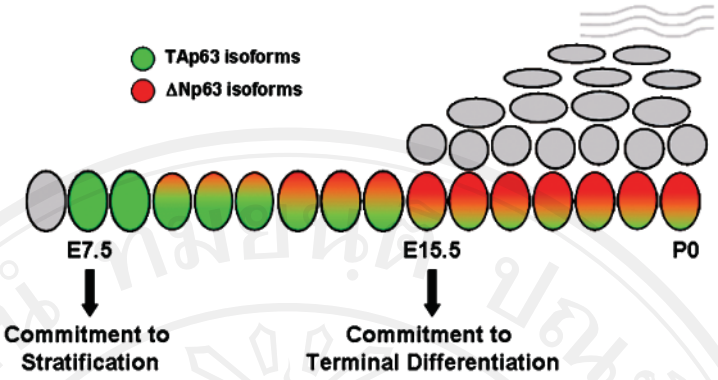


Figure 1.2 Transition of p63 isoforms at different stages of epidermal morphogenesis in mouse embryos. At E7.5, TAp63 isoforms are the first to be detected in the single-layered surface ectoderm. Subsequently, at E9.5, the ectoderm starts epithelial stratification and TAp63 isoforms are gradually replaced by ΔNp63 isoforms, which are required for epidermal maturation. In mature epidermis, ΔNp63α is required for the maintenance of basal keratinocytes so that they can proliferate efficiently (reproduced from Koster and Roop, 2004a).

1.2.2 Roles of p63 in development

The critical functions of p63 are epithelial stratification, and development and differentiation of ectodermal derivatives in vertebrates. These functions were discovered by the knockout of *p63* in mice. *p63* is normally expressed in the ectodermal surfaces of the limb buds, branchial arches and epidermal derivatives (which develop as a result of epithelial-mesenchymal interactions), such as hair follicles, whiskers, teeth and exocrine glands (which develop from adjacent epithelial and neural crest-derived mesenchymal tissues, including mammary, sweat, prostate,

salivary and lacrimal glands). Mice lacking all p63 isoforms die shortly after birth and exhibit an abnormal phenotype, including severely truncated forelimbs, absent hindlimbs and craniofacial malformations, such as cleft lip with/without palate (Mills et al., 1999; Thomason et al., 2008; Yang et al., 1999). Interestingly, *TP63* mutations in humans can lead to cleft lip with/without palate (Rinne et al., 2007). Attractively, all squamous epithelium in $p63^{-/-}$ mice remains single-layered, and the epithelial derivatives are missing. The abnormalities have been interpreted as resulting from either a lack of commitment of the simple ectoderm to epidermal lineages (Mills et al., 1999) or from loss of the self-renewing population of epithelial cells (Yang et al., 1999) **(Figure 1.3)**.

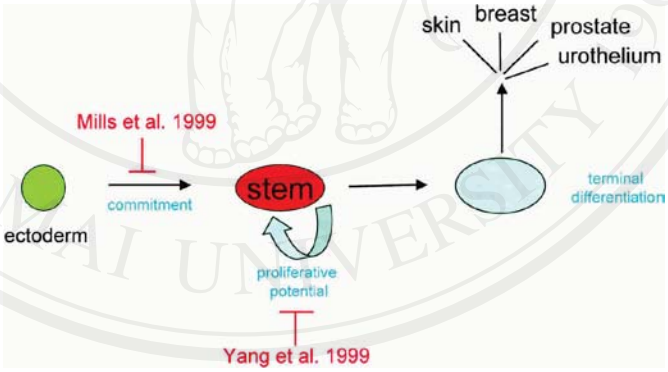


Figure 1.3 Different concepts of the role of p63 in epidermal morphogenesis between two independent studies which generated *p63* knockout mouse models (Mills et al., 1999; Yang et al., 1999). Mills et al. demonstrated that the role of p63 is essential for epithelial differentiation/stratification, whereas Yang et al. demonstrated that the role of p63 is for maintenance of stem cells (adapted from McKeon, 2004).

1.2.2.1 Knockout of *p63*$^{-/-}$ in mice

The study of the role of p63 in epidermal morphogenesis has been controversial. Two independent studies generated *p63* knockout mouse models with different phenotypes and proposed two different roles for p63 in epidermal morphogenesis **(Figure 1.3)**. The different genetic backgrounds of the mice and the different targeting strategies were thought to be accountable for the phenotypic difference (Koster et al., 2007; Mills et al., 1999; Yang et al., 1999). Both studies demonstrated that *p63* is normally expressed in the ectodermal surfaces of the limb buds, branchial arches, and epidermal appendages, which are the sites that have epithelial-mesenchymal interactions.

The study of Mills et al (1999) showed that mice lacking *p63* **(Figure 1.4)** were viable at birth, but died several hours later from severe dehydration, as the *p63*$^{-/-}$ mice lost approximately 30 times more water during the first few days of life than did normal mice, because of the single layer of epithelium in the skin. The visceral organs in the abdominal and thoracic cavities are unremarkable. However, the mice obviously display striking limb defects. The forelimbs are truncated and the hindlimbs are completely absent as a result of the absence of the apical ectodermal ridge (AER), a structure essential for limb outgrowth along the proximo-distal axis. The AER is located at the junction of the dorsal and ventral surfaces of the distal tip of the limbs. Teeth are completely absent. Using *Lef-1* as a marker for epithelial-mesenchymal interaction, it is apparent that its expression is absent in the ectoderm overlying the sites where the whisker follicles and mammary buds normally form, indicating that the epithelial-mesenchymal interactions are disturbed. The normal

epidermal structure of the skin is absent in newborn mutants and there is complete absence of hair follicles. Keratin-1 (K1), an early marker of epidermal differentiation, is absent in the homozygous mutants ($p63^{-/-}$), but not in the wild-type ($p63^{+/+}$) nor in the heterozygous mutants ($p63^{+/-}$). Several other epidermal differentiation markers are also absent in the homozygous mutants, including the early-differentiation markers Keratin-10 (K10) and the late-stage terminal differentiation markers filaggrin and loricrin. In addition to those markers, Keratin-14 (K14), Keratin-6 (K6), and Keratin-13 (K13) are not detectable in the oral epithelium of the homozygous mutants. Lack of stratification of the epithelium is demonstrated by a single layer of flattened skin cells, without the stratum spinosum, stratum granulosum, or stratum corneum. The epithelium of the tongue and oral cavity of the mutants consist of only a single cell layer, with no normal, differentiated structures. The study of Mills et al (1999) demonstrated that p63 had a crucial role in both epidermal morphogenesis and limb development, and homozygous mutant ($p63^{-/-}$) mice showed no evidence of development of a differentiated epidermis. The mutants were born with a single layer of epithelial cells covering their body surface. These cells were the uncommitted surface ectoderm, which initially covered the mutant mouse embryo. These epithelial cells were blocked in the transition to become keratinocytes (Mills et al., 1999).

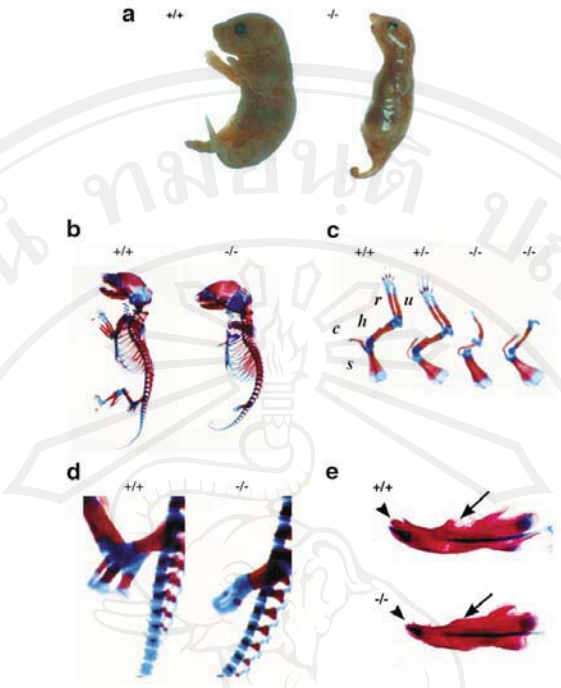


Figure 1.4 *p63* Knockout model of Mills et al. (a-d) The sagittal views of control ($p63^{+/+}$) and $p63^{-/-}$ newborn mice. $p63^{-/-}$ mice have skin defects and severe limb malformations. The forelimbs are severely truncated and the hindlimbs are completely absent. (e) Absence of incisors (arrowheads) in knockout mice (reproduced from Mills et al., 1999).

The study of Yang et al. (1999) demonstrated that $p63^{-/-}$ mice died within a day of birth. The phenotype of $p63^{-/-}$ mice **(Figure 1.5)** was similar to that in the study of Mills et al., including truncation of limbs, craniofacial abnormalities, and shiny, translucent skin. The phenotypic difference between the two studies was the presence of patches of differentiated keratinocytes found by Yang et al. The $p63^{-/-}$ mice expressed the markers involucrin, loricrin, and filaggrin in the dermis. These markers are the terminal differentiation markers of keratinocytes. This expression indicates

that differentiation of epidermis can take place in the absence of p63. The patches of differentiated cells were interpreted as the remnants of an epidermis that initially developed, but failed to be maintained, and it was concluded that p63 is required for epidermal stem cell maintenance, or self-renewal (Yang et al., 1999) **(Figure 1.2)**.

It is concluded that p63 has a predominant role in the regulation of epithelial commitment, differentiation and maintenance programs. During embryogenesis, p63 is required for the initiation of epithelial stratification, whereas in the adult tissues, it maintains the proliferative and regenerative potential of the basal keratinocytes. However, at least six of the ten isoforms of p63 (ΔNp63α, β, γ and TAp63α, β, γ) exist in a dynamic complex that counterbalances the effects of each other, thereby regulating growth, differentiation and apoptosis (Barbieri and Pietenpol, 2006; Koster and Roop, 2004a).

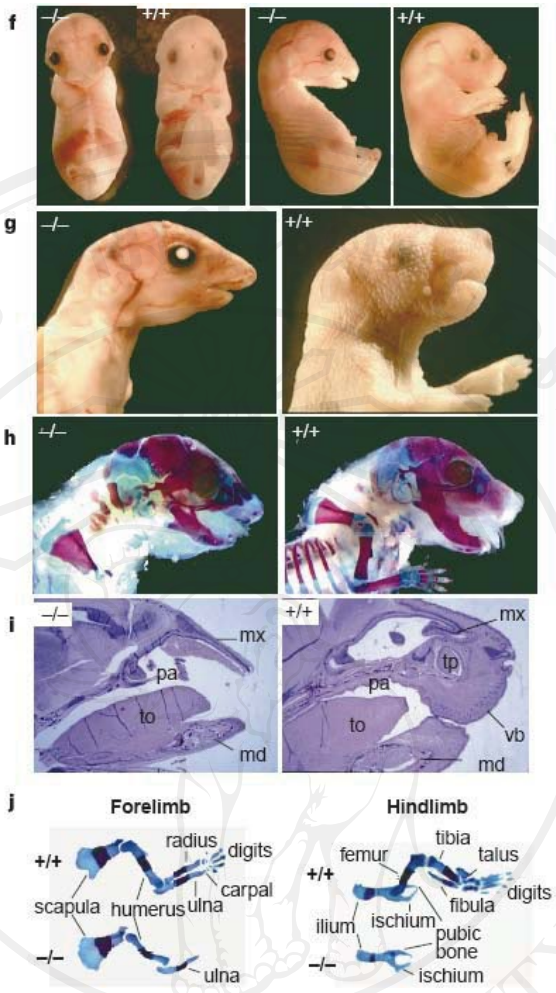


Figure 1.5 *p63*$^{-/-}$ Knockout model of Yang et al. (a-i) The frontal and sagittal views of control (*p63*$^{+/+}$) and *p63*$^{-/-}$ mice at E17 and at postnatal day 1 (P1). *p63*$^{-/-}$ mice have a small maxilla and mandible, and have no eyelids, whisker pads, skin or related appendages, such as vibrissae, pelage follicles and hair shafts all of which are present on the wild-type controls. Furthermore, the *p63*$^{-/-}$ mice reveal a truncated maxilla (mx), mandible (md), and secondary palate (pa) and an absence of vibrissae (vb), tooth primordia (tp) and soft-tissue structures. The fore- and hindlimbs lack distal components, including the radius, carpals and digits (reproduced from Yang et al., 1999).

