The Role of the Claudin 6 Cytoplasmic Tail in Epidermal Differentiation

and

The Role of Cdx in Endodermal Development

Adebola Enikanolaiye

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the Doctorate in Philosophy degree in
Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

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ABSTRACT

The mammalian skin provides a necessary barrier between the organism and the environment, defending against loss of water and solutes, preventing the invasion of pathogens as well as protecting against chemical and physical assault. Claudin (Cldn)-based Tight Junctions (TJs) are the main functional part of the skin barrier. In particular, Cldn6 through its cytoplasmic tail has been shown to be important for barrier function. In other to further investigate the role of the Cldn6 tail in TJ-function, we developed Cldn6 mouse mutants carrying varying truncations of the Cldn6 tail. Both of these mice present with epidermal differentiation perturbations and delayed barrier function that is repaired later in life. These studies support the importance of the tail portion of the Cldn molecules in epidermal differentiation and barrier function. In addition, both of these mouse models are useful for the study of barrier function in preterm infants and in aging, with the hope of developing novel therapeutics for the alleviation of barrier dysfunction.

Cdx is a family of homeodomain (HD) transcription factors (TFs) essential for many key developmental processes. In particular, Cdx2 is important for the establishment and maintenance of posterior identity in the developing endoderm. In spite of this, only a few Cdx targets in the developing endoderm have been discovered. In addition, the interplay between Cdx and its targets within the endoderm is poorly understood. In this study, we show that the *forkhead* box transcription factor, *Foxa2* is a Cdx2 target. We also show that Foxa2 and Cdx2 physically and genetically interact to regulate a subset of genes that are implicated in endodermal development. These studies help to further our understanding of endoderm biology with the goal of developing new strategies to diagnose and treat diseases associated with defective endoderm development.

RÉSUMÉ

La peau de mammifère fournit une barrière nécessaire entre l'organisme et l'environnement, forme la défense contre la perte d'eau et de solutés, prévient l'invasion de pathogènes ainsi que donne la protection contre les risques chimiques et les agressions physiques. Claudin jonctions serrées sont la partie fonctionnelle principale de la barrière de la peau. En particulier, Cldn6 par sa queue cytoplasmique a été montré pour être importants pour la fonction de barrière. Pour investiguer le rôle de la queue Cldn6 en TJ - fonction, nous avons développé des souris mutantes Cldn6 transportant différentes troncatures de la queue Cldn6. Ces deux souris présentent des perturbations épidermiques de différenciation et de la fonction de barrière retardé qui est réparé plus tard dans la vie. Ces études confirment l'importance de la partie de queue des molécules Cldn dans la différenciation épidermique et la fonction barrière. En outre, ces deux modèles de souris sont utiles pour l'étude de la fonction de barrière chez les prématurés et dans le vieillissement, avec l'espoir de les nouveaux produits thérapeutiques en développement pour la réduction de la dysfonction de la barrière.

Cdx est une famille de facteurs de transcription à homéodomaine essentielles pour de nombreux processus de développement clés. En particulier, Cdx2 est important pour l'établissement et le maintien de l'identité postérieure dans l'endoderme qui dévelop. En dépit de cela, seuls quelques objectifs Cdx dans les endoderme en développement ont été découverts. En outre, l'interaction entre Cdx et ses objectifs dans l'endoderme est mal comprise. Dans cette étude, nous montrons que la boîte de *forkhead* facteur de transcription, *Foxa2* est une cible de Cdx2. Nous montrons également que Foxa2 et Cdx2 physiquement et génétiquement interagissent pour régler un sous-ensemble de gènes qui

sont impliqués dans le développement endodermique. Ces études contribuent à approfondir notre compréhension de la biologie endoderme dans le but de développer de nouvelles stratégies pour diagnostiquer et traiter les maladies associées au développement de l'endoderme défectueux.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
AIP	Anterior Intestinal Portal
AJ	Adherens Junction
AP	Anterior-posterior
AP-MS	Affinity purification/Mass spectrometry
APC	Adenomatous polyposis coli
AQP	Aquaporin
α-SMase Acid	α-Sphingomyelinase
β-celerase	β-glucocerebrosidase
BioID	Proximity dependent Biotin Identification
BMP	Bone Morphogenetic Proteins
BSA	Bovine Serum Albumin
Ca2+	Calcium
cad	caudal
cAMP	cyclic Adenosine Monophosphate
CBP	CREB binding protein
cDNA	complementary DNA
CDRE	Cdx response element
Cdx	caudal related homeobox
Cdx1/2 DKO	$Cdx1^{-/-}$ $Cdx2^{-/-}$
CE	Cornified Envelope
ChIP	Chromatin immunoprecipitation
ChIP-seq	ChIP-sequencing
CIP	Caudal Intestinal Portal
Cl-	Chloride Ion
Cldn	Claudin
Co-IP	Co-Immunoprecipitation
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DBD	DNA Binding Domain
DKO	Double Knock-out
Dlg	Discs-large
Dll	Delta-like
DNA	Deoxyribonucleic acid
DPM	Dermal phase meter
Dsg	Desmoglein
DTT	Dithiothreitol
E	Embryonic days post-conception
EB	Embryoid Body
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGTA	Ethylene glycol tetraacetic acid
EMSA	Electrophoretic mobility shift assay
Eomes	Eomesodermin
EPB	Epidermal Permeability Barrier

EpCAM	Epithelial cell adhesion marker
Eph A2	Ephrin type-A receptor 2
EPU	Epidermal Proliferative Unit
ER	Estrogen receptor
ERK	Extracellular signal-Regulated Kinase
ES cell/ESCs	Embryonic Stem cell
FACS	Fluorescence activated cell sorting
FGF	Fibroblast Growth Factor
FHHNC	Familial hypomagnesemia, hypocalciuria and nephrocalcinosis
FIL	Filaggrin
FITC	Fluorescein isothiocyanate
FIZ1	Flt3 Interacting Zinc finger protein-1
flox	flanked by loxP
FLT-3	Fms-like tyrosine kinase 3
Fox	Forkhead box
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GI	Gastrointestinal
H	Histone
H&E	Hematoxylin and Eosin
HCl	Hydrochloric acid
HD	homeodomain
Hex	Hematopoietically expressed homeobox
HNF	Hepatocyte Nuclear Factor
Hox	Homeobox
HRP	Horseradish peroxidase
IAP	Intestinal alkaline phosphatase
ICM	Inner Cell Mass
IFABP	Intestinal fatty acid binding protein
IgG	Immunoglobulin Type G
Inv/INV	Involucrin
IP	Immunoprecipitation
IPAL	Intestinal Phospholipase A
JAM	Junctional adhesion molecules
K	Keratin
LEF	Lymphoid enhancer factor
LOR	Loricrin
MAGUK	Membrane-Associated Guanylate Kinases
MDCK	Madin Darby Canine Kidney Cells
Mix1	Mix-paired homeobox
mRNA	messenger Ribonucleic Acid
Muc	Mucin
MUPP1	Multi-PDZ Domain Protein 1
Na+	Sodium Ion
NHE1	Na+/H+ exchanger 1
NLS	Nuclear Localization Signal

Occ	Occludin
Oct	Octamer-binding transcription factor
P	Postnatal day
p63	Tumor protein 63
PAGE	Polyacrylamide gel electrophoresis
PATJ	PALS1- associated TJ protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pdx	Pancreatic and duodenal homeobox
PDZ	Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and Zonula occludens-1 protein (ZO1)
PFA	Paraformaldehyde
PKA	Protein Kinase A
Pol II	RNA Polymerase II
PPAR	Peroxisome Proliferator-Activated Receptors
qPCR	quantitative PCR
RA	Retinoic acid
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RT-PCR	Reverse transcriptase PCR
S1P	Sphingosine-1-phosphate
SC	Stratum Corneum
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Smad	sma/mothers against decapentaplegic
Smo	Smoothened
Sox	Sry-related HMG box
STAT	Signal Transducer And Activator Of Transcription
T	Brachyury
TA	transit-amplifying
TBS	Tris Buffered Saline
TBS-T	TBS-Tween
TCF	T-cell factor
TE	Trophectoderm
TER	Trans-Epithelial Resistance
TEWL	Trans-Epidermal Water Loss
TF	Transcription factor
TG1	Transglutaminase-1
TGF	Transforming Growth Factor
TJ	Tight Junction
TPA	12-O-tetradecanoyl-phorbol-13-acetate
TSS	Transcriptional start site
UV	Ultraviolet
WNK	WNK lysine deficient protein kinase
Wnt	Wingless
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ZO	Zonula Occludens
ZONAB	(ZO1)-associated nucleic acid binding protein

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CHAPTER 1 - GENERAL INTRODUCTION I

Importance of the Mammalian Skin

The mammalian skin is the outermost and the largest organ of the body accounting for up to 16% of body weight and a surface area of 1.8m^2 (Kanitakis, 2002). The skin and its appendages such as the hair follicles, sebaceous glands and sweat glands are indispensable for a number of critical functions. It is important for excretion, mediating the regulation of temperature, reducing the harmful effects of UV radiation and synthesis of vitamin D. The skin also plays a sensory role in that it allows organisms to perceive their environment. Through camouflage, the skin also protects against predators and serves as decoration for social and reproductive behavior. Finally, the skin functions in immunological surveillance as it contains critical elements of cellular immunity such as lymphocytes and mast cells. The single most critical function of the skin, however, is that it serves as a barrier to the outside environment, regulating the entry and exit of water, electrolytes and various other substances. In these capacities, it serves as a protective barrier against infections, toxic agents and mechanical, thermal and physical injury (Bickers and Athar, 2006;Bikle, 2011;Bikle, 2012;Fitzpatrick, 1993;Malissen et al., 2014;Tamoutounour et al., 2013;Wysocki, 1995).

There are a number of complex mechanisms responsible for the formation of the skin barrier during embryogenesis and for its maintenance throughout life. Critical to these processes is the ability of the skin to maintain a fine balance between proliferation and differentiation. Any dysfunction in these processes compromises the ability of the skin to regenerate and repair itself in response to external trauma. It also can result in a number of pathological conditions, which include various forms of dermatitis and the immune response-related psoriasis. Although not life threatening, skin conditions such as

psoriasis and dermatitis are highly prevalent and can significantly reduce quality of life. In addition, studies have shown that the absence of this barrier function is involved in the pathogenesis of more serious immune disorders such as inflammatory bowel disease and asthma (Cookson, 2004;Cork et al., 2009;Hammad and Lambrecht, 2008;Xavier et al., 2007). Finally, skin barrier dysfunction is also implicated in a number of cancers such as basal and squamous cell carcinomas (Dubas and Ingraffea, 2013).

Composition of the Mammalian Skin

The mammalian skin is made up of three layers namely the innermost hypodermis, the dermis and the outermost epidermis (Butnaru and Kanitakis, 2002) (Figure 1). The hypodermis is a loose layer of connective tissue and fat beneath the dermis that separates the skin from the underlying muscle. It is mostly made of adipocytes, is important for temperature control and provides a cushion against physical traumas. The vascularized hypodermis is also important for supplying nutrients to the rest of the skin (Kanitakis, 2002).

The dermis is made up of mesodermally-derived connective tissue and is found below the epidermis. Embedded within the dermis are the dermal vasculature, (blood and lymph vessels), nerve cells and fibers, sweat glands, sebaceous glands, hair follicles (which originate in the dermis and transverse the epidermis), nails and some striated muscle (Fuchs and Raghavan, 2002). The dermis is made of two layers, a thin papillary layer and a thicker reticular layer. The papillary dermis connects the epidermis and contains loosely arranged collagen fibers while the reticular layer is made up of thicker bundles of collagen, which extend to the hypodermis below. Fibroblasts within the

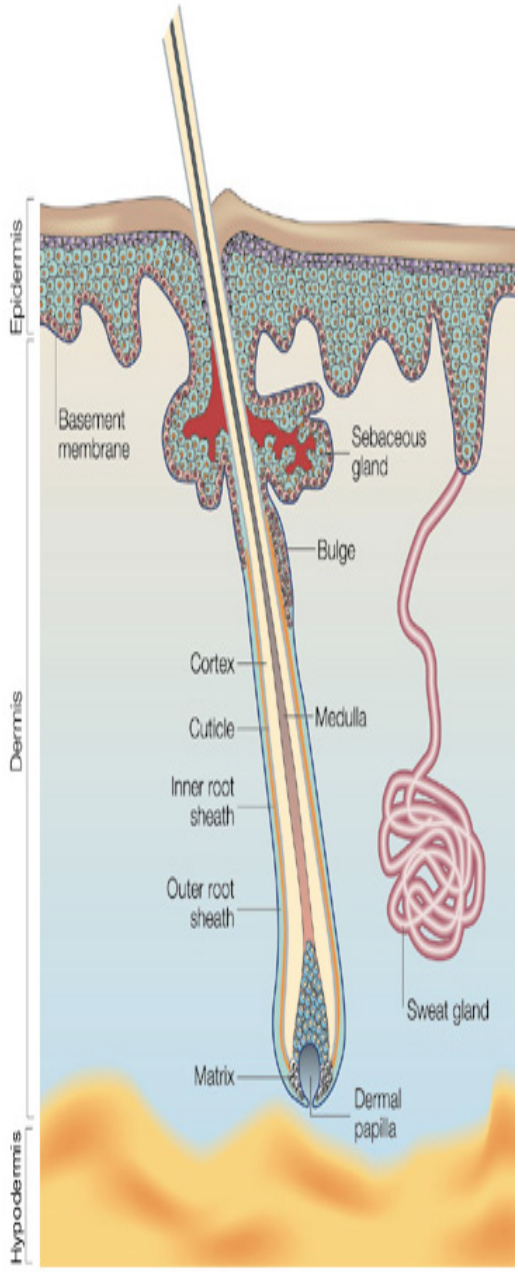

Figure 1: Overview of the Mammalian Skin. The mammalian skin is made up of the epidermis, dermis and hypodermis. The epidermal appendages, the hair follicle sebaceous gland and sweat gland, are also indicated here. Adapted with permission from Fuchs and Raghavan, 2002.

dermis produce collagen, elastin and other structural proteoglycans, immunocompetent mast cells and macrophages. Collagen gives the dermis its strength and toughness, elastin maintains elasticity and flexibility while the proteoglygcans provide viscosity and hydration (Burgeson and Christiano, 1997;Marinkovich et al., 1993;Nguyen-Trong et al., 2013).

The Epidermis

The mammalian epidermis is the outermost layer of the skin. It is a self-renewing stratified squamous epithelium that is separated from the underlying dermis by a basement membrane. The basement membrane consists of proteins secreted by epidermal keratinocytes and dermal fibroblasts (Breitkreutz et al., 2013). The basement membrane is also a source of nutrients for the non-vascularized epidermis. The epidermis itself is mostly made up of keratinocytes, which produce the structural protein Keratins (K) (Moll et al., 1982). Keratins are a large family of fibrous proteins that are co-expressed as pairs and function as obligate heteropolymers to provide structural support and tensile strength (Coulombe and Wong, 2004;Fuchs and Green, 1978;Fuchs, 1995;Steinert, 1993;Steinert et al., 1993;Steinert et al., 1994;Sun and Green, 1978). The epidermis also contains Langerhans cells, which are important for immune surveillance, melanocytes, which are responsible for pigmentation and protection from UV irradiation and Merkel cells, which are important for sensation (Holikova et al., 2001;Plonka et al., 2009;Slominski et al., 2005).

EPIDERMAL DEVELOPMENT AND STRATIFICATION

In mammals, epidermal development is a multistage process consisting of epidermal specification, commitment, stratification and terminal differentiation as well as the morphogenesis of epidermal derivatives. Distinct signaling patterns specify different developmental stages and ensure the correct morphogenesis of the epidermis and its appendages. Reciprocal interactions between the epidermis and the underlying dermis are also necessary for proper epidermal development.

Epidermal Specification

Gastrulation, the process by which the three primary germ layers are formed, begins with the formation of the primitive streak at the posterior of the epiblast (Bellairs, 1986;Lawson et al., 1986). Totipotent cells from the epiblast divide, differentiate and rearrange to form the ectoderm, mesoderm and endoderm. In the mouse, endoderm and mesoderm precursor cells of the epiblast undergo an epithelial to mesenchymal transition and migrate through the primitive streak (Lawson et al., 1991). These emerging cells go on to form mesoderm and definitive endoderm of the embryo while the remaining epiblast cells that do not migrate through the streak exist on the embryonic surface and form ectoderm (Figure 2).

After gastrulation, the ectoderm gives rise to the surface ectoderm, which is a single layer of simple epithelium consisting of flat cells expressing keratins (K) K8/K18 (Franke et al., 1979;Franke et al., 1979;Fuchs, 1995;Jackson et al., 1981;Landstrom and Lovtrup, 1979;Wolpert, 1998). The epidermal and neuronal lineage derive from a common progenitor, the neuroectoderm. The neuronal fate is thought to be the default

state of the ectoderm while the epidermal fate must be induced. Signaling molecules mostly produced by the dorsal mesoderm act on the ectoderm to determine cell fate (Chang and Hemmati-Brivanlou, 1998;Moreau and Leclerc, 2004). Bone morphogenetic protein (BMP) signaling, promotes epidermal development while it has an inhibitory effect on neural induction. Specifically, BMP4 mediates apoptotic cell death of neuronal cells (Botchkarev and Sharov, 2004;Gambaro et al., 2006). Wnt signaling mediates this fate decision by blocking the ability of the ectoderm to respond to FGFs. In the absence of FGF signaling, the ectodermal cells express BMPs and can then become specified towards the epidermal lineage. In addition, the switch to ectodermal fate occurs through the activation of ΔNp63, a p63 isoform (Romano et al., 2009;Romano et al., 2012;Wilson and Hemmati-Brivanlou, 1995). Epidermal specification occurs at about embryonic day (E) 8.5 in mice and at day 21 in humans. Studies show signals from the sub-ectodermal mesenchyme of the underlying dermis initiate epidermal specification and stratification. Although the exact nature of these signals is unknown (Ferraris et al., 2000), the other p63 isoform, TAp63 has been shown to be necessary for the initiation of epidermal stratification (Koster and Roop, 2004;Koster et al., 2004;Koster, 2010;Yao and Chen, 2012).

Embryonic Development of the Epidermis

After the epidermis has been specified, the embryonic epithelium is made up of a single layer of multipotent epidermal cells. These cells are able to maintain their population by cell division before withdrawing from the cell cycle and become

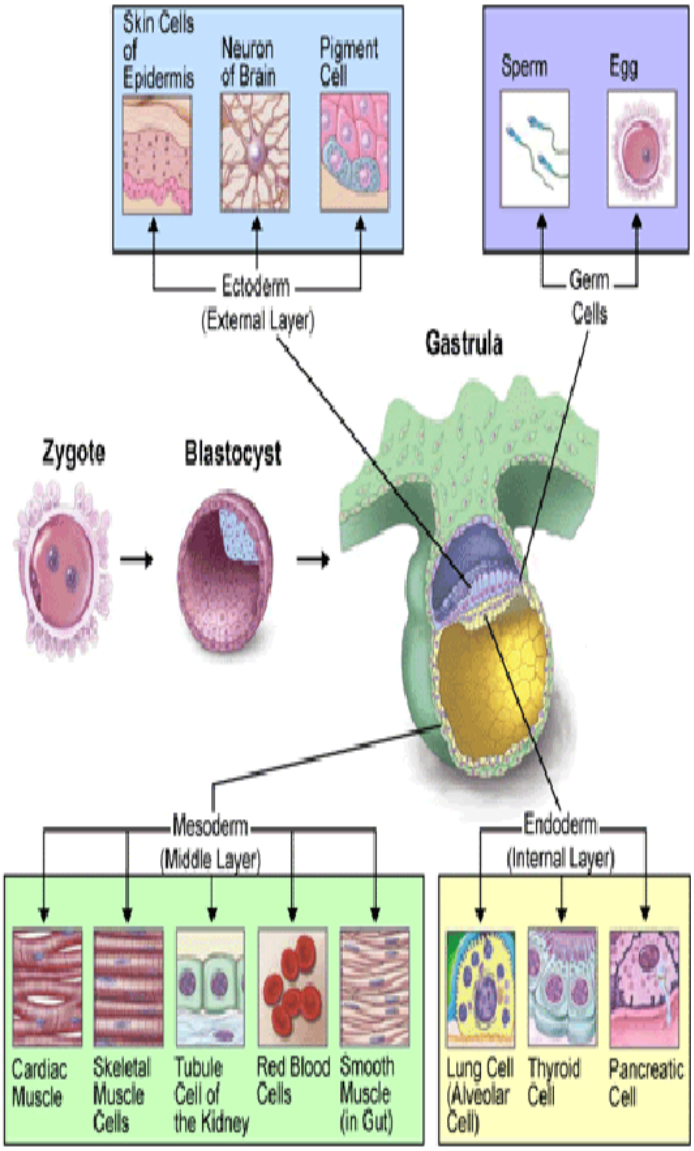

Figure 2: Formation of the three primary germ layers. Gastrulation is the process by which the three germ layers are formed. The innermost endoderm gives rise to the GI tract and its associated organs, the middle mesoderm gives rise to the muscles, skeletal system and the circulatory system and the outermost ectoderm forms the skin, brain, nervous system and other external tissues. Adapted from Wikimedia Commons, a public domain (http://commons.wikimedia.org)

irreversibly committed to terminal differentiation (Byrne et al., 1994;Byrne, 1997). The cells therefore detach from the basement membrane and begin an upward migration towards the skin surface to eventually form mature keratinocytes (Alonso and Fuchs, 2003a;Alonso and Fuchs, 2003b;Byrne, 1997;Kaur, 2006;Troy et al., 2005;Turksen and Troy, 1998).

The beginning of epidermal commitment is marked by a transition of K8/K18 expression to K5/K14 and p63 expression at E9.5 (Byrne et al., 1994;Mills et al., 1999;Yang et al., 1999). The embryonic epithelium then becomes the embryonic basal layer that gives rise to all structures of the future epidermis (Figure 3). Between E12-E14, the basal layer begins to proliferate to form an intermediate layer called the periderm (Byrne et al., 1994;M'Boneko and Merker, 1988;Smart, 1970). The periderm is a layer of protective tightly connected squamous endodermis-like cells. The periderm is shed once the epidermis becomes stratified and differentiated (Hardman et al., 1998). At E15.5 the epidermis forms an intermediate layer between the basal layer and the periderm, which is marked by the onset of K1/K10 expression (Bonneville, 1968;DuBrul, 1972;Hanson, 1947;Turksen and Troy, 1998). This layer corresponds to the spinous layer in the adult. The structural proteins loricrin (LOR) and filaggrin (FIL) are first expressed between E16.5 and E17.5 in what will become the granular layer. The stratum corneum (SC) is also formed at this time, creating a water impermeable layer (Bickenbach et al., 1995;Mehrel et al., 1990;Rothnagel et al., 1987). At E18.5 the epidermis is fully stratified, the periderm is shed and barrier acquisition is complete (Bonneville, 1968;Byrne et al., 1994;DuBrul, 1972;Hanson, 1947;Hardman et al., 1998;Weiss and Zelickson, 1975;Weiss and Zelickson, 1975) (Figure 3). The acquisition of the barrier is

therefore executed between E8.5 and E18.5 in mice, completed by the time the animal is born and maintained throughout life.

Epidermal Stratification

The adult epidermis is constantly renewed throughout life. This process is characterized by stratification and differentiation as well changes in cell morphology and gene expression patterns (Candi et al., 2005;Coulombe et al., 2004;Fuchs, 2007). The ordered and differential expression of keratins, as well as other differentiation-related molecules, allows us to accurately trace this process (Mack et al., 2005).

The adult epidermis is made up of four main layers: stratum basale (basal layer), stratum spinosum (spinous layer), stratum granulosum (granular layer), and stratum corneum (Figure 4). A fifth layer, the stratum lucidum, is a thin layer of translucent cells present in high-stress sites that require a thicker epidermis such as the soles of the feet [for comprehensive reviews of epidermal development and stratification refer to (Eckert et al., 1997;Eckert et al., 2013;Fuchs, 1995;Fuchs and Raghavan, 2002;Koster and Roop, 2007)].