1.2.3 Pathways of p63

This section is a summary of the pathways involved upstream and downstream of p63 in embryonic ectoderm **(Figure 1.6)**. Only genes with known or suspected roles in skin appendage development are indicated. Bone morphogenetic proteins (BMP)s and fibroblast growth factor10 (FGF10) can induce expression of *p63*, which also positively regulates its own expression (Mikkola, 2007). Although a large number of genes have been associated with p63-dependent regulation, only a few of these have been validated as direct p63 transcriptional targets (Perez and Pietenpol, 2007) **(Figure 1.7)**.

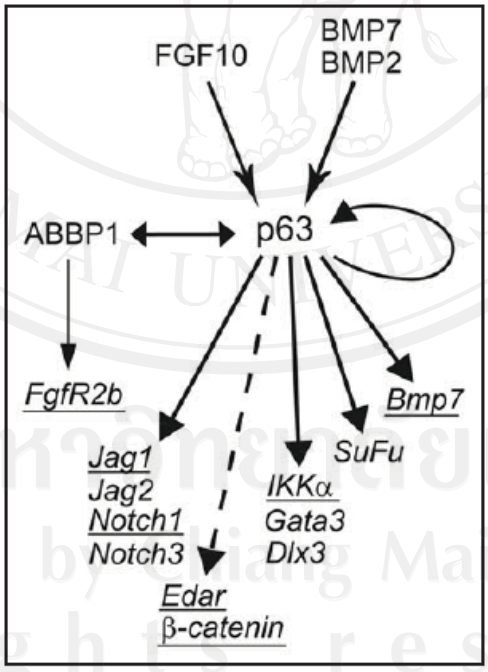


Figure 1.6 Pathways of p63 (reproduced from Mikkola, 2007).

Gene	Function	Validation
NOXA	Apoptosis	ChIP
BAX	Apoptosis	ChIP
p53AIP1	Apoptosis	ChIP
FAS (CD95)	Apoptosis	ChIP
GPX2	Detoxification	ChIP
REDD1 (DDIT4)	Detoxification	KO
MDM2	Prot. Degradation	ChIP
SMARCD3	Remodeling	ChIP
FASN	Metabolism	ChIP
FDXR	Metabolism	ChIP
CDKN1A (p21)	Cell Cycle	ChIP
CDKN1C (p57)	Cell Cycle	ChIP
14-3-3σ/SFN	Cell Cycle	ChIP
ADA	Cell Cycle	ChIP
SerpinB5 (Maspin)	Growth suppression	ChIP
IGFBP-3	Growth suppression	ChIP
S100A2	Growth suppression	ChIP
EGF-R	Growth	ChIP
FGF2-Rβ	Growth	KO
PEDF	Adhesion	ChIP
BPAG1	Adhesion	ChIP, KO
EVPL	Adhesion	ChIP
PERP	Adhesion	ChIP
ITGA3	Adhesion	ChIP
ITGA5	Adhesion	ChIP
ITGA6	Adhesion	ChIP
ITGB4	Adhesion	ChIP
Laminiγ2	Adhesion	ChIP
KRT14	Adhesion	ChIP, KO
JAG1	Development	ChIP
Wnt10b	Development	KO
EDAR	Development	KO
Notch1	Development	ChIP, KO
BMP7	Development	ChIP, KO
B-catenin	Development	KO
IKKa (CHUK)	Development	ChIP, KO
GATA3	Development	ChIP
Hes1	Development	ChIP
p63	Development	ChIP
CLCA2	Signaling	ChIP
VDR	Differentiation	ChIP

Figure 1.7 List of the published p63 target genes, experimentally validated by Chromatin Immunoprecipitation (ChIP) experiments and/or knockout (KO) mouse models (adapted from Vigano and Mantovani, 2007) (for review, please read Vigano and Mantovani, 2007).

1.2.3.1 Upstream regulatory genes

1.2.3.1.1 Bone morphogenetic proteins gene (*BMP*)s and fibroblast growth factors gene (*FGF*)

p63 regulates multiple signaling pathways, such as the BMP and FGF pathways. *p63* expression during development may be regulated by BMP signaling, especially BMP2/BMP7 (Barbieri and Pietenpol, 2006; Laurikkala et al., 2006; Radoja et al., 2007).

1.2.3.2 Direct downstream target genes

p63 is a transcription factor involved in the development of ectodermal tissues consisting of skin, teeth, hair, limbs and other exocrine glands. Chromatin Immunoprecipitation (ChIP) is generally used to identify the direct target genes and ChIP is used to locate the binding sites (Barski and Frenkel, 2004; Medeiros et al., 2009). ChIPs in primary keratinocytes reveal that p63 target genes are generally shared with those of p53, but some are specific to p63 (Testoni et al., 2006). Mutations in *TP63* lead to the multiple organ anomalies found in *TP63*-associated syndromes, such as EEC syndrome, AEC syndrome, RHS, ADULT syndrome, and LMS. These mutations indicate that *TP63* plays very crucial and indispensable roles in multiple pathways involved in the development and differentiation of the cells involved in the formation of those important structures, which are affected when the mutations take place. These important structures include hair, teeth, skin, sweat, salivary, and prostate glands. These roles imply that there are numerous downstream

target genes and pathways of *p63*. Recently, it has been reported that there are approximately 2,000 downstream target genes (Yang et al., 2006).

1.2.3.2.1 Distal-less homeobox 3 gene (*DLX3*)

Dlx3 is a homeodomain transcription factor and a member of the vertebrate Distal-less family (Morasso et al., 1999). The Distal-less gene family comprises of six members, in mice and humans, which are organized in three convergently transcribed pairs. In humans, *DLX3* is paired with *DLX4* on Chromosome 17. The functions of both Dlx3 and p63 are crucial parts of the transcriptional regulatory pathways relevant in organogenesis of the ectodermal derivatives. The mutations of either gene cause ectodermal dysplasia syndromes (Radoja et al., 2007). *Dlx3* is expressed throughout development in a series of structures, derived from epithelial-mesenchymal interactions, such as hair follicles, teeth, limb buds (distal limb), branchial arches, labyrinthine layer of the placenta, osteoblasts, mammary glands, fungiform papillae, genital tubercle, and epidermis (Morasso et al., 1995; Morasso et al., 1999). *Dlx3* is, clearly, a distinct member of the *Dlx* family and it is the only member that is not expressed in the central nervous system (Hassan et al., 2004; Morasso et al., 1995; Morasso et al., 1999; Morasso and Radoja, 2005). Dlx3 *is* also involved in the processes of osteogenesis, hematopoiesis, and epidermal stratification. It is very highly expressed in differentiated osteoblasts and osteocytes, implying that it may play an important role in the late stage of osteoblast differentiation and osteocyte function (Li et al., 2008). Dlx3 also plays an unique and important role in

placental morphogenesis, a role that cannot be provided by other *DLX* genes (Morasso and Radoja, 2005).

Dlx3 is required for the maintenance of *Esx1* expression, normal placental morphogenesis, and embryonic survival (Morasso et al., 1999). The embryonic phenotype of *Dlx3*$^{-/-}$ cannot be investigated, as inactivation of *Dlx3* in mice results in placental failure (Morasso et al., 1999). Mutations in *DLX3* have been reported to be responsible for developmental abnormalities in humans, that causing syndromes such as Tricho-Dento-Osseous (TDO) syndrome **(Figure 1.8)**, which is characterized by defects in the development of hair and teeth, increased bone density in the cranium, absence of overt limb malformations (Haldeman et al., 2004; Price et al., 1998a; Price et al., 1998b; Price et al., 1999) and Amelogenesis Imperfecta Hypoplastic-Hypomaturation with Taurodontism (AIHHT), which is characterized by dental enamel defects and enlarged pulp chambers with no abnormalities of hair or bone (Dong et al., 2005). It is interesting to note that mice with heterozygous mutation of *Dlx3* do not have the characteristic features of TDO syndrome, suggesting that the dominant pattern of inheritance of TDO syndrome in humans is the result of the formation of nonfunctional complexes involving the frame-shifted/truncated DLX3 protein, as opposed to haploinsufficiency (Morasso et al., 1999).

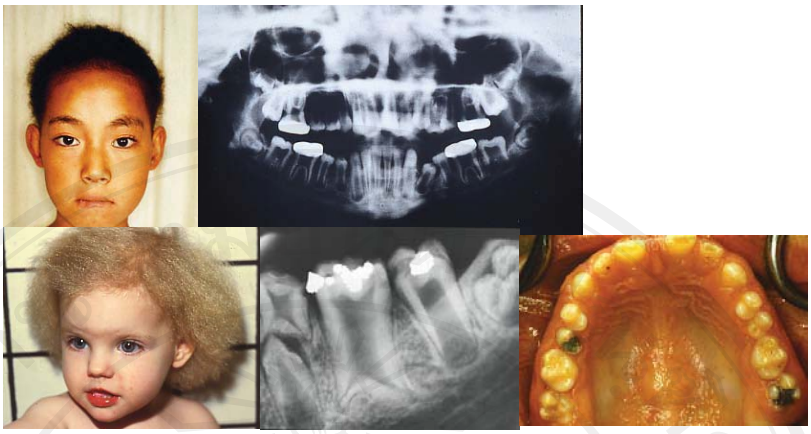


Figure 1.8 Tricho-Dento-Osseous Syndrome (TDO) is caused by mutations in the *DLX3* gene. Characteristic features are kinky hair, amelogenesis imperfecta (AI), and taurodontism (Courtesy of Prof. Timothy Wright, Chapel Hill, North Carolina).

The Dlx and p63 families of transcriptional effectors are essential for the development of the epidermis and/or embryonic appendages (Koster and Roop, 2004a; Morasso and Radoja, 2005; Radoja et al., 2007). Dlx3 is regulated by p63 as part of a transcriptional regulatory pathway relevant to specific EDs (Barbieri and Pietenpol, 2006; Laurikkala et al., 2006; Radoja et al., 2007). The ectodermal dysplasia syndromes caused by mutations in *DLX3* and *TP63*, have been defined as a group of pathological conditions that share common anomalies in epithelial- and mesenchymal-derived organs such as hair, teeth, nails and sweat glands, and have been associated with abnormalities in other organs (Radoja et al., 2007). The transcription of Dlx3 is abolished by mutations in the sterile α-motif (SAM) domain of *TP63* that are associated with AEC syndrome. This may be the reason why dental anomalies, especially enamel hypoplasia, are more common in patients with Rapp-

Hodgkin Syndrome and AEC syndrome, as both syndromes are caused by mutations in the SAM domain (Radoja et al., 2007). Due to the pathogenesis of *TP63* molecular lesions in AEC syndrome, the abnormalities found in AEC syndrome/RHS might be the results of the misregulation of *Dlx3* (Radoja et al., 2007).

Dlx proteins act as transcriptional activators, while Muscle segment homeobox (Msx) proteins act as transcriptional repressors (Zhang et al., 1997). Therefore Dlx proteins have antagonistic interaction with Msx proteins (Zhang et al., 1997). Missing the C terminal of the MSX1 homeodomain disrupts teeth and nail formation, and the complete absence of the MSX1 homeodomain causes orofacial clefts and tooth agenesis (Satokata and Maas, 1994). These mutations may interrupt DLX-MSX interactions (Vieira, 2003).

Due to the fact that *DLX3* mutations are the cause of TDO syndrome and of AIHHT, Dlx3 may be crucial in enamel and tooth formation (Dong et al., 2005; Morasso and Radoja, 2005; Price et al., 1998a; Price et al., 1998b; Radoja et al., 2007; Wright et al., 1997).

1.2.3.2.2 IκB kinase alpha gene (*Ikkα*)

Ikkα is a component of the IκB kinase (Ikk) complex that plays an important role in the activation of NF-κB. During embryogenesis, Ikkα is essential for NF-κB activation in the limb and skin development (Shimada et al., 1999). In *Ikkα* mutant mice and in mice expressing a transdominant negative mutant of *IκBα* ($c^{IκBαΔN}$), molars have abnormal tooth cusps, indicating that Ikkα is involved in cusp formation

through the NF-κB pathway (Ohazama et al., 2004; Pummila et al., 2007) **(Figure 1.9).**

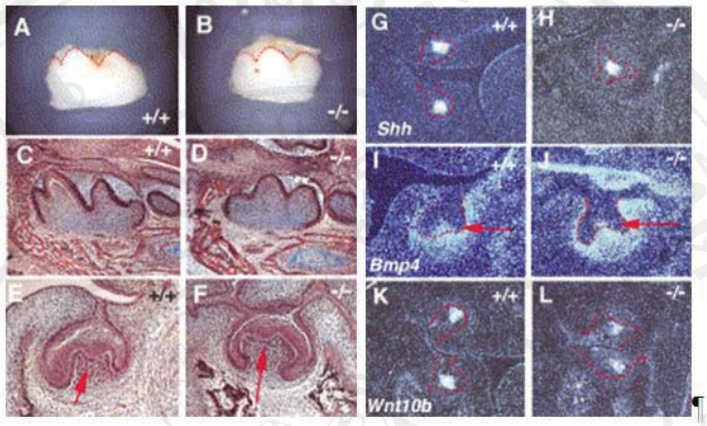


Figure 1.9 Molar tooth phenotype of *Ikkα* mutant mice; First lower molar at E14 (G–L), E15.5 (E and F), and E18 (A and D) of wild-type (A, C, E, G, I, and K) and mutant (B, D, F, H, J, and L) mice (reproduced from Ohazama et al., 2004).

This abnormal tooth cusp phenotype is similar to that of *EdaA1*, *Edar* and *Edaradd* mutants (Candi et al., 2006; Pummila et al., 2007). Interestingly, $Ikk^{-/-}$ mice and $p63^{-/-}$ mice display similar developmental defects, although skin and epithelial appendage development arrest at a later stage in $Ikk^{-/-}$ than in $p63^{-/-}$ mice. The abnormalities have been demonstrated to be caused by an aborted epidermal differentiation (Koster et al., 2007). Therefore Ikkα is also required for normal whisker and tooth development **(Figure 1.10)**, which begins with the invagination of the epithelium into the underlying mesenchyme (Candi et al., 2006).

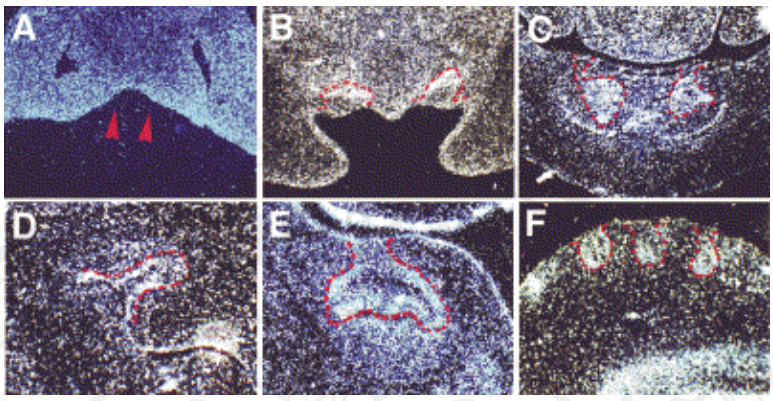


Figure 1.10 Expression pattern of *Ikkα* during early tooth and whisker development (reproduced from Ohazama et al., 2004).

Ikkα has both protein-kinase-dependent and -independent effects. The kinase-independent function is required for epidermal keratinocyte differentiation and for skeletal, and craniofacial morphogenesis (Hu et al., 1999). Ikkα prevents terminal differentiation of keratinocytes by blocking the expression of late differentiation markers, such as loricrin and filaggrin (Candi et al., 2006).

The role of p63 is directly upstream of Ikkα in epithelial development. The action of TAp63 is mediated by direct transactivation of Ikkα. ΔNp63 upregulates Ikkα indirectly, through GATA-3 (Candi et al., 2006).

1.2.3.2.3 Fibroblast-growth-factor-receptor-2 gene (*FgfR2b*)

The SAM domain of the wild-type ΔNp63α protein is able to bind to Apobec-1-binding protein-1 (ABBP1), a protein of the RNA splicing machinery leading to the preferential production of the IIIb splice isoform of FgfR2, FgfR2b (Mikkola, 2007;

Rinne et al., 2007). FgfR2b mRNAs have been reported to be detected in the epithelial cells of developing teeth (E11-P4) (Kettunen et al., 1998).

However, AEC mutations in the SAM domain abolish the binding of the domain to ABBP1, which, most probably, leads to changes in FGFR2 RNA splicing (Fomenkov et al., 2003). Interestingly, gain-of-function mutations in the *FGFR2* gene have been reported in a number of craniosynostosis syndromes, which are also characterized by distal limb malformations. Recently, loss-of-function mutations in *FGFR2* have been found in lacrimo-auricolo-dento-digital syndrome (LADD), an ectodermal dysplasia syndrome characterized by dominant inheritance of limb defects in association with abnormal lacrimal ducts, ear cups and teeth. Interestingly, LADD has phenotypes that overlap with EEC syndrome, and was earlier also presumed to be caused by mutations in *TP63* (Rinne et al., 2007). AEC-associated mutations completely abolish this physical interaction, leading to strongly reduced levels of FgfR2b *in vitro* (Fomenkov et al., 2003). So, *p63* regulates gene expression at the post-transcriptional level. A link between p63 and FgfR2b is also supported by the similarity of phenotypes in *p63* and *FgfR2b* knockout mice. The limb defects of *FgfR2b* and *p63* mutants are very similar. In addition, epidermal proliferation is grossly impaired in *FgfR2b* mutants causing severely hypoplastic epidermis. FgfR2b is also a major regulator of skin appendage development: *FgfR2b* mutants have reduced numbers of hair follicles, tooth development is arrested at the bud stage and of the five pairs of mammary placodes, only one pair is formed, which subsequently regresses (Mikkola, 2007).

1.2.3.2.4 *β*-catenin gene/ wingless related protein gene *(Wnt)*

β-catenin/Wnt signaling is essential for development of all skin appendages from the early stages. Hair and mammary placodes cannot be detected in mice overexpressing *Wnt inhibitor Dkk1* and tooth development arrests at the bud stage (Mikkola, 2007). The regulation of *β-catenin* level and its localization is important for the regulation of Wnt signaling. Decreased *β-catenin/Wnt* expression is less clear in the oral/dental epithelium than in the surface ectoderm. The downregulation of *β-catenin/Wnt* may be critical for the consequent loss of *p63* function. *AXIN2 i*s a Wnt-signaling regulator. The mutations in *AXIN2* lead to the rare syndrome of hypodontia with adenomatous colorectal polyps and/or cancer. The abnormal AXIN2 proteins are suspected to activate Wnt signaling. Normally *Axin2* is highly expressed in the mesenchyme underlying the oral and dental epithelium. At later stages of tooth development its expression is intense in the odontoblasts and enamel knots, which are epithelial signaling centers that regulate tooth formation (Lammi et al., 2004).

Wnt7b has a reciprocal pattern of expression to *Shh*, in that it is expressed in the non-dental oral epithelium. Overexpression of *Wnt7b* in dental epithelium leads to a loss of *Shh* expression, and tooth development is arrested (Tucker and Sharpe, 2004).

Interestingly, *Wnt4* is an example of a gene that has been identified as upregulated upon disruption of *p63* levels in squamous cell lines, is responsive to *p63* regulation in an overexpressed kidney cell system, and is reported to have promoter elements that are *p63*-responsive in reporter assays (Osada et al., 2006). *Wnt4* has a recognized function in regulating patterning, such as sex determination and kidney

morphogenesis, and in regulating cell fate during embryogenesis (Perez and Pietenpol, 2007).

1.2.3.2.5 Ectodysplasin receptor gene (*Edar*)

The Eda signaling pathway consists of Ectodysplasin-A (Eda), Ectodysplasin-A receptor (Edar) and Ectodysplasin-A receptor-associated death domain (Edaradd). Ectodysplasin (EDA) is a tumor necrosis factor (TNF) ligand. In mice, disruption of Eda (Tabby), its receptor Edar (Downless) and intracellular adaptor protein Edaradd (Crinkled), cause abnormal numbers of teeth. Loss of function of *Eda* or *Edar* reduces tooth number. The aberrant development of $Eda^{-/-}$ molars is observed from the bud stage onwards and results in few shallow cusps and small size of teeth (Mikkola and Thesleff, 2003). In rare cases, overexpression of *Edar* also reduces tooth number. So, the number of teeth that is formed seems to depend on a balance of the level of EDA signaling (Tucker and Sharpe, 2004).

The mutation in EDA signaling pathway causes ectodermal dysplasia in humans **(Figure 1.11)** with notable similarities to *TP63*-associated EDs, and absence of primary hair placodes and defective development of the tooth from the bud stage in mice (Mikkola, 2007). Sparse scalp hair, eyebrows and eyelashes, hypodontia, abnormally shaped teeth and a reduced number of sweat glands are the phenotype shared between *TP63*-associated syndromes and hypohidrotic ED, suggesting that p63 and EDA signaling share some common pathways (Pummila et al., 2007; Rinne et al., 2007).

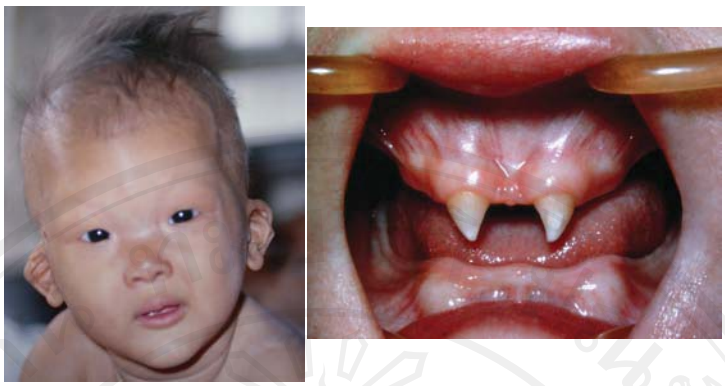


Figure 1.11 Hypohidrotic ectodermal dysplasia is caused by mutation in the *EDA* gene. Characteristic features are sparse hair, hypodontia, abnormally shaped teeth and a reduced number of sweat glands (Courtesy of Dr. Piranit Kantaputra, Chiang Mai, Thailand).

1.2.3.2.6 *Jag1* and *Notch1*

Jag1 and *Jag2* are ligands for Notch receptors. They are co-expressed with *Notch1* and *Notch2* in wild-type dental epithelium at E11-E12 (Laurikkala et al., 2006). Jagged1 (JAG1) is known to be directly regulated by p63 proteins (Barbieri and Pietenpol, 2006). *Notch ligand Jagged-1* is believed to be regulated by *p63* but not by *p53* (Sasaki et al., 2002). However, normal *Jag2* expression has been found in $p63^{-/-}$ embryos suggesting compensatory regulation in the absence of *p63* (Mikkola, 2007). *TP63* may be a modifier of the mutant *JAG2* phenotype, and, at the same time *JAG2* may be a modifier of the mutant *TP63* phenotype (van Bokhoven and Brunner, 2002).

Notch1, *Notch2* and *Notch3* are all co-expressed in the suprabasal cells of the oral and dental epithelium of wild type embryos at E11-E12. Interestingly, expression of *Notch2* and *Notch3* is unaffected in *p63* mutants, indicating that downregulation of *Notch1* is specific and not simply due to the absence of the cell population where it is normally expressed (Laurikkala et al., 2006). In addition to *Notch1*, also *Notch3* has been identified as a putative *p63* target (Vigano and Mantovani, 2007). The mutations of *JAG1* and *NOTCH2* cause Alagille syndrome (Arteriohepatic dysplasia), which is characterized by deep-set eyes, a broad forehead, posterior embryotoxon, pulmonary circulation defects, butterfly-like vertebral arches, paucity of intrahepatic interlobular bile ducts and chronic cholectasis (McDaniell et al., 2006; Ropke et al., 2003). The role of Notch signaling during tooth development is not well understood, but it appears to be essential for the initiation stage of hair follicle formation (Mikkola, 2007). *p63* has been described to trigger the Notch pathway in neighboring cells by inducing *Jag1* and *Jag2* (van Bokhoven and Brunner, 2002).