The innermost layer of the epidermis, the basal layer, is a proliferative layer of undifferentiated columnar cells attached to the basement membrane (Fuchs and Horsley, 2008;Lavker and Sun, 1982). The basal layer is the only mitotically active epidermal layer and the basal layer, along with the hair follicle bulge and base of the sebaceous gland are the main epidermal stem cell niches (Figure 5) (Blanpain and Fuchs, 2006;Blanpain and Fuchs, 2009;Goldstein and Horsley, 2012;Tumbar et al., 2004). Epidermal homeostasis depends on these reservoirs of self-renewing stem cells that are

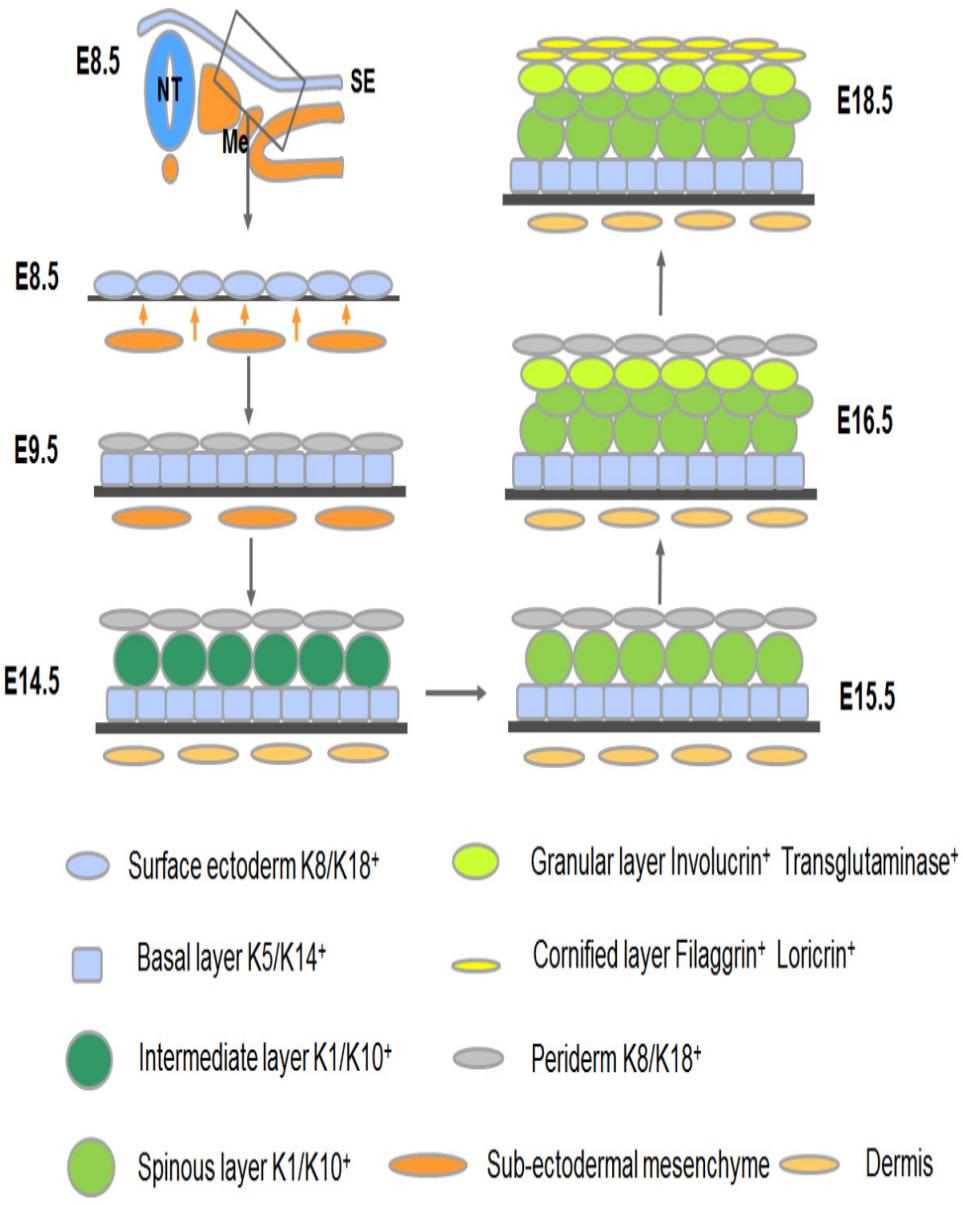

Figure 3. The Embryonic Epidermis. The epidermis is derived from embryonic surface ectoderm. After epidermal commitment, the surface ectoderm becomes the embryonic epidermal basal layer, which subsequently gives rise to the intermediate layer, spinous layer, granular layer and cornified layer. Different layers are labeled in different colors, with their specific markers annotated. Arrows at E8.5 indicate inductive cues from the meschencyme leading to stratification. NT, neural tube; Me, mesoderm; SE, surface ectoderm. Adapted with permission from Lu et al, 2013.

necessary for a lifetime of regeneration and maintenance by replacing cells that are shed from the outermost layers of the skin (Fuchs, 2007;Fuchs and Horsley, 2008;Kaur, 2006). Although the mechanism by which a single proliferative layer repopulates multiple layers of suprabasal cells is still unclear there are two models that describe the behavior of these stem cells within the basal layer (Cotsarelis, 2006;Jensen et al., 1999;Watt et al., 2006). The first model, the epidermal proliferative unit (EPU) model postulates that epidermal keratinocytes are organized into a column-like structure where one of the basal cells is the stem cell and the other cells are transit-amplifying (TA) cells that proliferate, differentiate, and move outwards to repopulate all the layers of the epidermis (Mackenzie, 1997;Potten, 1974). The second model is the asymmetric division model which suggests that the stem cells undergo asymmetric division (lateral or perpendicular to the basement membrane) to generate both a self-renewing daughter stem cell and a daughter cell committed to differentiation; in this model there is no intermediate "TA" cell (Clayton et al., 2007;Doupe and Jones, 2012;Fuchs, 2008;Fuchs and Horsley, 2008).

The basal layer also produces and assembles an extracellular matrix (ECM), which is mostly made of laminin5 and assembled by $\alpha 3\beta 1$-integrin. Cells in the basal layer express K5/K14. As the cells leave this layer, they exit the cell cycle, turn off expression of laminin5, integrin and K5/K14 and turn on K1/K10, which is maintained throughout the suprabasal layers (Byrne et al., 1994;Fuchs and Green, 1980;Fuchs, 1995;Turksen and Troy, 1998). In addition, keratinocyte cell shape begins to change from columnar to squamous. As the keratinocytes progress into the spinous layer they express proteins necessary for the formation of the insoluble hydrophobic outer layer of the SC

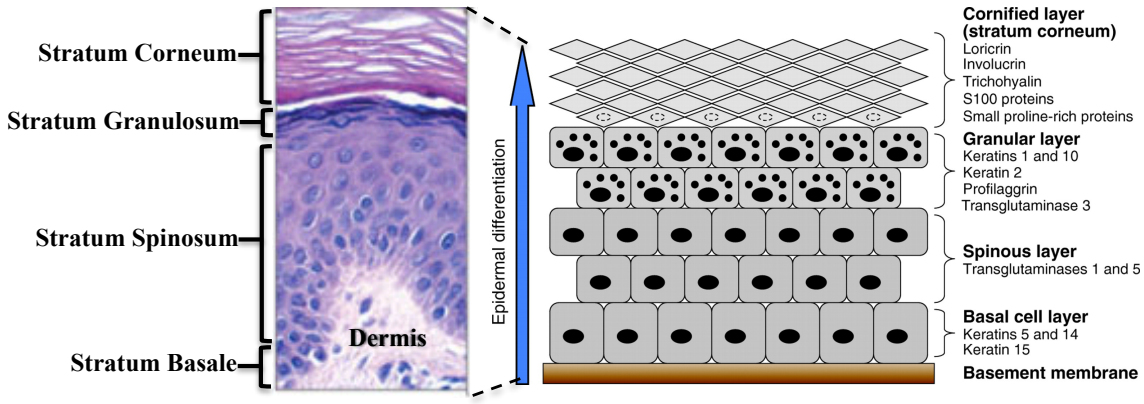

Figure 4. Epidermal Differentiation and Stratification. Keratinocytes within the epidermis proliferate within the basal cell layer. They then withdraw from the cell cycle to begin differentiation and an upwards migration through the different epidermal layers. As they progress, they become anucleated and increasingly compacted in size, before being eventually shed from the skin surface by desquamation. Each stage of epidermal differentiation is characterised by the expression of specific proteins as indicated in the figure. Larger dots represent the nucleus while the smaller black dots in the cells of the granular layer represent keratohyalin granules. Adapted with permission from Sandilands A et al., 2009.

(Banks-Schlegel and Green, 1981). These include the structural protein Involucrin (INV), a critical component of the insoluble cornified envelope (CE) (Eckert et al., 2004;Steinert and Marekov, 1997). In this layer, the cells also contain lamellar bodies rich in ceramides, cholesterol and free fatty acids. The spinous layer is also home to Langerhans cells that are important for immunity. Next, the keratinocytes progress to the granular layer (Jackson et al., 2005;Mack et al., 2005) where they acquire keratohyalin granules and lipid containing lamellar bodies (Odland, 1960). The granules contain Filaggrin, which is important for the aggregation of keratin filaments (Dale, 1985;Gan et al., 1990;Markova et al., 1993;Rothnagel et al., 1987;Rothnagel et al., 1987;Steinert and Marekov, 1995). Epidermal cells then enter the cornified layer (Byrne et al., 2003;Mack et al., 2005) where deposition of structural proteins Keratins, INV, FIL and LOR, small proline rich proteins (Cabral et al., 2001), Ca^{2+} binding S100 proteins and late envelope proteins (Steinert and Marekov, 1995) leads to the formation of the CE. At this layer, the keratinocytes are now terminally differentiated keratinocytes or corneocytes. Cornification also involves nuclear extrusion, the destruction of organelles, release of lipids into the intercellular space (Eckert et al., 2005;Mack et al., 2005;Matoltsy, 1966) and cross-linking of the structural proteins via the activity of the transglutaminase enzyme (TG1) (Kim et al., 1995;Marshall et al., 2001;Steinert et al., 1996;Yuspa et al., 1989). This arrangement of corneocytes sealed together by a lipid matrix in a "bricks and mortar" fashion helps provide the skin with its permeability barrier. It is at this point that the SC is fully formed and the barrier function of the skin is achieved (Byrne et al., 1994;Downing, 1992;Elias, 2005;Hardman et al., 1998;Kalinin et al., 2001;Kalinin et al., 2002;Mack et al., 2005) (Figure 4).

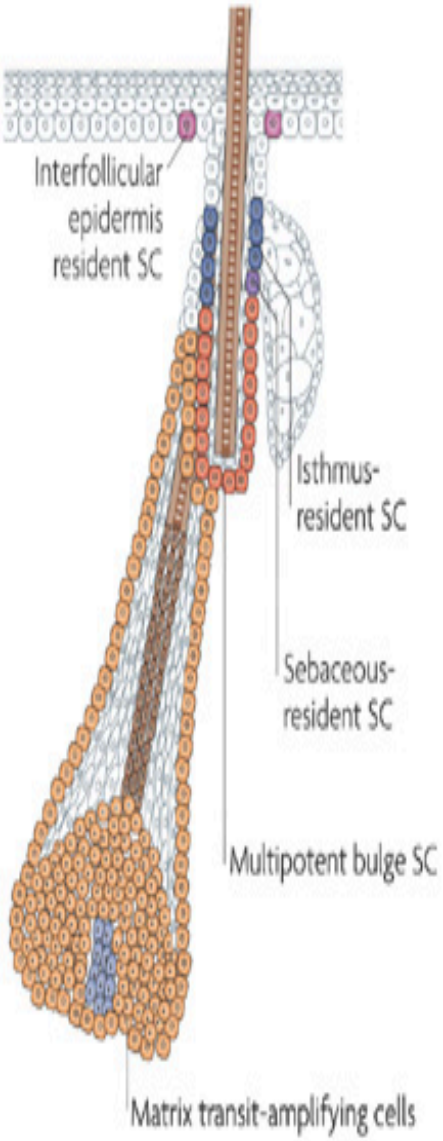

Figure 5. Stem Cell Niches of the Epidermis. Schematic of the epidermis showing the different stem cell niches in the basal layer, hair follicle bulge, sebaceous glands and isthmus. Adapted with permission from Blanpain and Fuchs, 2009.

JUNCTIONAL COMPLEXES OF THE EPIDERMIS

The ultimate consequence of terminal differentiation in the epidermis is therefore the formation of the skin barrier. The SC is the major physical component of this barrier and was previously considered to be an impenetrable wall in healthy mammals. However current literature suggests that a significant portion of barrier function is mediated by intrinsic junctional structures within the epidermis (Brandner et al., 2002;Brandner et al., 2006;Schluter et al., 2004;Schluter et al., 2007).

There are three types of junctional complexes, namely desmosomes, adherens junctions (AJ) and tight junctions (TJ) (Figure 6) (Niessen, 2007). Adherens and tight junctions were first identified as part of a tripartite junctional complex bordering the apico-basolateral membrane in a variety of polarized simple epithelia (Elias et al., 1977;Farquhar and Palade, 1965;Morita et al., 2004;Morita et al., 2011). Desmosomes form the third structure of this complex. These complexes generally have three main components: 1) structural proteins critical for initiation of the junctions (2) plaque proteins associated with the cytoskeleton and (3) signaling/polarity proteins that transduce signals from the membrane. For the purposes of the thesis, discussion will be restricted to structural proteins necessary for barrier formation.