1.2.4 *TP63*-associated human syndromic and non-syndromic malformations

All developmental abnormalities caused by *TP63* mutations in humans, although less severe, are resemble those seen in $p63^{-/-}$ mice (Mikkola, 2007). There is a correlation between the position of the mutations and the observed abnormal phenotype (McGrath et al., 2001; Radoja et al., 2007; van Bokhoven et al., 2001; van Bokhoven and Brunner, 2002). Mutations in *TP63* are the causes at least five different syndromes including Ectrodactyly-Ectodermal dysplasia-Cleft lip/palate syndrome (EEC3, MIM 604292), Ankyloblepharon-Ectodermal defects-Cleft lip/palate syndrome (AEC, MIM 106260), Limb-Mammary Syndrome (LMS, MIM 603543), Acro-Dermato-Ungual-Lacrimal-Tooth syndrome (ADULT, MIM 103285) and Rapp-Hodgkin Syndrome (RHS, MIM 129400). Furthermore, two non-syndromic human disorders are caused by *TP63* mutations: non-syndromic split hand/foot malformation (SHFM4, MIM 605289) and newly non-syndromic cleft lip (NSCL) (Rinne et al., 2007) **(Figure 1.12).** All of these disorders are transmitted in an autosomal dominant pattern (Bamshad, 2008).

Mutation	Exon	Domain	Isoform		Syndrome
N6H	3'	TA2	dN	alpha, beta, gamma	ADULT
R58C	3	TA	TA	alpha, beta, gamma	SHFM
G76W	4	-	dN, TA	alpha, beta, gamma	LMS
S90W	4	-	dN, TA	alpha, beta, gamma	LMS
3'ss intron 4	intron 4/ exon 5	-	dN, TA	alpha, beta, gamma	SHFM
G134D	4	-	dN, TA	alpha, beta, gamma	LMS
G134D	4	-	dN, TA	alpha, beta, gamma	ADULT
L162P	5	DBD	dN, TA	alpha, beta, gamma	EEC
Y163C	5	DBD	dN, TA	alpha, beta, gamma	EEC
Y192C/D	5	DBD	dN, TA	alpha, beta, gamma	EEC
K193E	5	DBD	dN, TA	alpha, beta, gamma	SHFM
K194E	5	DBD	dN, TA	alpha, beta, gamma	SHFM
V202M	5	DBD	dN, TA	alpha, beta, gamma	EEC
R204L/Q/W	6	DBD	dN, TA	alpha, beta, gamma	EEC
H208Y	6	DBD	dN, TA	alpha, beta, gamma	EEC
R227Q	6	DBD	dN, TA	alpha, beta, gamma	EEC
C269Y	7	DBD	dN, TA	alpha, beta, gamma	EEC
S272N	7	DBD	dN, TA	alpha, beta, gamma	EEC
C273Y	7	DBD	dN, TA	alpha, beta, gamma	EEC
R279C/H/Q	7	DBD	dN, TA	alpha, beta, gamma	EEC
R279H	7	DBD	dN, TA	alpha, beta, gamma	RHS
R280C/H/S	7	DBD	dN, TA	alpha, beta, gamma	EEC
R280C/H	7	DBD	dN, TA	alpha, beta, gamma	SHFM
R298G/Q	8	DBD	dN, TA	alpha, beta, gamma	ADULT
R304P/Q/W	8	DBD	dN, TA	alpha, beta, gamma	EEC
C306Y/R	8	DBD	dN, TA	alpha, beta, gamma	EEC
C308S/Y	8	DBD	dN, TA	alpha, beta, gamma	EEC
P309S	8	DBD	dN, TA	alpha, beta, gamma	EEC
D312G/H/N	8	DBD	dN, TA	alpha, beta, gamma	EEC
R313G	8	DBD	dN, TA	alpha, beta, gamma	EEC
R313G	8	DBD	dN, TA	alpha, beta, gamma	NSCL
A315E	8	DBD	dN, TA	alpha, beta, gamma	EEC
3'ss intron 10	intron 10/ exon 11	-	dN, TA	alpha, beta, gamma	AEC
I510T	13	SAM	dN, TA	alpha	RHS
I510T	13	SAM	dN, TA	alpha	AEC
L514F/S/V	13	SAM	dN, TA	alpha	AEC
G518V	13	SAM	dN, TA	alpha	AEC
C522G/W	13	SAM	dN, TA	alpha	AEC
1572 Ins A	13	SAM	dN, TA	alpha	EEC
1576 Del TT	13	SAM	dN, TA	alpha	LMS
G530V	13	SAM	dN, TA	alpha	AEC
T533P	13	SAM	dN, TA	alpha	AEC
534 Ins TTC	13	SAM	dN, TA	alpha	AEC
Q536L	13	SAM	dN, TA	alpha	AEC
I537T	13	SAM	dN, TA	alpha	AEC
S541F	13	SAM	dN, TA	alpha	AEC
S541P/Y	13	SAM	dN, TA	alpha	RHS
R555P	14	SAM	dN, TA	alpha, beta	AEC
I558T	14	SAM	dN, TA	alpha, beta	AEC
L563P	14	SAM	dN, TA	alpha, beta	EEC
1709 Del A	14	TI	dN, TA	alpha, beta	RHS
1721 Del C	14	TI	dN, TA	alpha, beta	RHS
1742 Del C	14	TI	dN, TA	alpha, beta	AEC
1743 Del AA	14	TI	dN, TA	alpha, beta	LMS
1787 Del G	14	TI	dN, TA	alpha, beta	RHS
1859 Del A	14	TI	dN, TA	alpha, beta	RHS
1859 Del A	14	TI	dN, TA	alpha, beta	AEC
K632X	14	TI	dN, TA	alpha, beta	LMS
Q634X	14	TI	dN, TA	alpha, beta	SHFM
E639X	14	TI	dN, TA	alpha, beta	SHFM

Figure 1.12 Pathogenic *TP63* mutations in seven allelic diseases (reproduced from Rinne et al., 2007) (for review, please read Rinne et al., 2007).

1.2.4.1 Ectrodactyly-Ectodermal Dysplasia-Cleft Lip/Palate (EEC) syndrome

EEC syndrome **(Figure 1.13)** is the prototype of all *TP63*-associated syndromes and is characterized by one or more features of ectodermal dysplasia, which present as defects of hair, skin, nails, teeth and sweat and mammary glands. The severity and type of the ectodermal features are highly variable. Only few patients show defects in all of the described ectodermal structures above. EEC patients, occasionally, also have mammary gland/nipple hypoplasia (14%) and hypohidrosis (11%). About two-thirds of these patients have a deep median cleft in the hands and feet, missing digits (ectrodactyly) and fusion of the remaining digits (syndactyly) is also frequent (43%). Cleft lip/palate (CL/P) is present in about 40% of EEC patients, mostly as cleft lip with or without cleft palate (Rinne et al., 2007) (for review, please read Rinne et al., 2007).

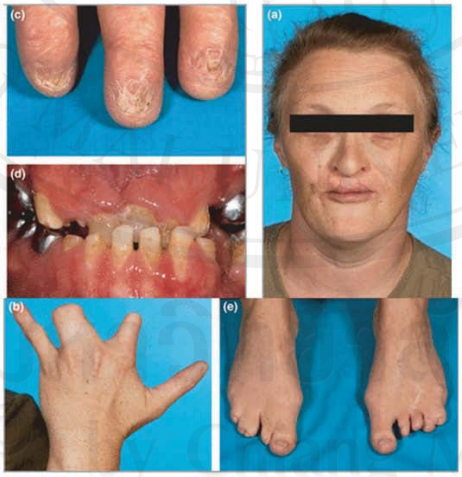


Figure 1.13 EEC syndrome is caused by mutations in the DNA-binding domain of the *TP63* gene. Characteristic features are ectrodactyly, sparse hair, dry skin, and cleft lip/palate (reproduced from Clements et al., 2009).

The majority of EEC mutations are caused by *TP63* point mutations in the DNA binding domain (DBD). Approximately 34 different mutations have been reported (Rinne et al., 2007; van Bokhoven et al., 2001; van Bokhoven and Brunner, 2002). No thorough studies of dental phenotype have been performed.

1.2.4.2 Ankyloblepharon-Ectodermal Defects-Cleft Lip/Palate (AEC) syndrome

AEC syndrome **(Figure 1.14)**, which is known as "Hay-Wells syndrome", is characterized predominantly by ankyloblepharon, but has little or no limb involvement. The ankyloblepharon presents as partial/complete fusion of the eyelids, and is very rare in other EEC-like syndromes (McGrath et al., 2001; van Bokhoven and Brunner, 2002).

The AEC syndrome phenotype differs from the others in the severity of the skin phenotype, the occurrence of eyelid fusion at birth and the absence of limb malformations. Approximately 80% of the patients at birth have severe skin erosion, which usually heals during the first year of life. The ankyloblepharon is present in about 45% of AEC patients. Other ED symptoms, such as nail and tooth defects, are present in more than 80% of patients, and hair defects are almost constant features (94%). Lacrimal duct obstruction is seen in 50% of patients, whereas mammary gland hypoplasia and hypohydrosis occur occasionally (both 13%). Interestingly, almost 40% of patients have hearing impairment and genito-urinary defects. Cleft lip is present in 44% and cleft palate in about 80% of patients. Limb malformations are rare. Ectrodactyly has never been reported, but mild syndactyly is found in

approximately 25% of cases (Rinne et al., 2007). The presence of skin fragility, manifested as erosive skin lesions in body areas in addition to the scalp, is thought to be an important diagnostic feature of AEC syndrome (Payne et al., 2005).

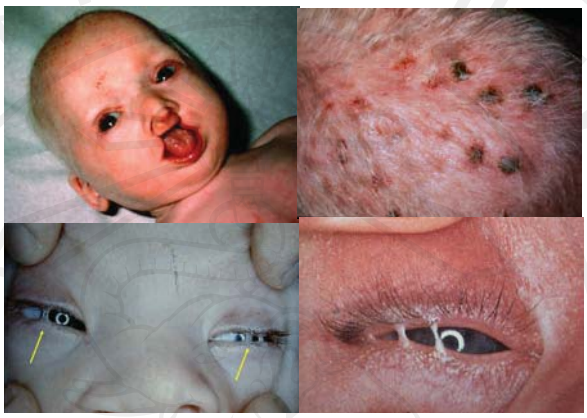


Figure 1.14 AEC syndrome is caused by mutations in the SAM domain of *TP63* gene. Characteristic features are ankyloblepharon, scalp dermatitis, sparse hair, hypodontia, enamel hypoplasia and cleft lip/palate (Courtesy of Prof. Robert J. Gorlin, Minneapolis, Minnesota).

AEC mutations are located in the C-terminus of the p63 protein. They are either point mutations in the SAM domain or deletions in the SAM or TI domains. The SAM domain is known to be involved in protein-protein interactions and, therefore, mutations in this domain are most probably hampering its binding to interacting proteins that may be disrupted p63 transcriptional activity (McGrath et al., 2001; Rinne et al., 2007). No thorough studies of dental phenotype have been performed.

1.2.4.3 Rapp-Hodgkin Syndrome (RHS)

RHS, also called Rapp-Hodgkin ectodermal dysplasia, was first described over 30 years ago in an affected mother, son, and daughter with a combination of anhidrotic ectodermal dysplasia, cleft lip, and cleft palate (Rapp and Hodgkin, 1968). The clinical syndrome is characterized by facies (narrow nose and small mouth), wiry, slow-growing, and uncombable hair, sparse eyelashes and eyebrows, obstructed lacrimal puncta/epiphora, bilateral stenosis of external auditory canals, palmoplantar keratoderma, microsomia, hypodontia, cone-shaped incisors, enamel hypoplasia, dystrophic nails, and cleft lip/palate (Kantaputra et al., 1998; Kantaputra et al., 2003) **(Figures 1.15-16)**.

In RHS patients, the *TP63* mutations are located in the C-terminus of the p63 protein similar to those found in AEC patients. They are either point mutations in the SAM domain or deletions in the SAM or TI domains. The SAM domain is known to be involved in protein-protein interactions, and, therefore, mutations in this domain are most probably hampering its binding to interacting proteins (Kantaputra et al., 2003; Rinne et al., 2007). No thorough studies of dental phenotype have been performed.

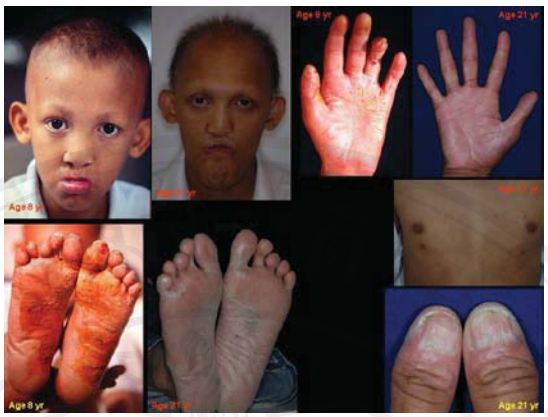


Figure 1.15 Thai patient with RHS at ages 8 and 21 years. Note sparse hair, repaired cleft lip, microsomia, palmoplantar keratoderma, dystrophic nails, and supernumerary nipples. Keratoderma improves with age (Courtesy of Dr. Piranit Kantaputra, Chiang Mai, Thailand).

Figure 1.16 Dr. Piranit Kantaputra, Thai patient with RHS, and Dr. Warissara Sripathomsawat (Courtesy of Dr. Piranit Kantaputra, Chiang Mai, Thailand).

1.2.4.4 Limb-Mammary Syndrome (LMS)

Limb-Mammary Syndrome (LMS) was first reported in a large Dutch family. It is an autosomal-dominant syndrome, characterized by severe hand and/or foot anomalies, and mammary gland and nipple hypoplasia/aplasia. Less frequent findings include lacrimal-duct atresia, nail dysplasia, hypohidrosis, hypodontia, and cleft palate with or without bifid uvula (van Bokhoven et al., 1999). Approximately 30% of patient with LMS have cleft palate (Rinne et al., 2007).

The LMS phenotype resembles the EEC syndrome phenotype, but the ectodermal manifestations are milder in LMS. A consistent feature of LMS is the mammary gland and/or nipple hypoplasia (100%). Lacrimal duct obstruction and dystrophic nails are frequently observed (59 and 46% respectively), hypohidrosis and tooth defects are detected in about 30%, but other ectodermal defects, such as hair and skin defects, are rarely detected. About 70% of LMS patients have similar limb malformations as in EEC syndrome, and about 30% have orofacial clefting, especially cleft palate (Rinne et al., 2007).

The differential diagnosis includes UMS (Ulnar-Mammary Syndrome), EEC syndrome (Ectrodactyly, Ectodermal Dysplasia, and Cleft Lip/Palate) Syndrome, ECP (ectrodactyly with cleft palate) and ADULT syndrome (Acro-Dermato-Ungual-Lacrimal-Tooth) syndrome (van Bokhoven et al., 1999). No thorough studies of dental phenotype have been performed.

1.2.4.5 Acro-Dermato-Ungual-Lacrimal-Tooth (ADULT) syndrome

ADULT syndrome, (Acro-Dermato-Ungual-Lacrimal-Tooth Syndrome) a rare autosomal dominant disorder, is characterized by ectrodactyly, syndactyly, fingernail and toenail dysplasia, hypoplasia of the breast and nipple, excessive freckling, lacrimal duct atresia, frontal alopecia, primary hypodontia, and/or early loss of permanent teeth, neurodermatitic signs, prominent ectodermal dysplasia and normal lip and palate (Reisler et al., 2006; Rinne et al., 2006b; Rinne et al., 2007). Currently, four ADULT syndrome families and three sporadic cases have been reported (Rinne et al., 2007).

ADULT syndrome has clinical overlap with other *TP63*-associated syndromes, such as EEC syndrome (Ectrodactyly, Ectodermal Dysplasia, and Cleft Lip/Palate Syndrome), LMS (Limb-Mammary Syndrome), AEC syndrome (Ankyloblepharon-Ectodermal Defects-Cleft Lip/Palate Syndrome), RHS (Rapp-Hodgkin syndrome) and SHFM4 (Isolated Split Hand/Foot Malformation) (Rinne et al., 2006b). No thorough studies of dental phenotype have been performed.

1.2.4.6 Non-syndromic (Isolated) Split Hand/Foot Malformation (SHFM4)

SHFM4 (Non-syndromic (Isolated) Split Hand/Foot Malformation) is a pure limb malformation condition, a human developmental defect, is characterized by ectrodactyly and syndactyly, and without orofacial clefting or ectodermal dysplasia (Rinne et al., 2007; Temtamy and McKusick, 1978). SHFM4 is caused by several mutations, which are dispersed throughout the *TP63*. Interestingly, several SHFM4

mutations are reported to cause alteration in p63 protein activation and stability (Rinne et al., 2007; van Bokhoven and Brunner, 2002).

1.2.4.7 Non-Syndromic Cleft Lip (NSCL)

NSCL is a non-syndromic orofacial clefting type. R313G is the only amino acid change that has been reported to cause non-syndromic cleft lip, by Leoyklang et al. This mutation was also observed in a sporadic EEC syndrome phenotype (Leoyklang et al., 2006; Rinne et al., 2007).

1.2.5 Genotype-phenotype correlations of *TP63*

The specific pattern of mutations in *TP63* displays a noticeable relationship between the molecular defects in *TP63* and the clinical appearance of each *TP63*-associated syndrome/condition. The mutation patterns in the *TP63* establish a clear genotype-phenotype correlation **(Figure 1.17)**: DBD mutations are associated with EEC syndrome and SAM domain mutations are associated with AEC syndrome and RHS (Mikkola, 2007; Rinne et al., 2007; van Bokhoven et al., 2001; van Bokhoven and Brunner, 2002). The mutations are thought to involve both dominant negative and gain-of-function effects (Mikkola, 2007). Seventy-four different mutations have been found in 250 patients worldwide who were affected with *TP63*-associated syndromes (Rinne et al., 2007).

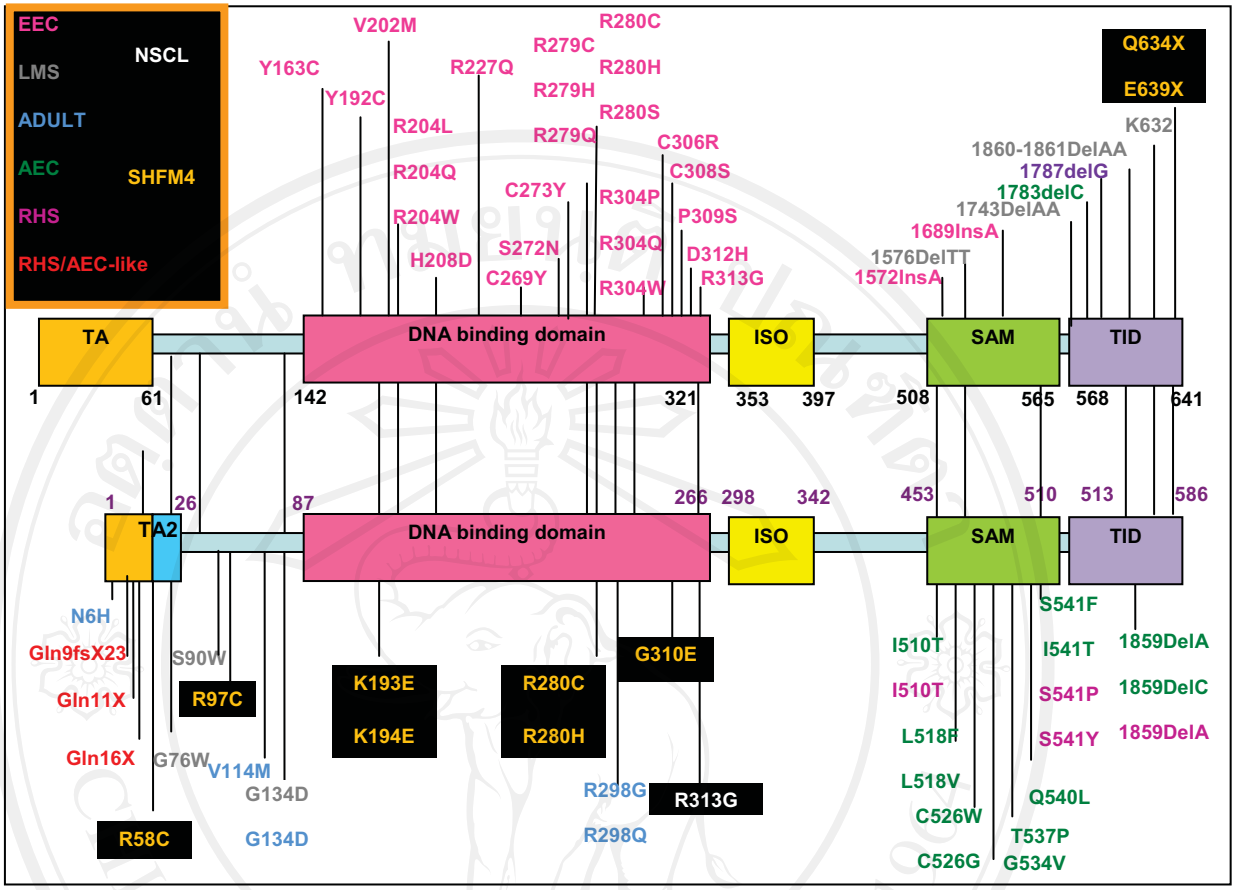


Figure 1.17 Genotype-Phenotype Correlations of *TP63*: Mutations in the DBD are associated with EEC syndrome, and mutations in the SAM domain are associated with AEC syndrome and RHS. However the nonsense mutations (p.Gln11X, p.Gln16X) and a deletion mutation (p.Gln9fsX23) in some RHS/AEC-like patients introduce premature termination codons (PTC) in the N-terminal part of the p63 protein. These mutations produce smaller p63 protein by translational re-initiation at next methionine after PTC. The second initiation codon is flanked by a strong Kozak sequence. The mutations that labelled with different colors show different syndrome/disorders, as followed: pink represents EEC syndrome, gray represents LMS, blue represents ADULT syndrome, green represents AEC syndrome, purple represents RHS, white represents NSCL, orange represents SHFM4, red represents RHS/AEC-like syndrome (adapted from Rinne et al., 2007).

1.2.5.1 Relationship between AEC syndrome and RHS

AEC syndrome and RHS are allelic disorder. AEC syndrome is very similar to RHS. The clear differences between AEC syndrome and RHS are the absence of ankyloblepharon in RHS and the more severe skin phenotype in AEC syndrome. However, the ankyloblepharon is present only in about 45% of patients with AEC syndrome, and it cannot be used as a discriminating feature between the two conditions (Payne et al., 2005).

AEC syndrome and RHS are associated with mutations in the SAM domain of P63 and they have similar phenotypes. There are either point mutations in the SAM domain or deletions in the SAM or TI domains. Several examples show that the same mutation can lead to different clinical conditions. AEC syndrome and RHS share three mutations: I510, S541 and 1859 Del A (Rinne et al., 2007).

1.2.5.2 Relationship between EEC, LMS and ADULT syndrome

EEC syndrome, LMS and ADULT syndrome are allelic disorders. There is a considerable overlap between EEC syndrome, LMS and ADULT syndromes (Amiel et al., 2001; McGrath et al., 2001; van Bokhoven et al., 1999).