Desmosomes

Desmosomes are adhesive intercellular junctions between cells that anchor the intermediate filament network to the plasma membrane thereby providing mechanical strength to tissues (Delva et al., 2009;Garrod and Chidgey, 2008;Kowalczyk and Green, 2013). They are composed of the membrane-spanning cadherins desmocollin and

desmoglein, desmoplakin, which interact with keratin intermediate filaments, as well as plakoglobin, plakophilin and other accessory proteins that are not vital for its adhesive function. Desmosomes are critical for adhesion and the maintenance of tissue integrity.

Adherens Junctions

AJs are junctional structures that encompass two apposing membranes that run parallel over a distance of 0.2-0.5μm and are separated by 20nm (Hirokawa and Heuser, 1981;Miyauchi et al., 2008). AJs can also be found outside of these junctional complexes in both epithelial and non-epithelial cells. AJs consist of two important units: the nectin-afadin complex and the cadherin-catenin complex, with the type of nectin or cadherin expressed within a tissue dictating the strength and adhesive specificity of the AJ (Baum and Georgiou, 2011;Boggetti and Niessen, 2012;Harris and Tepass, 2010;Harris, 2012;Niessen, 2007;Niessen and Gottardi, 2008). Nectins are IgG-like adhesion receptors that form lateral homo-dimers that can engage in homophilic and heterophilic adhesion with other Nectins or Nectin-like proteins (Irie et al., 2004;Irie et al., 2004). Nectins are directly linked to the cytoskeleton via its interaction with the acting binding proteins afadin or "AF-6" (Katata et al., 2003;Mandai et al., 1997;Mandai et al., 2013;Tachibana et al., 2000;Takai and Nakanishi, 2003;Takai et al., 2008). Type I classical cadherins are a large super family of proteins that typically form homophilic interactions; they initiate intercellular contacts through pairing with cadherins on opposing cells(Aberle et al., 1996;Tinkle et al., 2004;Zhu and Watt, 1996). Classical cadherins form a basic complex with the catenins, α-, β-, γ- and p120- catenin (Oda and Takeichi, 2011). β-catenin bridges the gap between the actin-binding protein, α-catenin, and cadherin thereby

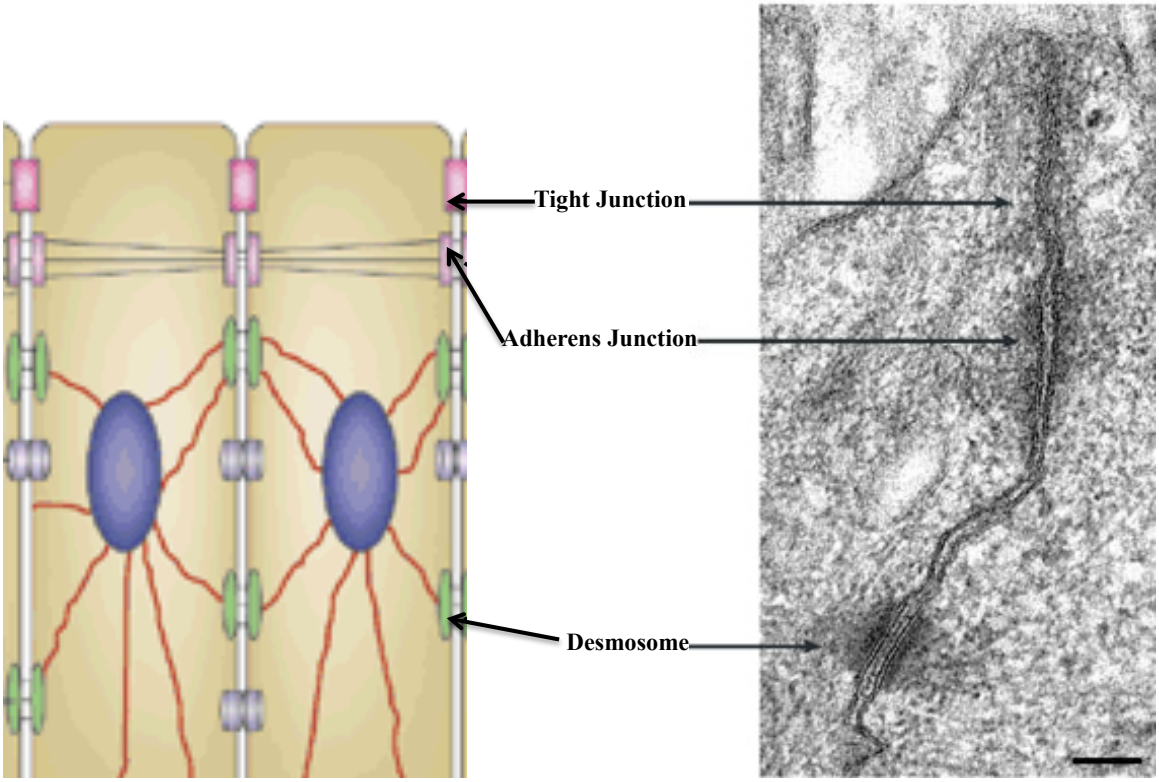

Figure 6. Junctional Complexes of the Epidermis. Schematic drawing and Electron Micrograph of the three main junctional complexes of epithelial tissue, Tight Junctions, Adherens Junctions and the Desmosomes. Adapted with permission from Tsukita et al., 2001.

mediating the adhesive function of the AJ (Aberle et al., 1996). β-catenin can also directly bind to several signaling proteins, for example, the EGF receptor or various tyrosine phosphatases. Finally, β-catenin is the central player in canonical Wnt signaling which regulates cell fate determination. In this manner, it enables the coordination of morphogenetic movements with cell fate determination (Brembeck et al., 2006;Nelson and Nusse, 2004). AJs can therefore function as signaling platforms that regulate a diverse range of cellular processes including cytoskeletal dynamics, cell polarity, shape, division, growth, apoptosis and barrier function (Hartsock and Nelson, 2008).

Tight Junctions

Tight junctions are the most apical structure of the junctional complexes. In a number of tissues, TJ formation requires and is preceded by, the formation of AJs. TJs are intercellular contacts that exist between adjacent cells that associate laterally (Figure 7). They define apical and basolateral membrane domains (Cereijido et al., 1998) thereby creating cell polarity.

There are two fundamental transport pathways across an epithelium, the transcellular (across cells) and paracellular (between cells) pathways. Transcellular transport is mediated by energy-dependent transporters and channels, which are found on basolateral and apical membranes. Paracellular transport is mediated by TJs, which span the apical intercellular space. They form a regulated, semipermeable barrier controlling the passive diffusion of ions and small non-charged solutes through the paracellular space. When examined by electron microscopy, the paracellular space at TJ sites is almost eliminated; with adjacent membranes nearly fusing at sites termed ‘kissing points’

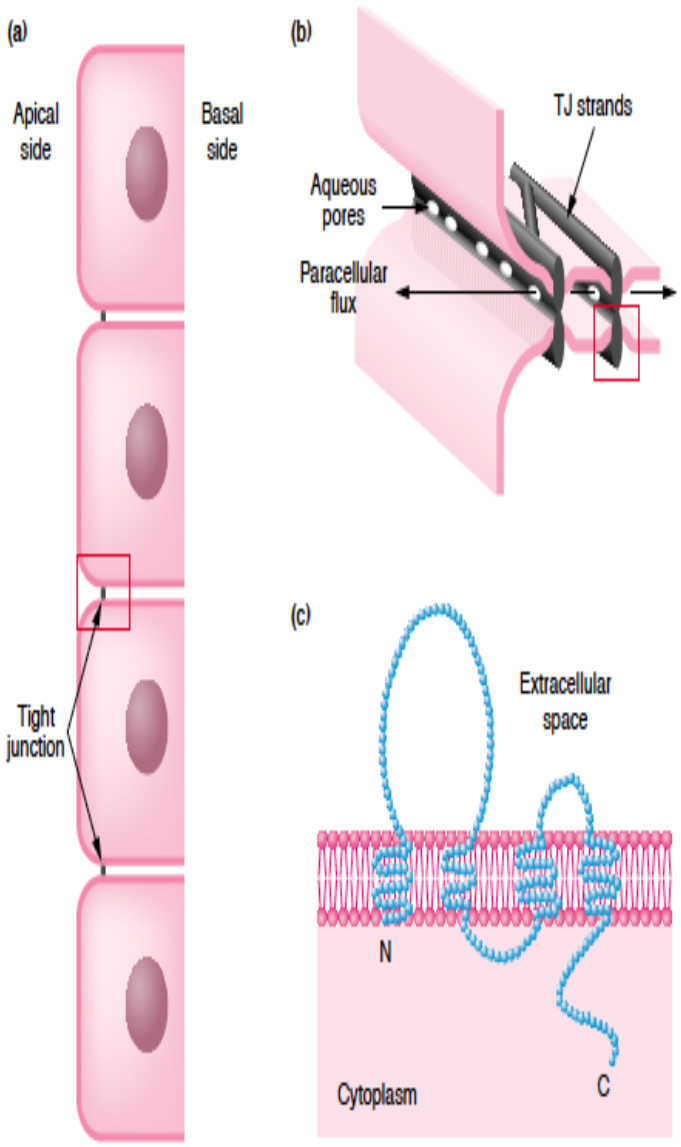

Figure 7. Tight Junctions. a. TJ strands are found in the most apical region of the lateral membrane embedded within the lipid bilayer. Each strand associates with another in the apposing membrane strand. b. Aqueous pores are thought to exist within paired TJ strands to mediate the material transport through the paracellular pathway. c. Claudins are the main structural and functional component of TJs. Adapted with permission from Tsukita and Furuse, 2002.

or “zonulae occludens” (Farquhar and Palade, 1965;Furuse et al., 2001). TJs therefore seal these intracellular spaces to allow selective permeability to and transport of small, hydrophilic molecules and ions (Angelow and Yu, 2007;Balda and Matter, 1998;D'Atri and Citi, 2002).

The extent of paracellular permeability depends on the specific demands of different epithelia at different times. This is in turn dependent on the protein composition of the TJs within the epithelia (Tsukita et al., 2001;Van Itallie and Anderson, 2006). TJs must therefore be dynamic to allow the distinct turnover of proteins leading to rapid changes in permeability as a response to physiological needs/stimuli (Nusrat et al., 2000;Shen et al., 2008;Tsukita et al., 2001;Van Itallie and Anderson, 2006;Yamazaki et al., 2011). In addition to their more traditional roles of paracellular transport, TJs function in adhesion, barrier function, cellular polarity and in signal transduction leading to cell differentiation, proliferation and modulation of gene expression (Aijaz et al., 2006;Balda and Matter, 2000;Fanning et al., 2012;Itoh et al., 1999;Morita et al., 2003;Niessen, 2007;Schneeberger and Lynch, 2004;Tsukita et al., 2001;Turksen and Troy, 2002;Van Itallie and Anderson, 2013). Although TJ-protein complexes consist of a number of transmembrane proteins, only Claudins (Cldn), Occludins (Occ) and the IgG-like family of junctional adhesion molecules (JAMs) have the potential to mediate cell–cell adhesion (Furuse and Tsukita, 2006;Furuse and Tsukita, 2006;Furuse and Moriwaki, 2009;McCarthy et al., 1996;Schneeberger and Lynch, 1984;Schneeberger and Lynch, 1992;Schneeberger and Lynch, 2004).

Tight Junction Components

JAM is a member of IgG superfamily and consists of JAM-A, -B, -C and -4 (Ebnet et al., 2004). JAMs can engage in homophilic and heterophilic adhesions but are not able to induce TJ formation when overexpressed in fibroblasts. JAMs are not found exclusively at TJs but are also expressed on the surface of circulating leukocytes, erythrocytes and platelets and have been shown to function not only in barrier function but also in transendothelial migration, platelet activation, angiogenesis and retrovirus binding (Bazzoni, 2003;Mandell and Parkos, 2005;Martin-Padura et al., 1998). JAMs also regulate polarization and differentiation of spermatids (Gliki et al., 2004). Another most recently identified TJ protein Tricellulin, is specifically enriched at vertically oriented TJ strands at tri-cellular contacts (Ikenouchi et al., 2005;Krug et al., 2009). Tricellulin has been implicated in recessive, non-syndromic deafness (Nayak et al., 2013;Riazuddin et al., 2006). Interestingly, knockdown of Occludin in epithelial cells leads to mis-localization of tricellulin to bi-cellular TJs (Ikenouchi et al., 2008). The function of tricellulin in TJs currently remains unclear.

Occludin, the first component of tight junctions to be identified (Ando-Akatsuka et al., 1996;Ikenouchi et al., 2003) is a ~65kDa transmembrane protein with a cytoplasmic carboxyl tail that binds to cytoplasmic TJ proteins (Furuse et al., 1993). When overexpressed in mouse L-fibroblasts that normally lack TJs and cell-cell adhesion, Occludin assembles into microscopically visible cell-cell contacts (Furuse et al., 1996;Furuse et al., 1998). When expressed in other cell types, Occludin induces an increase in the strength of TJs, as indicated by increased trans-epithelial resistance (TER) (McCarthy et al., 1996;Medina et al., 2000). Although Occludin-deficient mice exhibit

several different phenotypes suggestive of barrier impairment, such as growth retardation, mineral deposits in the brain, male sterility, and gastritis, they still formed functional tight junctional strands and showed intact barrier function (Saitou et al., 1998;Saitou et al., 2000).

CLAUDINS

The existence of TJ strands and barrier function in Occludin knockout mice suggested that there must be additional components within the complex that contribute to TJ formation and barrier function. This led Tsukita *et al* to identify two other tight junctional transmembrane proteins, named Claudin-1 and -2 (Furuse et al., 1998). Around the same time, other groups found that mutation of certain proteins now known as Claudins (Cldns) resulted in diseases such as hypomagnesaemia (Claudin-16) (Simon et al., 1999), deafness (Claudin-14) (Wilcox et al., 2001) and absence of central nervous system myelin and sertoli cell tight junction strands (Claudin-11) (Gow et al., 1999;Gow et al., 2004). It is now well established that the most important functional component of these TJs are Cldns, a 21-34kDa family of 27 highly conserved tetraspan membrane proteins (Figure 8).

The word claudin comes from the Latin word claudere, 'to close'. They were identified as proteins that co-fractionated with Occludin by sucrose density gradient centrifugation of TJ/AJ-enriched membrane fractions (Furuse et al., 1998;Furuse et al., 1998). Cldns are members of the pfam00822 or PMP-22/EMP/MP20/Claudin superfamily. Although structurally similar, they are functionally divergent and only a subset appear to function in barrier formation (Anderson and Van Itallie, 2009).

Structure of Claudins

Cldns are integral membrane proteins that have four hydrophobic transmembrane domains and two extracellular loops (Figure 9). The first loop contains the highly conserved signature motif (W-x (15,20)-[Gn]-L-Wx (2)-C-x (8,10)-C- x(15,16)-[qR]) (Gunzel and Yu, 2013). It mediates the 'electrostatic selectivity filter' of the TJ(Colegio et al., 2002;Colegio et al., 2003;Van Itallie et al., 2009) and determines paracellular permeability and thus overall resistance and charge selectivity (Anderson and Van Itallie, 2009). The second loop mediates Cldn-Cldn interaction through an unknown mechanism (Piontek et al., 2008). Within the Cldn family, the cytoplasmic tail domain, although relatively constant in length, is divergent in sequence (Gunzel and Yu, 2013). It has been shown to play a role in trafficking of Cldns to the TJ as well as in protein degradation (Gunzel and Yu, 2013). The end of the tail usually contains a post-synaptic density protein 95, Dlg (Discs-large) and ZO1 (Zonula Occludens 1) (PDZ)-binding motif (Schneeberger, 2003) (Ikenouchi et al., 2007;Itoh et al., 1999;Tsukita et al., 2009;Turksen and Troy, 2004;Willott et al., 1993) with which Cldns interact with the PDZ domains of TJ-associated plaque proteins such as the ZO family, MUPP1 and PATJ (Adachi et al., 2009;Hamazaki et al., 2002;Itoh et al., 1999). The Cldn tail undergoes post-translational modifications of functional consequences. For example, there are conserved cysteine residues whose palmitoylation has been suggested to be important for Cldn trafficking to the TJ (Van Itallie et al., 2005). In addition, there are phosphorylation sites that have been implicated in regulation of both Cldn localization and TJ formation (Aono and Hirai, 2008;D'Souza et al., 2005;Ikari et al., 2006;Ishizaki et al., 2003;Van Itallie et al., 2012;Van Itallie et al., 2012).

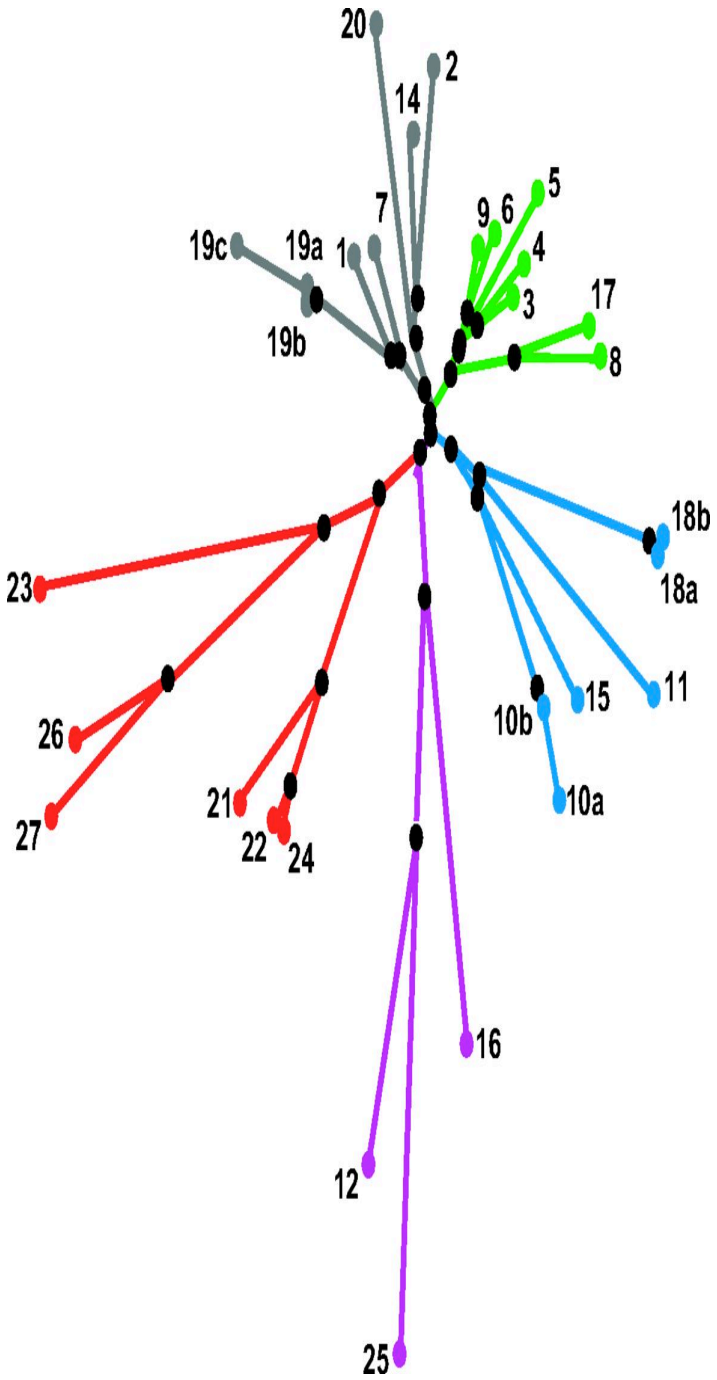

Figure 8. Phylogenetic Tree of the Human Claudin Family. Human Claudins are grouped into four main clusters represented by the colours above. Cluster I consists of Cldn3, -4, -5, -6, -8, -9 and -17; Cluster II consists of Cldns1, -2, -7, -14, -19 and -20, Cluster III consists of Cldns 10, -11, -15, -18 and Cluster IV consists of Cldns 12, -16, -21, -22, -23, -24, -26, -27. Adapted with permission from Gunzel and Yu, 2013

Claudins and Paracellular Permeability

Claudins are critical to TJ function. Overexpression of Claudins in fibroblasts results in the formation of TJ fibers and induces Ca^{2+}-independent cell–cell adhesion. The type of Claudin expressed also affects paracellular ion and/or size selectivity (Nitta et al., 2003). When Cldn2 was introduced into MDCK cells (which do not express the protein), TER decreased 20-fold, to levels characteristic of MDCK II cells that do express Cldn2 (Furuse et al., 2001). The nephron is an excellent naturally occurring example of Cldn expression profiles determining regional paracellular properties, with Cldns 1, 2, 3, 4, 8, 10, 11 and 16 displaying segment-specific patterns of expression with concomitant alteration in paracellular trafficking (Kiuchi-Saishin et al., 2002). The extracellular loops in claudins are charged and their isoelectric points vary widely between the different claudins; this determine the nature of the barrier. For example, changing the charge in the first EC loop of Claudin-15 has been shown to alter barrier ion specificity (Colegio et al., 2002). Overexpression of Cldn chimeras containing switched extracellular domains alters trans-epithelial resistance TER and permeability to Na^- relative to Cl^- (Colegio et al., 2003). Isoform-specific organ and tissue distribution of Cldns therefore dictates the tissue-specific barrier properties of TJs (Angelow et al., 2008;Furuse et al., 2001;Furuse, 2006;Furuse and Tsukita, 2006;Gunzel and Yu, 2013;Van Itallie and Anderson, 2006). The proportion of the various Cldns also affects the specificity of barrier properties. For example, co-culturing of fibroblasts singly transfected with Cldn 1, 2 or 3 showed that Cldn1 strands associate with Cldn3 but not with Cldn2 strands (Furuse et al., 1999). It is

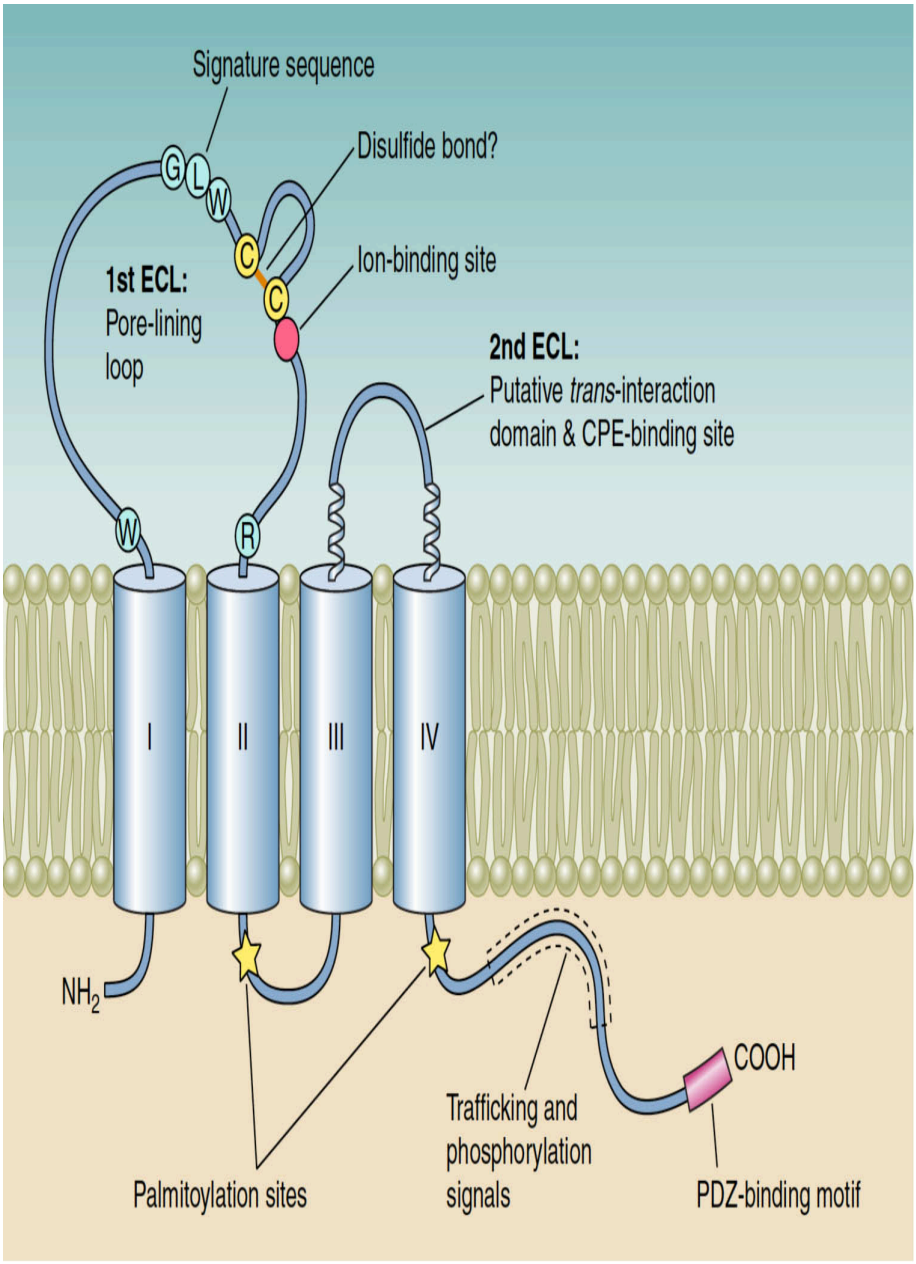

Figure 9. Claudin Structure. Predicted topology, secondary structure and putative functional domains of the Claudin protein family. Roman numerals indicate **α**-helical transmembrane domains. It bears four transmembrane domains with a short cytoplasmic tail and two extracellular loops. Adapted with permission from Gunzel and Yu, 2013.

now widely recognized that TJ strands are a mosaic of multiple Cldn types that form homotypic and heterotypic interactions between cells (Anderson and Van Itallie, 2009;Furuse, 2010) and that the large variety in strength, size, and ion specificity of tight junctional barriers in different epithelia and endothelia is largely due to the type of claudin(s) found at specific TJs (Table 1) (Colegio et al., 2002;Furuse and Tsukita, 2006;Van Itallie and Anderson, 2004;Van Itallie and Anderson, 2014).