The phenotype of ADULT syndrome is most similar to that of LMS. However, there are clear differences. ADULT syndrome is characterized by the absence of orofacial clefting and by the presence of hair and skin defects, especially hyperpigmentation of skin. Teeth, skin and nail defects are constantly present in ADULT syndrome (100, 93 and 100%, respectively), but only rarely in LMS. Hair

(53%) and lacrimal duct defects (67%) are observed more frequently in patients with ADULT syndrome than in those with LMS (Propping et al., 2000; Rinne et al., 2006a; Rinne et al., 2007; van Bokhoven and Brunner, 2002).

However, LMS differs from EEC syndrome in at least three findings. Firstly, mammary gland and nipple hypoplasia are consistent features of LMS but are only occasionally seen in EEC syndrome. Secondly, patients with LMS have no hair and skin defects that are seen in EEC syndrome. Thirdly, patients with LMS have CP, while those with EEC syndrome have CL/P but never have CP only (van Bokhoven et al., 2001). The distinction between CL/P and CP is most relevant, because the affected structures (the primary palate and the secondary palate, respectively) develop independently (Ferguson, 1988; van Bokhoven et al., 1999; van Bokhoven et al., 2001).

1.2.6 Orofacial clefts and *TP63*-associated syndromes

Cleft lip, with or without cleft palate, and cleft palate only are distinct entities. In general, they are not found in the same family, except for families affected with Van der Woude Syndrome (VWS), which is an autosomal dominant disorder caused by mutations in *IRF6*. Mixed types of orofacial clefts can also be found in *TP63*-associated syndromes **(Table 1.1)**. Recently it has been demonstrated that p63 interacts with Irf6 (Dixon, personal communication at Epistem Conference 2007, Ghent, Belgium).

Table 1.1 Incidence of orofacial clefts in *TP63*-associated syndromes (Rinne et al., 2007).

Types of cleft / *TP63* syndromes	Cleft lip (CL)	Cleft palate (CP)	Cleft lip/palate (CL/P)
EEC	NA	NA	40%
AEC	44%	80%	NA
RHS	NA	NA	NA
LMS	0%	30%	0%
ADULT	0%	0%	0%

NA; data not available

1.2.7 Tooth development in mouse and human

1.2.7.1 Stages of tooth morphogenesis

Early epithelial-mesenchymal interactions, a common organ differentiation and similar gene networks regulate the development of teeth and other organs, and initiate the tooth morphogenesis (Hu and Simmer, 2007). The first sign of human tooth development starts as a thickening of the oral ectoderm that occurs at around the day 11.5 of embryonic development (E11.5) in mice and at approximately 7 weeks in humans. Subsequently the thickening oral ectoderm transforms itself into invaginating epithelium, forming a tooth bud at E13.5, into the underlying neural crest-derived mesenchyme. Subsequently this mesenchyme condenses around the

invaginating epithelium. Later the epithelium starts to wrap itself around the condensing mesenchyme to form a cap (at E14.5), and later a 'bell'-stage tooth germ (at E18.5). This process is controlled by a signaling centre that develops at the tip of the tooth bud at the late bud stage, known as the enamel knot. During the bell stage of tooth development, cytodifferentiation occurs in the epithelial cells adjacent to the dental mesenchyme, which differentiate to become enamel-forming ameloblasts, and the adjacent dental mesenchymal cells differentiate to become dentin-forming odontoblasts (for review, please see Morasso and Radoja, 2005; Tucker and Sharpe, 2004) **(Figure 1.18)**.

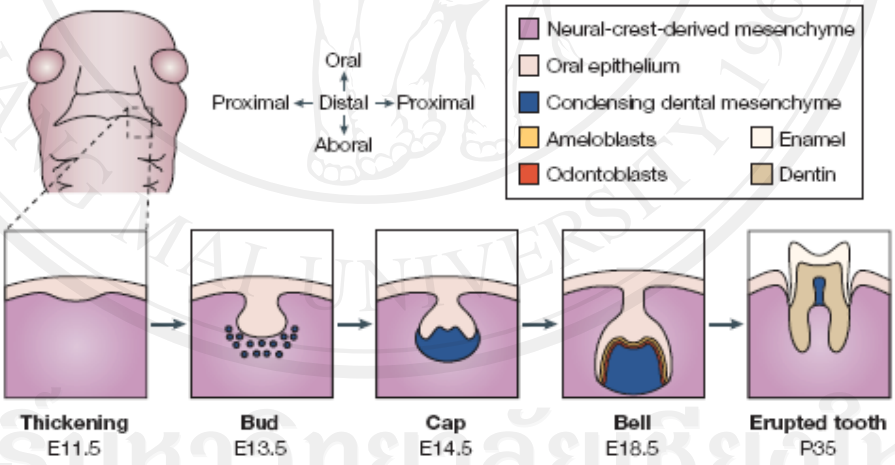


Figure 1.18 Stages of tooth development (reproduced from Tucker and Sharpe, 2004).

1.2.7.2 Molecular interactions in odontogenesis

Complicated growth factors and transcription factors have been recognized as critical regulators in all stages of tooth development (http://bite-it.helsinki.fi). Bone morphogenetic protein (BMP), fibroblast growth factor (FGF), sonic hedgehog (SHH), and Wnt have been identified as the critical signaling molecules that are important in the development of tooth structures (Pispa and Thesleff, 2003). Before the initiation of tooth development in the mouse embryo, the mandibular ectoderm determines the size of the tooth fields and the type of tooth, and can be separated into two domains: the proximal domain which expresses *Fgf8* and gives rise to molars, and the distal domain, which expresses *Bmp4* and gives rise to incisors. Essentially, *Fgf* and *Bmp* act antagonistically to limit mesenchymal *Barx1* and *Dlx2* expression to the proximal domain and mesenchymal *Msx1* and *Msx2* expression to the distal domain, respectively. *Fgf8* is obviously responsible for controlling the expression of both *Gsc* and *Lhx*. In the aboral region, *Gsc* is expressed while the expression of *Lhx6* and *Lhx7* disappear. Thus the mesenchyme can be divided into the Lhx-positive rostral (oral) domain and the Gsc-positive caudal (aboral) domain (for review, please, see Tucker and Sharpe, 2004) **(Figure 1.19)**. During the initiation of the tooth bud, there are four areas of *Shh* (sonic hedgehog) expression in the epithelium that specify the sites of the developing tooth germs at E11.5. *Shh* regulates the proliferation of dental epithelial cells to form the tooth bud. The boundary between the oral epithelium and the dental epithelium is set up through an interaction between SHH and members of the WNT family. *Wnt7b* has a reciprocal pattern of expression with *Shh*. It is also expressed in the non-dental oral epithelium. In the mesenchyme, the positioning of the tooth fields is directed by the expression of *Pax9*. In mice, expression of *Pax9* is

limited to four small patches of mesenchyme that mark the positions of the tooth germs. As with *Barx1*, *Pax9* is positively regulated by *FGF8* and negatively regulated by *BMP4*. *MSX1* and *BMP4* act together in a positive-feedback loop (for review, please read Tucker and Sharpe, 2004; Tucker et al., 1998) **(Figure 1.20)**.

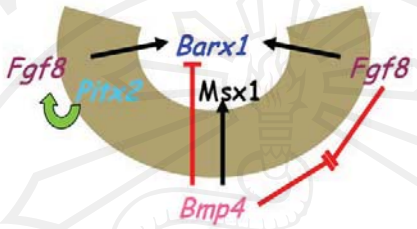


Figure 1.19 Role of gene expression in early tooth development. In epithelium (brown), *Pitx2* regulates *Fgf8*, which in turn upregulates *Barx1* in mesenchyme. *Bmp4* in epithelium activates *Msx1* while it inhibits *Barx1* in mesenchyme. *Bmp4* is antagonistic against *Fgf8*.

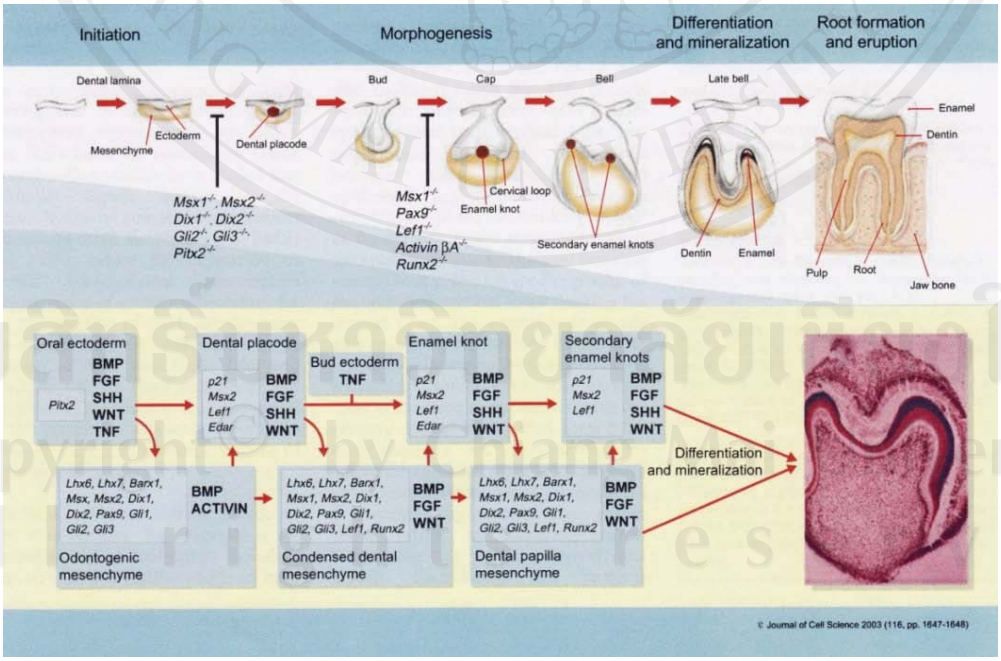


Figure 1.20 Molecular signaling in tooth development (reproduced from Thesleff, 2003).

1.2.7.3 Hypodontia (Tooth agenesis)

Hypodontia is one of the most common developmental problems in man. The congenitally missing teeth result from disturbances during the initial stages of tooth formation; initiation and proliferation. Hypodontia may occur in isolation, or as part of syndromes. Hypodontia has a much higher prevalence in children who have a cleft lip, cleft palate or both, ectodermal dysplasia, Down syndrome, and Rieger syndrome (Das et al., 2002; Fekonja, 2005; Vieira, 2003). Hypodontia is the phenomenon of congenitally missing teeth in general. The terms oligodontia, anodontia, aplasia of teeth, congenitally missing teeth, absence of teeth, agenesis of teeth and lack of teeth have been used to describe the reduction in number of teeth (Das et al., 2002; Fekonja, 2005). Remaining teeth can vary in size, shape, or rate of development (Vieira, 2003).

In humans, hypodontia is more common in the permanent dentition than in the primary dentition (Tucker and Sharpe, 2004; Vieira, 2003). However, the fact that mice have only one dentition, equivalent to the primary dentition in humans, makes it rather difficult to compare mice and humans (Tucker and Sharpe, 2004). In the primary dentition, hypodontia is more common in the maxilla and is frequently associated with the lateral incisors (Fekonja, 2005). There is no difference in the frequency of hypodontia between boys and girls (Endo et al., 2006; Fekonja, 2005). If the primary tooth is missing, the successional tooth is more likely to be absent (Fekonja, 2005). In the permanent dentition, hypodontia occurs with equal frequency in the maxilla and mandible and most frequently affects the third molars (Fekonja, 2005). The type of missing teeth and the population prevalence vary between racial

groups (Fekonja, 2005). Excluding the third molars, hypodontia is most commonly found in the maxillary lateral incisors and mandibular second premolars (Altug-Atac and Erdem, 2007; Fekonja, 2005; Mattheeuws et al., 2004; Sisman et al., 2007). Besides the third molars, population prevalences across the world vary between 1.6 and 9.6 percent (Fekonja, 2005). Absence of the third molar is commonly found in most population studies, with prevalences reported of 9–37 percent (Fekonja, 2005). The incidence of missing permanent teeth, excluding the third molar, is 1.74 percent in Turkish orthodontic patients (Altug-Atac and Erdem, 2007), 5.5 percent in Jordanian dental patients (Albashaireh and Khader, 2006), 6.1 percent in Swedish children (Thilander and Myrberg, 1973), 6.5 percent in Norwegian children (Aasheim and Ogaard, 1993), 6.9 percent in Southern Chinese children in Hong Kong (Davis, 1987), 7.54 percent in Turkish orthodontic patients (Sisman et al., 2007), 8.5 percent in Japanese children (Endo et al., 2006), 11.2 percent in Koreans (Chung et al., 2008),.and 11.3 percent in orthodontically treated children in Slovenia (Fekonja, 2005).