Scaffolding Proteins

The incorporation and association of TJ proteins at TJ strands require the clustering of these proteins. As no direct interactions between occludins, claudins, and JAMs have been documented, cytoplasmic binding partners must achieve this scaffolding function. Indeed, the cytoplasmic face of the TJ contains aggregates of protein complexes that participate in protein associations mainly through PDZ domain-mediated protein-protein binding (Ponting et al., 1997;Ponting et al., 1997). These cytoplasmic proteins interact with the cytoskeleton through their C-terminus and their PDZ domains (Guillemot et al., 2008;Schneeberger and Lynch, 2004). Such scaffolding proteins include zonula occludens proteins ZO-1, ZO-2, and ZO-3, and certain members of the membrane-associated guanylate kinase-like homolog family (Anderson et al., 1995;Itoh et al., 1999;Van Itallie et al., 2009). ZO-1 and ZO-2 have been shown to be crucial for clustering of claudins, strand formation, and barrier function (Umeda et al., 2006). Several other PDZ-containing scaffolding proteins, such PATJ, MUPP1 and MAGUK proteins are also associated with TJs and can directly interact with one or more of their trans-membrane components (Schneeberger and Lynch, 2004). They selectively

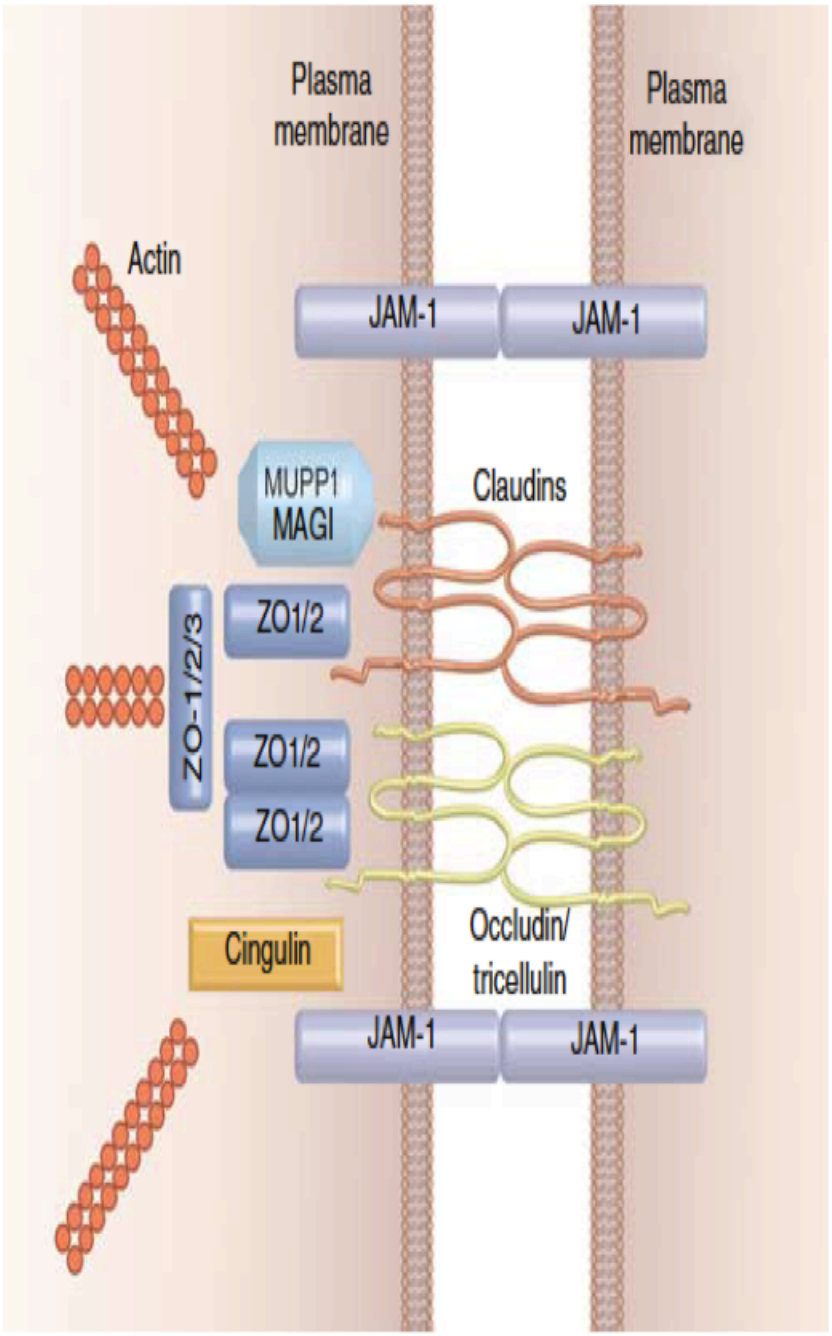

Figure 10: Protein Interactions at TJs. Schematic representation of the basic structural transmembrane components of tight junctions and the various protein-protein interactions and connections to the actin cytoskeleton. Adapted with permission from Niessen, 2007.

recognize and bind the PDZ-binding domains of TJ proteins such as Cldns (Hamazaki et al., 2002;Itoh et al., 1999;Lemmers et al., 2002;Roh et al., 2002;Tsukita et al., 2001;Van Itallie et al., 2004;Van Itallie et al., 2009) (Figure 10). TJ complexes also contain proteins that lack PDZ domains. These proteins regulate signaling but do not contribute to TJ structure. An example is Cingulin, a non-PDZ tight junction protein that interacts with ZOs, JAMs, actin and myosin (Clayburgh et al., 2005;Cordenonsi et al., 1999;Guillemot et al., 2012). In spite of increasing evidence that TJs mediate signaling through their plaque proteins (Forster, 2008;Kirschner et al., 2009), we are only beginning to understand the complex roles these structures play in epithelia.

CLAUDINS AND THE EPIDERMAL PERMEABILITY BARRIER (EPB)

As discussed above, mutations in Cldns underlie several human diseases, the bases of which are aberrant barrier function (Heiskala et al., 2001). In addition, a number of mouse models provide further insight into Claudin function in barrier formation (Table 2). Mice lacking Claudin-1 exhibit wrinkled skin with dehydration from markedly increased trans-epidermal water loss (TEWL) and death within 1d of birth (Furuse et al., 2002). The expression of structural proteins that contribute to the SC such as LOR, INV and TG1 were unaffected and lamellar lipid structures in the SC also appeared normal in these animals. This suggested that while the physical barrier of SC was intact the contribution of Cldn1-based TJs to barrier function was significant.

Studies in the Turksen lab have clearly demonstrated that Cldn 6 is intricately involved in the epidermal differentiation program and that Cldn6-based TJ function, and thus barrier integrity, is modified in response to Cldn modulation (Table 3). Using an *in*

vitro system, in which embryonic stem cells (ESCs) were differentiated into embryoid bodies (EBs), Cldn6 was initially identified as one of the earliest proteins expressed during commitment of ESCs to the epithelial cell fate as it was expressed concomitantly with the early epithelial marker K8 (Turksen and Troy, 2001). This finding led us to hypothesize that Cldn6 may play a role in epidermal development and differentiation. The overexpression of Cldn 6 to the suprabasal layer of the epidermis using the INV promoter lead to EPB defects with changes in both the TJ and SC barrier (Turksen and Troy, 2002) (Figure 11). These mice also died within two days from dehydration as a result of epidermal differentiation perturbations and inadequate skin barrier function. Interestingly, this phenotype was dependent on the levels of Cldn6 overexpression as the heterozygous counterparts displayed a less severe EPB defect (Troy et al., 2005). Although the Cldn6 knockout mice had no gross phenotypically phenotype (Anderson et al., 2008), these studies suggest that a precise balance of Cldn6 protein is important for proper epidermal function.

Role of the Claudin 6 Tail in Barrier Function

The mechanism by which Cldn6 mediates epidermal differentiation and EPB function was as yet unknown. Given that Cldn6 resides at the cell membrane within a large protein complex, we hypothesized that potential interactions with other TJ-proteins within the complex could be important for mediating its barrier function. To determine how Cldn6 could interact with other proteins we looked to its structure. As discussed above, Cldn6 has two extracellular loops and an intracellular cytoplasmic tail. While the

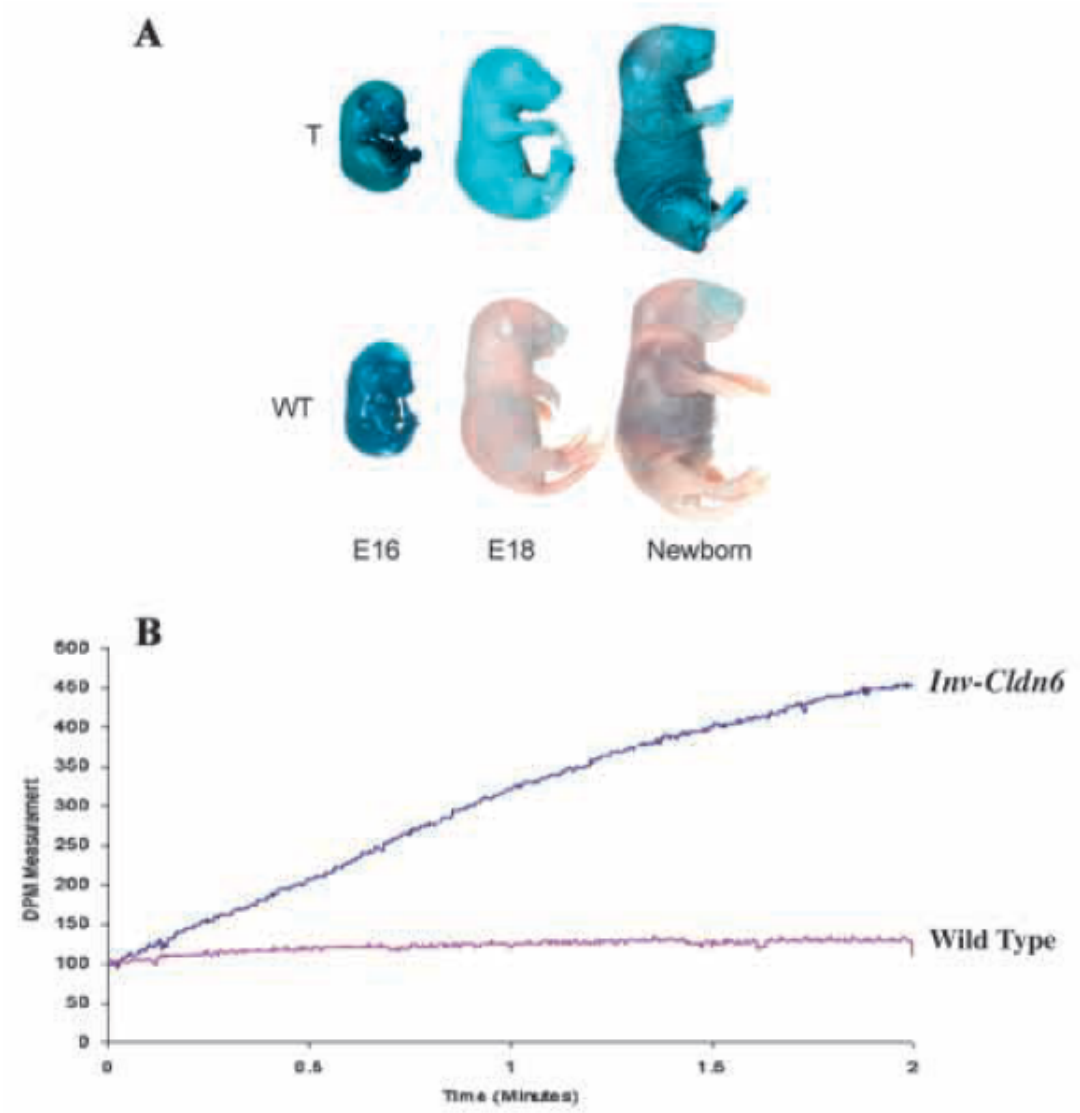

Figure 11. Defective EPB in Inv-Cldn6 Transgenic Mice. A. Under normal conditions, an intact EPB exists at E17.5 days thereby preventing the penetration of substances such as X-gal. When X-gal enters the epidermis a striking blue staining results. At E16.5 neither the wild-type nor the transgenic embryos possessed an intact EPB, yet at E18.5 there was no penetration of X-gal through the EPB of the wild-type embryos while transgenic embryos remained blue. (B) Dermal Phase Measurement show increased trans-epidermal water loss in the transgenic neonates as compared to the wild type. WT; wildtype, T;transgenic. Adapted with permission from Turksen and Troy, 2002.

loops are conserved, within the Cldn family, the amino acid sequences of the tail are divergent. The tail also contains a number of putative functional protein domains, such as phosphorylation sites and a PDZ-binding domain. This provided clues into the manner in which Cldn6 might function in cell signaling.

Further support for the role of the Cldn tail in mediating paracellular permeability was provided when swapping of tails between different Cldns resulted in disrupted barrier properties due to effects on protein stability (Van Itallie et al., 2004). In other studies where the entire cytoplasmic domain of Cldn1 was replaced with a GFP tag, the truncated protein does not localize to the plasma membrane but accumulates in intracellular vesicles (Ruffer and Gerke, 2004). With respect to Cldn6, experiments in the Turksen lab showed that the over-expression of a whole tail Cldn6 deletion mutant to the suprabasal layer of the epidermis resulted in postnatal and lifelong hyperplasia due to increased proliferation and epidermal differentiation perturbation. This suggests that the Cldn6 tail is important for epidermal barrier function. The improper protein folding and inefficient targeting to the membrane of Cldn6 observed in the epidermis of these mice, although suggestive of a role for Cldn6 in cell signaling during epidermal differentiation, limits the applicability of this model (Arabzadeh et al., 2006). In addition, the nature of the functional domains important for Cldn6 function is still unknown.

There is some evidence for the effects of post-translational modifications on Cldn localization and barrier function. Van Itallie et al. showed that Cldns are palmitoylated at conserved cysteine residues near the cytoplasmic end of the second and fourth transmembrane domains (Van Itallie et al., 2005). In Cldn 14, mutation of either or both of these sites abolished palmitoylation and reduced its TJ localization. There are also

serine and/or threonine phosphorylation sites in the C-terminal end of Cldns, and Cldn5 has been shown to be phosphorylated in response to cAMP, an event that is correlated with improved barrier function in cultured endothelial cells (Ishizaki et al., 2003). Phosphorylation of Cldns1-4 by the threonine-serine kinase WNK4 leads to an increase in paracellular permeability (Ohta et al., 2006;Tatum et al., 2007), while in ovarian cancer cells expressing a constitutively phosphorylated form of Cldn3, decreased TER and increased paracelleular permeability is observed (D'Souza et al., 2005). It therefore appears that post-translational modification of Cldn tails affects Cldn localization and incorporation in TJs and therefore barrier function. However the importance of posttranslational modifications in Cldn6 function is unknown.

RATIONALE I

The evidence strongly suggests that the cytoplasmic tail portion of Cldn6 is important for protein-protein interactions and cell signaling necessary for epidermal homeostasis. However, the mechanism by which it plays this role is largely unknown. In addition, the nature of the Cldn6 tail domains necessary for EPB function are yet to be determined. I therefore **hypothesize** that the portion of the cytoplasmic tail of the tight junction protein Cldn 6 carrying the PDZ-binding domain and potential phosphorylation sites is important for the epidermal differentiation and barrier function. In order to investigate this, my main **objective** is to analyze the epidermal development, differentiation and EPB function of two transgenic mice models carrying deletions of varying lengths in the Cldn6 cytoplasmic tail.

The evidence suggests that the Cldn6 tail contributes to the maintenance of epidermal homeostasis (Turksen and Troy, 2002). To further address the role of the tail, we overexpressed a Cldn6 mutant protein carrying a deletion of the last 23 amino acids (Inv-Cldn6-CΔ196). The results of the analysis of this transgenic mouse model are reported in Chapter 2. In order to more closely map the tail region important for this function, we overexpressed a mutant carrying a shorter truncation of 14 amino acids (Inv-Cldn6-CΔ206). This region contains 3 potential phosphorylation sites and a PDZ-binding domain. There is increasing evidence that phosphorylation of Cldns affect their ability to fold properly and to be targeted to the membrane (Tanaka et al., 2005a; Rigbolt et al., 2011). Also, the PDZ-binding domain in other Cldns have been shown to interact with scaffolding proteins such as ZO-1 (Schneeberger and Lynch, 2004; Umeda et al., 2006). The results of these studies are reported in Chapter 3.

CHAPTER 2

Dermatitis and Aging-Related Barrier Dysfunction in Transgenic Mice Overexpressing an Epidermal-Targeted Claudin 6 Tail Deletion Mutant

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*Corresponding Author

AUTHOR CONTRIBUTIONS

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Tammy-Claire Troy made the initial observations regarding barrier dysfunction, performed analysis of barrier integrity, contributed reagents and wrote the initial manuscript for submission. Azadeh Arabzadeh made the initial observations regarding the dermatitis and performed some of the initial H&E staining. AE and NL collected and processed samples and performed further immunohistochemistry. AE and NL also performed all experiments required for revisions. KT was responsible for experimental conception and design, as well as final editing and approval of the manuscript with contributions from all authors.

ABSTRACT

The barrier function of the skin protects the mammalian body against infection, dehydration, UV irradiation and temperature fluctuation. Barrier function is reduced with the skin's intrinsic aging process, however the molecular mechanisms involved are unknown. We previously demonstrated that Claudin (Cldn)-containing tight junctions (TJs) are essential in the development of the epidermis and that transgenic mice overexpressing Cldn6 in the suprabasal layers of the epidermis undergo a perturbed terminal differentiation program characterized in part by reduced barrier function. To dissect further the mechanisms by which Cldn6 acts during epidermal differentiation, we overexpressed a Cldn6 cytoplasmic tail deletion mutant in the suprabasal compartment of the transgenic mouse epidermis. Although there were no gross phenotypic abnormalities at birth, subtle epidermal anomalies were present that disappeared by one month of age, indicative of a robust injury response. However, with aging, epidermal changes with eventual chronic dermatitis appeared with a concomitant barrier dysfunction manifested in increased trans-epidermal water loss. Immunohistochemical analysis revealed aberrant suprabasal Cldn localization with marked down-regulation of Cldn1. Both the proliferative and terminal differentiation compartments were perturbed as evidenced by mislocalization of multiple epidermal markers. These results suggest that the normally robust injury response mechanism of the epidermis is lost in the aging Involucrin-Cldn6-CΔ196 transgenic epidermis, and provide a model for evaluation of aging-related skin changes.

INTRODUCTION

Formed during development by a series of cell commitment, mesenchymal-epithelial cell interactions, and terminal differentiation, the mammalian epidermis undergoes continuous self-renewal in a tightly regulated process of epidermal cell proliferation and differentiation (Fuchs and Raghavan, 2002;Fuchs, 2007;Turksen and Troy, 1998). As the end result of terminal differentiation, the robust barrier function of the skin protects against microorganism invasion and UV irradiation, inhibits water loss, regulates body temperature and is an important part of the host defense system (Pouillot et al., 2008). These important functions decline in efficiency with aging, leading to an inefficient epidermal injury response and dermatitis (Elias and Ghadially, 2002;Elias, 2008;Ghadially, 1998), for reasons that are not yet understood.

Tight junctions (TJs) are essential not only for dividing epidermal cells into apical and basolateral compartments to create cell polarity (Farquhar and Palade, 1963), but also for the existence of skin barrier function by regulating the selective permeability of the paracellular pathway (Chiba et al., 2008;Krause et al., 2008;Paris et al., 2008). The selectivity function of TJs is imparted by Claudins (Cldns), a family of 23 highly conserved tetraspan membrane proteins whose heterogeneity stems in large part from distinctly charged amino acid sequences in the first external loop (Chiba et al., 2008;Katoh and Katoh, 2003;Turksen and Troy, 2004). Cldn type and mixing ratio thus provide for the specific permeability requirements of different epithelia (Turksen, 2004). The importance of Cldns in epidermal differentiation and barrier function has been confirmed by experiments in which Cldn expression has been perturbed in epidermal cells; for example, Cldn1 knockout mice die shortly after birth due to skin barrier

dysfunction (Furuse et al., 2002). Involucrin-Cldn6 (Inv-Cldn6) transgenic mice also suffer skin barrier dysfunction, the severity/lethality of which is dependent upon the level of Cldn6 overexpression (Troy et al., 2005;Turksen and Troy, 2002). Further, Inv-Cldn6-CΔ187 transgenic mice overexpressing a cytoplasmic tail-ablated Cldn6 display epidermal hyperproliferation, apparently due to an inefficiency of Cldn protein membrane targeting, as a result of the unfolded protein response pathway (Arabzadeh et al., 2006).

The latter data suggest the importance of the cytoplasmic tail portion of Cldn molecules in cell signaling during epidermal differentiation. The cytoplasmic tail of different Cldns, while relatively constant in length, is divergent in sequence, but a number of putative functional protein domains are present in many family members (Gonzalez-Mariscal et al., 2003;Turksen and Troy, 2004). To address the activities of the functional domains in more detail, we again used the Involucrin promoter (Inv) this time to target a shorter deletion in the cytoplasmic tail (Cldn6-CΔ196) to the differentiative compartment of the epidermis. The Inv-Cldn6-CΔ196 transgenic mice possess subtle epidermal differentiation abnormalities at birth that by 1-month of age are completely normalized. However, with aging, Inv-Cldn6-CΔ196 mice suffered dermatitis, often manifested as patent wounds in repetitive grooming areas. Normal hydration levels were not maintained in the aging skin, and immunohistochemistry revealed perturbations in the expression and localization of multiple Cldns, as well as various classical markers of epidermal differentiation. These results suggest that the normally robust injury response mechanism of the epidermis is lost in the aging Inv-Cldn6-CΔ196 transgenic epidermis and provides a model for evaluation of chronic dermatitis and aging-related skin changes.

METHODS

Generation of Inv-Cldn6-CΔ196 transgenic mice

The Inv-Cldn6-CΔ196 construct (Troy and Turksen, 2007) was injected into ova collected from superovulated CD1 mice to generate viable lines at the Ottawa Hospital Research Institute (OHRI) Transgenic Mouse Facility as previously described (Troy et al., 2007). Genotyping was performed by PCR using genomic DNA and primers specific for Cldn6 (5'-ATG GCC TCT ACT GGT CTG CA-3') and the FLAG® tag (5'-TCA CTT GTC ATC GTC GTC CTT G-3'). Age-matched wild type and Inv-Cldn6-CΔ196 transgenic mice were photographed using a Nikon COOL-PIX950 digital camera (Nikon) for image processing with Adobe Photoshop version 7.0 (Adobe Systems). All research complied with the principles and guidelines of the Canadian Council on Animal Care and was approved by the Ottawa Hospital Research Institute Animal Care Committee. Once the dermatitis phenotype became unmanageable, Inv-Cldn6-CΔ196 transgenic mice were euthanized according to institutional and legislative policies.