1.2.7.4 Genes known to cause non-syndromic hypodontia in mice and humans

To date, the number of genes identified as having a role in tooth development exceeds 100. All these genes are potential candidates for tooth agenesis in humans (http://biteit.helsinki.fi/). Tooth agenesis is probably caused by several independent defective genes, acting alone or in combination with other genes, leading to a specific phenotypic pattern (Vieira, 2003).

Mutations in muscle segment homeobox (*MSX1*), paired box 9 (*PAX9*), Axis inhibition protein 2 (*AXIN2*) and Ectodysplasin-A (*EDA*) are associated with tooth agenesis in humans and in mice, but interestingly in humans, these genes are also associated with isolated or non-syndromic tooth agenesis (Das et al., 2002; Tucker and Sharpe, 2004; Vieira, 2003).

A non-syndromic form of selective tooth agenesis is associated with mutations in human *MSX1* and *PAX9*, leading to haploinsufficiency. The lack of Msx1 and Pax9 in mice leads to complete tooth agenesis. In conclusion, Msx1 and Pax9 play critical roles in early tooth development.

Msx1, a transcription factor, is expressed in several embryonic structures, including the dental mesenchyme. *Msx1*$^{-/-}$ mice exhibit cleft palate, deficient mandibular and maxillary alveolar bones, and failure of tooth development. These mice also have abnormalities of the nasal, frontal and parietal bones, and of the malleus in the middle ear. The phenotype is similar to that of human cleft palate and oligodontia in which the defective gene is known. Thus Msx1 plays a critical role in epithelial-mesenchymal interactions during craniofacial and tooth development (Satokata and Maas, 1994; Vieira, 2003).

PAX9 is a contributor to one specific type of tooth agenesis. *Pax* gene products are thought to function primarily by binding the enhancer DNA sequences and by modifying transcriptional activity of downstream genes. *Pax9*$^{-/-}$ mice have craniofacial and limb anomalies, and failure of tooth development at the bud stage. Molar agenesis is associated with a defective *PAX9* (Vieira, 2003).

Axin2, a negative regulator of Wnt signaling pathway, is directly controlled by Wnt/beta-catenin. *AXIN2* mutation has been identified in an unrelated young patient with severe tooth agenesis (Lammi et al., 2004). The mutation is expected to inhibit and stimulate of Wnt signaling, which may lead to tooth agenesis. During tooth development, *Axin2* is expressed in the mesenchyme underlying oral and dental epithelium, enamel knot, dental papilla mescenchyme, and odontoblasts (Lammi et al., 2004). *Axin2* is also expressed in the tail bud, presomitic mesoderm (PSM), dorsomedial lip of the dermomyotome of mature somites, lateral plate mesoderm, limb buds, brain, spinal cord, and branchial arches I and II (Aulehla et al., 2003).

EDA, a signaling molecule of the tumor necrosis factor superfamily, is required for normal development of ectodermal derivatives, such as teeth, hair, nails, or sweat glands. *Eda* expression is detected in several organs during embryogenesis and also is detected in the epithelium in developing murine tooth and hair. $Eda^{-/-}$ mice have defective ectodermal organ development, such as abnormal primary hair follicle, and defects of molar tooth crowns, which consist of missing of the third molar, and reduced size and number of cusps of first and second molars (Mikkola and Thesleff, 2003). The missing teeth in mice are similar to congenitally missing teeth in patient with non-syndromic tooth agenesis with *EDA* mutations (Han et al., 2008).

1.2.8 p63 and tooth development

p63 isoforms start to express at E10 throughout the simple epithelium during development of structures in the mouse embryo. *p63* expression is detected in enamel organ/differentiated ameloblasts at different stages of tooth development and in basal cells of epidermis. During tooth development, p63 isoforms are detected throughout the epithelium at initiation (E11), placode (E12), bud (E13) and cap (E14-E15) stages (Mikkola, 2007). The pan-p63 hybridization signal (**Figure 1.21)** is seen throughout the dental epithelium, and its expression later extends into the oral epithelium (B-D). At the bell stage (E17), because p63 mRNAs are reduced in differentiating cells, p63 mRNAs are more intense in the outer enamel epithelium than in the inner enamel epithelium, which subsequently give rise to enamel-secreting ameloblasts and stellate reticulum (Laurikkala et al., 2006).

The study of p63 protein expression during tooth morphogenesis in the mouse embryo has shown that p63 proteins are detected in the undifferentiated ectoderm (enamel organ), and in the differentiated epithelial cells where it colocalizes with K5 and Ki67, both of which are epithelial markers for differentiation (Rufini et al., 2006).

The lack of p63 affects tooth development. In $p63^{-/-}$ embryos, tooth development ceases at the dental lamina stage. Dental placodes are not present in either E12 or E13 embryos and the thickened epithelium of the dental lamina appears to regress in $p63^{-/-}$ embryos during later stages of development (Laurikkala et al., 2006).

ΔNp63 isoforms are detected at high levels in embryonic ectoderm at all stages of tooth development. In contrast, TAp63 isoforms are not detected at all at any stages of tooth development (Laurikkala et al., 2006) (**Figure 1.22)**.

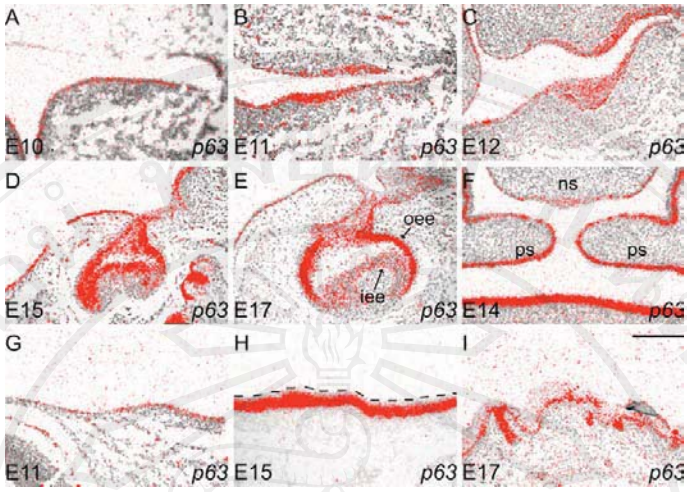


Figure 1.21 pan-p63 hybridization signal in tooth development. (A-B) p63 mRNAs are demonstrated in the simple epithelium and dental placode respectively. (C-E) At all stages of tooth formation, p63 mRNAs are expressed in the enamel organ, which differentiates from ectoderm. (F) p63 mRNAs are expressed in the epithelium during palate formation. (G) At E11, p63 mRNAs are present in simple surface ectoderm. (H) p63 mRNAs continue in the basal epithelial cell layer (whereas they are downregulated in the superficial epithelial cell layer) at E15, (I) and in the skin and basal epithelial cell layer at E17 (reproduced from Laurikkala et al., 2006)

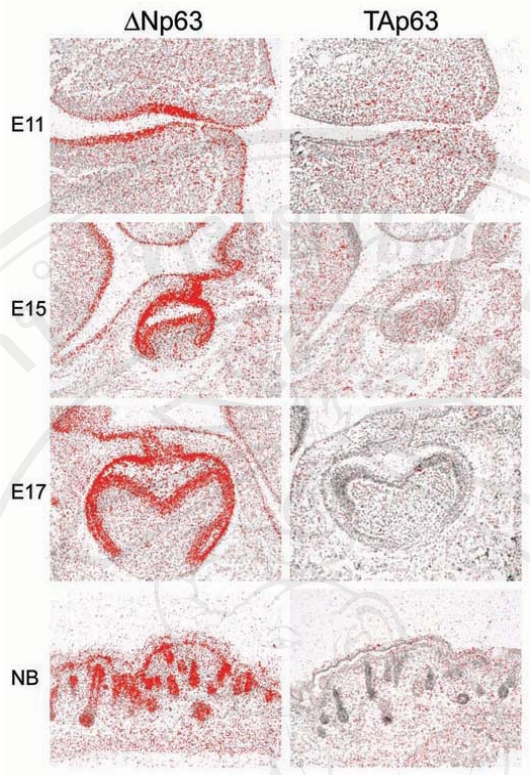


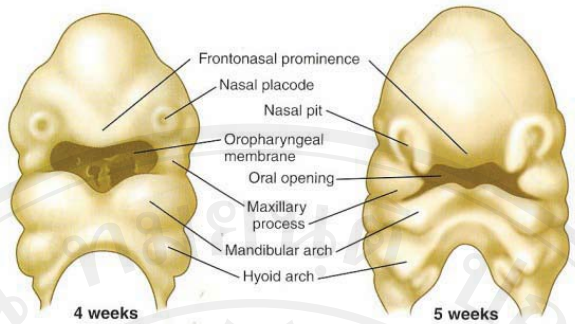
Figure 1.22 p63 isoforms in tooth development. Intense expression of ΔN isoforms at all stages of skin, hair and tooth development (E10 to P3), whereas TA isoforms cannot be observed at any time except in some other, non-ectodermal tissues (reproduced from Mikkola, 2007).

1.2.9 Facial development and palate development

1.2.9.1 Facial development

The face develops from five facial processes: two mandibular processes, two maxillary processes and the frontonasal process. During the fourth gestational week the mandibular processes fuse in the midline to form the lower jaw. Following the formation of the nasal placodes the medial and lateral nasal processes develop within the frontonasal process. During the sixth and seventh weeks, the medial nasal processes fuse with each other and with the maxillary processes thus forming the upper lip and the primary palate. Failure of the fusion of facial processes can result in an orofacial cleft. The most common is a failure of the maxillary process and the frontonasal process causing a cleft lip or a failure of the two palatal shelves of the maxillary processes causing a cleft palate (for review, please read Rice, 2005) **(Figure 1.23)**.

A



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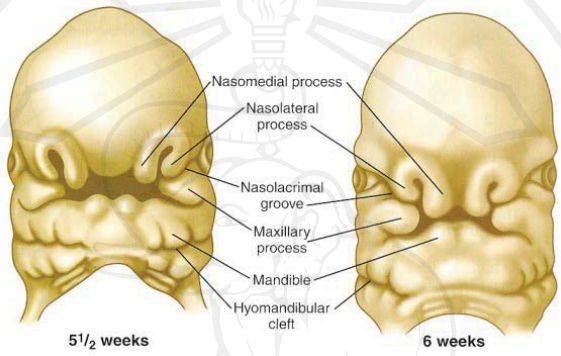

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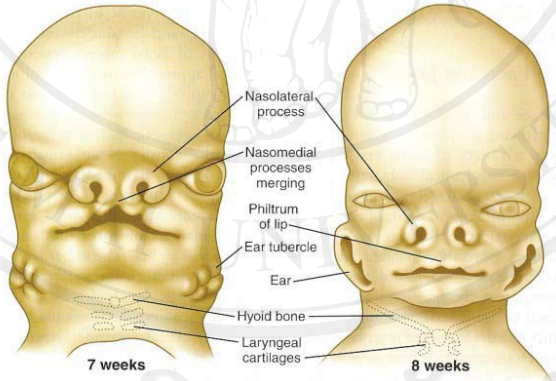

Figure 1.23 Facial development: (A) At 4-5 weeks, five facial processes begin to develop: two maxillary processes, two mandibular processes (fused together at 4 weeks), and one frontonasal process. (B) At 5½-6 weeks, the nasomedial process and nasolateral process are formed. (C) At 7-8 weeks, the medial nasal processes fuse with each other and with the maxillary processes (reproduced from Carlson, 2004).

1.2.9.2 Palate development

The secondary palate develops late during organogenesis of higher vertebrates. The process begins on 45th day in humans and E12in mice (Ferguson, 1988). Arising as bilateral outgrowths of the maxilla, the palatal shelves stretch from the anterior to the posterior, along the lateral walls of the oropharynx. These palatal shelves are formed of mesenchyme mainly of neural crest as well as mesodermal origin in association with ectoderm. In mammals, growth of the palatal shelves causes their vertical extension on each side of the tongue. Under both mechanical and morphogenetic forces, the palatal shelves elevate at a definite time and become horizontally positioned over the tongue. Continued directed growth causes the approximation of the horizontally occurring palatal shelves. Palatogenesis culminates when the shelves make contact, adhere and fuse along the midline, forming an epithelial seam, later replaced by mesenchyme, to form the definitive palate **(Figure 1.24)**. The definitive palate fuses with the primary palate and the nasal septum in the anterior to middle regions and thus separates the oral and the nasal regions. Disruption of palate development can occur at any stage of the morphogenesis (for review, please read Ferguson, 1988; Rice, 2005).

Retarded growth of the palatal shelves, impaired shelf elevation, failure of the bilateral shelves to contact, adhere or fuse medially, post-fusion rupture and failure of the mesenchyme to differentiate appropriately are the commonly recognized causes of a cleft palate (Ferguson, 1988; Rice, 2005).

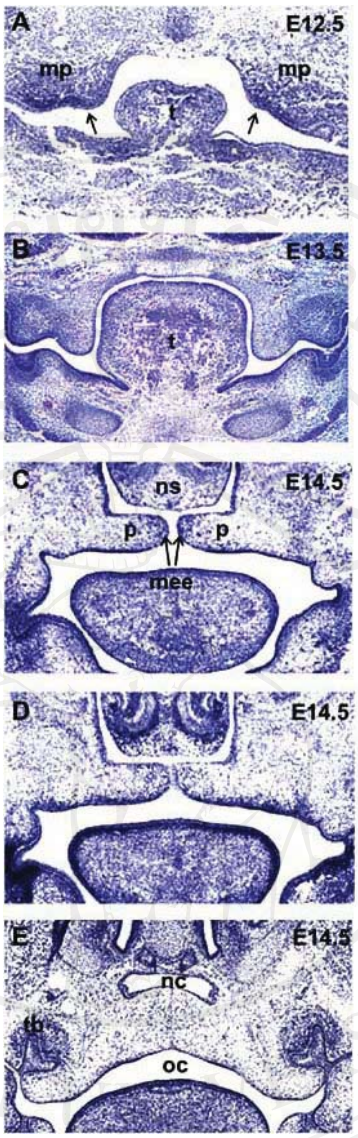

Figure 1.24 Palate development: (A) At E12.5, budding of palatal shelves from the maxillary processes. (B) At E13.5, elongation of the palatal shelves on the sides of the tongue. (C-D) At E14.5, elevation of the palatal shelves and epithelium of the palatal shelves are in contact. (E) Palatal shelves are fused (reproduced from Rice, 2005).

Epithelial-mesenchymal interactions control early palate morphogenesis. Bmp and Fgf signals from the mesenchyme induce the epithelial cells to proliferate. Then, Shh signals from the epithelium stimulate the underlying mesenchyme to proliferate, setting up a morphogenetic cycle.

1.2.9.3 Orofacial clefts

Orofacial clefts include syndromic and non-syndromic forms of cleft lip with or without cleft palate (CL/P) and cleft palate only (CP), respectively. A highly prevalent congenital anomaly, orofacial clefts can result from genetic and environmental disturbances. Mutations in *DHCR7*, *EFNB1*, *FGFR1*, *IRF6*, *OFD1*, *MID1*, *MSX1*, *PVRL1*, *TP63*, *SIX3*, *TGIF*, *PTCH1*, *GLI2*, *COL II*, *COL XI*, *DTDST*, *FGFR2*, *TGFβR1*, *TGFβR2*, *TBX1*, *TBX22*, *TCOF1*, *TWIST* are known to cause orofacial clefts in humans (for review, please read Rice, 2005). The known human teratogens of orofacial clefts are diphenylhydantoin (dilantin), thalidomide, dioxin, ethyl alcohol, trimethadione, retinoids, aminopterin and methotrexate, as well as hyperthermia (Gorlin et al., 2001; Rice, 2005). There are other involved factors, such as geographical origin, racial and ethnic backgrounds, and socio-economic status. Non-syndromic forms of cleft palate are found in 50% of cases (Koillinen et al., 2005). Non-syndromic forms of cleft palate occur in 1:1000 live human births, whereas cleft lip with or without cleft palate occurs in 1:700 live human births in the Caucasian population (Koillinen et al., 2005; Rice, 2005).

1.2.10 p63 and palate development

p63-mediated signals are important for the outgrowth and patterning of the facial processes and in the initial stages of palatal development. The roles of p63 in the regulation of Bmp signaling that controls the growth, modeling and fusion events underlying facial development have been reported (Thomason et al., 2008). Absence of p63 results in increased Bmp signaling in several areas of the facial processes. Consequently, down-regulation of the key signaling molecules Fgf8 and Shh leads to a reduction of mesenchymal cell proliferation, regional growth defects and altered morphogenesis of the nasal processes, resulting in a bilateral cleft of the upper lip. In addition, a decreased proliferation in the mesenchyme of the maxillary processes at E11.5 results in absence of the anterior region of paired palatal shelves and, subsequently, cleft palate (for review, please read Thomason et al., 2008).

p63 transcripts/proteins are detected in the ectoderm of the facial processes and of the maxillary and mandibular components of the first branchial arch, during facial development. p63 transcripts/proteins are highly detected in the fusion regions between the medial nasal process and maxillary process on each side, which lead to the formation of the upper lip and primary palate. p63 transcripts/proteins have been found to be detected at all stages of development of the secondary palate (Thomason et al., 2008) **(Figure 1.25)**. Besides, p63 mRNAs are intense in the epithelium of the palatal shelves, which develop from bilateral outgrowths of the maxillary process (Laurikkala et al., 2006) **(Figures 1.21.F and 1.25.L)**.

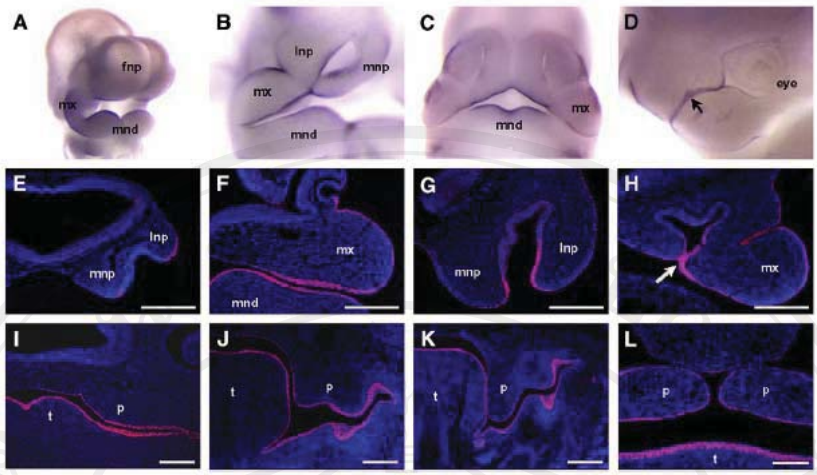

Figure 1.25 Expression of p63 transcripts/proteins during facial morphology: (A) At E9.5, p63 transcripts are detected in the ectoderm of the facial processes and the maxillary and mandibular components of the first branchial arch. (B) At E10.5, p63 transcripts are detected in the ectoderm surrounding the nasal pit and in the maxillary processes. (C) At E11.75, p63 transcripts are detected in the leading edges of the mandible and the fused regions of the medial nasal processes. (D) At E12.5, intense p63 transcripts are detected in the nasolacrimal groove. (E and F) At E10.5, p63 proteins are detected in basal cells of the ectoderm in the most distal portions of the medial and lateral nasal processes (E) and in the ectoderm of the mandibular and maxillary process (F). (G and H) At E11.5, p63 proteins are detected in the ectodermal component of the nasal pit (G) and highly in the fusion region between the medial nasal process and maxillary process (arrowed) (H). (I–L) During development of the secondary palate, p63 proteins are detected in the ectoderm in the oral cavity (I), the developing tooth germs (J), and the palatal shelves (J–L). fnp, frontonasal process; mnd, mandible; mx, maxillary process; mnp, medial nasal process; lnp, lateral nasal process; p, palate; t, tongue; tg, tooth germ (reproduced from Thomason et al., 2008).

p63$^{-/-}$ embryos exhibit bilateral cleft lip and a complete cleft of the secondary palate **(Figure 1.26)**, suggesting that the defect in palatogenesis occurs as a result of abnormal growth and patterning during early embryogenesis (Thomason et al., 2008).

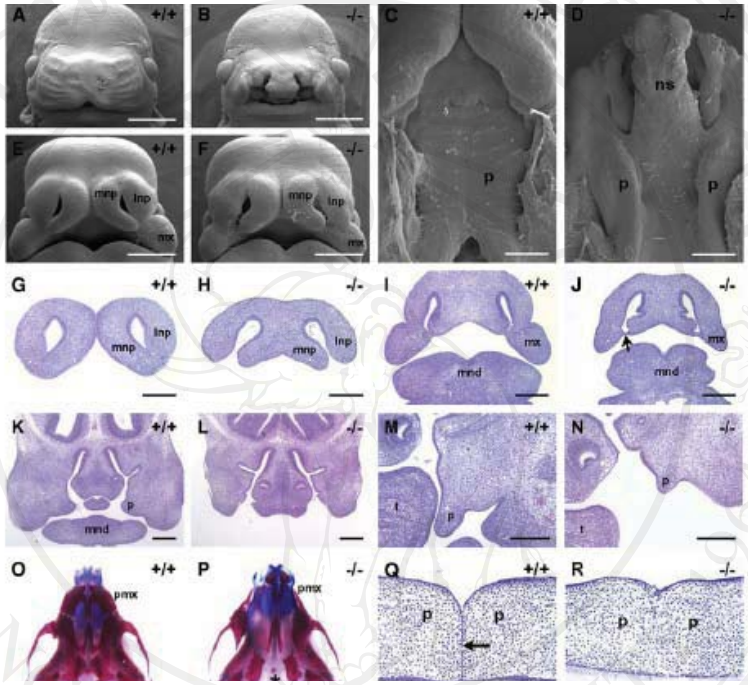

Figure 1.26 Bilateral cleft lip (B) and complete cleft of the secondary palate (D) phenotype of *p63*$^{-/-}$ mice. (E and F) At E11.5, the phenotype is initially apparent by the maxillary processes of *p63*$^{-/-}$ embryos appearing smaller and the medial nasal processes appearing rounded at the tips and bulging medially (F). (G–N) Abnormally-shaped facial processes and absence of palatal outgrowth. (O and P) Abnormalities of the nasal capsule, absence of the premaxilla, palatal processes of the maxilla and palatine bone. (Q and R) palatal shelves of *p63*$^{-/-}$ fuse in the midline with no evidence of an epithelial seam. mnd, mandible; mx, maxillary process; mnp, medial nasal process; lnp, lateral nasal process; p, palate; t, tongue; ns, nasal septum; pmx, pre-maxilla (reproduced from Thomason et al., 2008).

1.3 OBJECTIVES

1.3.1 To study if *TP63* mutations are associated with non-syndromic hypodontia.

1.3.2 To study if *TP63* mutations are associated with non-syndromic orofacial clefts.

1.3.3 To study if *TP63* mutations are associated with syndromic hypodontia with/without orofacial clefts.

1.4 HYPOTHESIS

H_0: *TP63* mutations are not associated with patients with non-syndromic hypodontia, non-syndromic orofacial clefts and syndromic hypodontia with/without orofacial clefts.

H_1: *TP63* mutations are associated with patients with non-syndromic hypodontia, non-syndromic orofacial clefts and syndromic hypodontia with/without orofacial clefts.