Sample preparation, histology and immunohistochemistry

a) Sample collection. Freshly dissected skin samples (~1 cm2) were collected at various postnatal time points (birth, 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 5 months, 6 months) from either the mid-dorsal region or areas of frequent grooming (e.g. the neck and behind the ears) of Inv-Cldn6-CΔ196 transgenic mice and their age-matched CD1 counterparts.

b) Frozen sections. Frozen sections were required for immunolocalization with FLAG® antibodies, whereas histology and all other immunostaining procedures were performed using paraffin-embedded sections. For frozen sections, skin samples were embedded in HistoPrep (Fisher Scientific), solidified using dry ice-chilled isopentane and 5 μm sections were mounted onto Superfrost®/Plus slides (Fisher Scientific) as described (Arabzadeh et al., 2006). Samples were fixed for 10 minutes in methanol (-20°C) and washed with PBS before immunohistochemistry (see below).

c) Paraffin sections and histology. For paraffin sections, skin samples were fixed overnight in Bouin's solution (75% saturated picric acid, 20% formaldehyde, 5% glacial acetic acid) and dehydrated via a graded series of ethanol washes (from 30% to 100%) before paraffin embedding and sectioning (5 μm). Sections mounted onto Superfrost®/Plus slides (Fisher Scientific) were dewaxed using toluene and rehydrated in a reverse graded series of ethanol washes to water. Following antigen unmasking and washing in PBS, sections were either stained with hematoxylin and eosin (H&E) as described (Troy et al., 2005) or further processed for immunohistochemistry (see below).

d) Immunohistochemistry. After blocking for non-specific antibody binding (10% goat serum, 0.8% BSA, 1% gelatin in PBS), skin samples were incubated for 1 hour in antibodies appropriately diluted in incubation buffer (1% goat serum, 0.8% BSA, 1% gelatin in PBS)(Troy et al., 2005;Troy et al., 2005). Antibodies recognizing the following proteins were used: FLAG® (M2 monoclonal) (1:440; Sigma), K15 (1:100; rabbit #UC55), K5 (1:100; rabbit #5054), K14 (1:100; rabbit #199), K1 (1:100; rabbit #UC81), K6 (1:200; BabCO), K17 (1:500; a gift from Dr. Pierre Coulombe, Johns Hopkins), involucrin (1:100; BabCO), filaggrin (1:100; BabCO), loricrin (1:100; rabbit #UC84),

Cldn1 (6:100; Zymed Laboratories), Cldn2 (1:200; Zymed Laboratories), Cldn3 (1:50; Zymed Laboratories), Cldn5 (1:100; Zymed Laboratories), Cldn6 (1:100; hen #3677), Cldn11 (1:100; hen #3680), Cldn12 (1:100; hen #5186) and Cldn18 (1:100; rabbit #A9953). Following washes (0.8% BSA, 1% gelatin in PBS), skin samples were incubated for 1 hour at room temperature with FITC-conjugated secondary antibodies against mouse, rabbit and chicken (1:50; Jackson ImmunoResearch) diluted in incubation buffer. After washes in wash buffer and PBS, sections were mounted with Moviol 4–88 (Calbiochem) containing 2.5% 1,4 diazobicyclo-[2,2,2]-octane (DABCO; Sigma) for observation on a Zeiss Axioplan 2 fluorescence microscope equipped with an AxioCam camera and Axio Vision 2.05 software (Carl Zeiss); digital images were processed using Adobe Photoshop version 7.0 (Adobe Systems).

Protein isolation and immunoblotting

Skin samples (0.4 g) were dissected from either the mid-dorsal region or areas of frequent grooming (e.g. the neck and behind the ears) of Inv-Cldn6-CΔ196 transgenic mice and their age-matched CD1 counterparts and whole cell protein extracts were prepared by homogenizing in SDS extraction buffer (62.5 mM Tris pH 6.8, 25% glycerol, 2% SDS and 2% β-mercaptoethanol with pepstatin A and a complete mini protease inhibitor cocktail tablet [Roche]) followed by centrifugation. Following a 30-minute incubation in sample reducing buffer (62.5 mM Tris pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue and 2% β-mercaptoethanol), boiling and centrifugation, 10 μg of soluble proteins were separated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose (Arabzadeh et al., 2006). After blocking for non-specific antibody binding

(5% skim milk, TBS/0.1% tween-20 [TBS-T]), blots were incubated at 4°C overnight with diluted (5% skim milk, TBS-T) antibodies against FLAG® (polyclonal) (1:500;Sigma) and GAPDH (1:20,000; Abcam). After several washes in TBS-T, blots were incubated in diluted (5% skim milk, TBS-T) HRP-conjugated antibodies against mouse and rabbit IgG (1:20,000; Amersham) for 1 hour at room temperature and washed withTBS-T before incubation with chemiluminescent HRP substrate (Millipore) and visualization on BioMax XAR autoradiography film (Kodak). Films were scanned and digital images were prepared using Adobe Photoshop version 7.0 (Adobe Systems).

Barrier integrity assay

Trans-epidermal water loss (e.g. impedence) of both Inv-Cldn6-CΔ196 transgenic and wild type mouse skin was measured using a dermal phase meter (DPM) (NovaTechnology Corporation) as described (Turksen and Troy, 2002). Diminished barrier integrity is reflected in higher DPM values over time as depicted digitally with EDWINA software (Nova Technology Corporation) and converted into graphical form with Excel software (Microsoft).

Cornified envelope extracts

Using dorsal skin samples of CD1 and Inv-Cldn6-CΔ196 transgenic mice, purified cornified envelope extracts were prepared as described (Hohl et al., 1991;Troy et al., 2005;Turksen and Troy, 2002). Briefly, skin samples were immersed in hot extraction buffer (0.1 M Tris-HCl pH 8.5, 2% SDS, 20 mM dithiothreitol, 5 mM EDTA), followed by 15-minute incubation at 95°C and gentle centrifugation before observation using an

Olympus CK2 inverted microscope (Olympus) equipped with a Nikon COOLPIX 4500 digital camera (Nikon). Images were produced with Adobe Photoshop version 7.0 (Adobe Systems).

RESULTS

Generation and Expression Analysis of Inv-Cldn6-CΔ196 Transgenic Mice

We previously reported that overexpression of a Cldn6 lacking the C-terminal half of the cytoplasmic tail domain (e.g. truncation after amino acid 196; Cldn6-CΔ196) (Figure 12A-C) in the suprabasal compartment of the mouse epidermis results in marked hyperproliferative squamous invaginations/cysts replacing hair follicles and lethal epidermal barrier dysfunction (Troy and Turksen, 2007). The rationale for generating the Δ196 mutation was based on two main factors. First, we already knew that tailless Cldn6 was not targeting to the membrane appropriately. Second, we wanted to delete the PDZ domain that is at the tip of the cytoplasmic tail as well as putative phosphorylation sites, which could be accomplished by deletion from amino acid 196. The choice of 196 rather than 195 or 197, both of which would also have accomplished removal of the PDZ and putative phosphorylation sites, was one of convenience for the steps required to make our mutant insert. Because we have demonstrated previously that the level of Cldn6 overexpression correlates to the severity of the phenotype (Troy et al., 2005;Turksen and Troy, 2002), we re-derived F0s with lower Cldn6-CΔ196 expression to avoid lethality and explore the function of the Cldn6 tail in postnatal animals; levels of expression of the mutant Cldn6 is half of that observed in our previously generated mice. Nine Inv-Cldn6-CΔ196 positive F0 transgenic mice (five females and four males) were generated and

three viable lines exhibiting indistinguishable phenotypes were successfully established. Cldn6-CΔ196 expression was confirmed to be confined to the upper spinous and granular layers of the epidermis and localized to cell membranes, mimicking endogenous Cldn6 expression (Figure 12D). Immunoblotting confirmed a ~19.5kDa band corresponding to the transgene product in skin samples from Inv-Cldn6 CΔ196 but not wild type littermates (Figure 12E).

Inv-Cldn6-CΔ196 neonates displayed no gross phenotypic anomalies compared to their wild type counterparts; trans-epidermal water loss (TEWL) measurements (DPM in the range of 98–105) (Figure 12F) and the absence of the X-Gal (5-bromo-4-chloro-3-indolyl-β–D-galactopyranoside) penetration (not shown) confirmed no skin barrier dysfunction in the transgenic mice at birth. Extracts from Inv-Cldn6-CΔ196 mouse skin revealed normal looking cornified envelopes, with a rigid shape and uniform size (Figure 12G), providing further evidence that early postnatal barrier function was not perturbed. As observed in our Inv-Cldn6 and Inv-Cldn6-CΔ187 mouse models (Arabzadeh et al., 2008;Troy et al., 2005) however, the emergence of hair fibers provided a diagnostic identifier for the Inv-Cldn6-CΔ196 transgenic epidermis. In comparison to the sleek and lustrous appearance of wild type mice, the coat of Inv-Cldn6-CΔ196 mice was frizzed and lackluster, a phenotype that persisted throughout life (Figure 12H); however since it does not appear to have any direct relevance to the aging-related barrier defects reported herein, it has not been further explored.

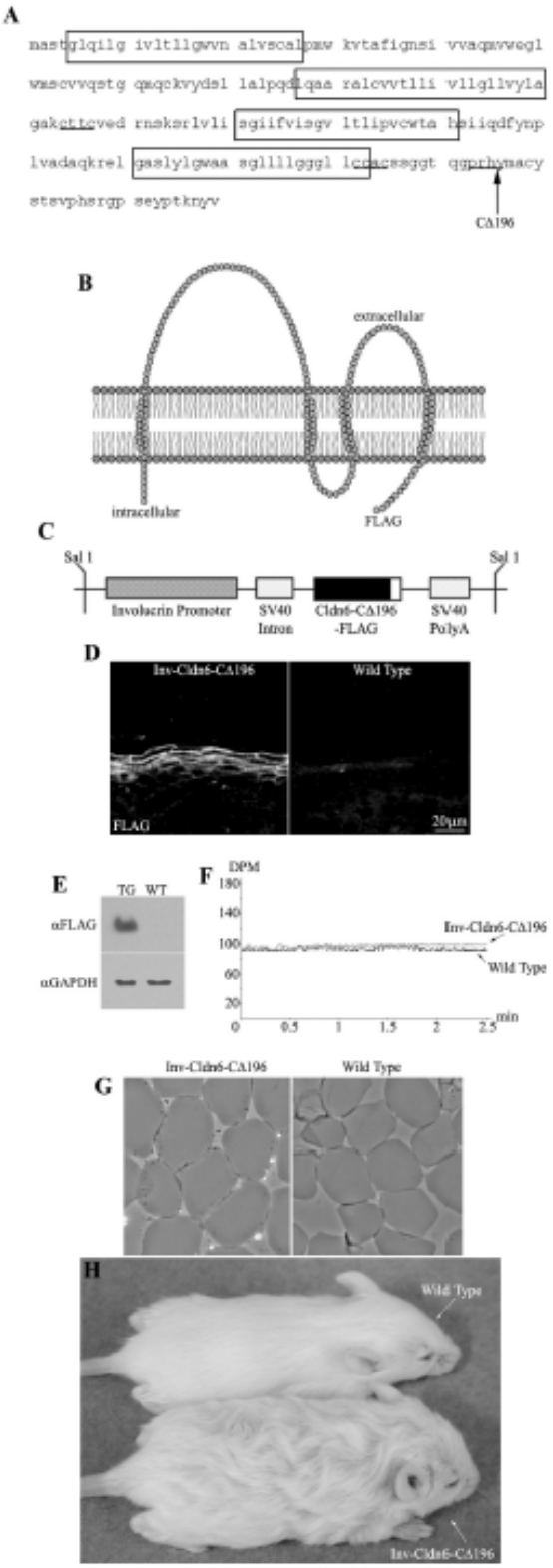

Figure 12. Inv-Cldn6-CΔ196 Transgenic Mice. The Cldn6 protein sequence is shown; transmembrane spanning regions are encased within boxes, the CXXC motifs are underlined, and the truncation site is indicated with an arrow (A). The Inv-Cldn6-CΔ196 mutant was created by deleting the C-terminal half of the cytoplasmic tail domain of Cldn6 after amino acid 196 (B). The Inv promoter was use to drive transgene expression to the suprabasal compartment of the transgenic mouse epidermis, where TJs are localized (C). Transgene expression was confirmed to be restricted to the upper spinous and granular layers of the epidermis as visualized by immunohistochemistry using anti-FLAG antibodies (D) and immunoblotting confirmed a ~19.5kDa band corresponding to the transgene product in skin samples from transgenic (TG) but not wild type (WT) skin samples using anti-GAPDH as a loading control (E). Trans-epidermal water loss measurements confirmed no skin barrier dysfunction in the Inv-Cldn6-CΔ196 transgenic mice at birth (F). This was further supported by evaluation of cornified envelopes; which were characterized by a rigid shape and uniform size comparable to that of the wild type (G). Inv-Cldn6-CΔ196 mice were easily identifiable by their frizzed and lackluster coat appearance as compared to the wild type, a phenotype that persisted throughout life (H).

Epidermal Maturation is Delayed in Inv-Cldn6-CΔ196 Mice

Although in the epidermis there were no gross phenotypic abnormalities or barrier dysfunction apparent at birth, histological examination revealed that the newborn (Figure13A) and 1 week- old (not shown) transgenic epidermis was moderately thicker than that of the wild type, with a thicker stratum corneum, a less-compacted granular layer and an overall expanded suprabasal compartment. By 2-weeks of age, the Inv-Cldn6-C**Δ**196 epidermis displayed a more compact and organized granular layer, but it remained less mature and thicker than that in the wild type animals (Figure 13B). Typical epidermal thinning had commenced in the transgenic epidermis by 3-weeks of age (~1 week later than wild type; not shown) and by 1-month of age the Inv-Cldn6-C**Δ**196 transgenic epidermis was histologically indistinguishable from that of the wild type (Figure 13C).

Consistent with the histological results, abnormalities in the expression of epidermal differentiation markers were present in neonatal Inv-Cldn6-C**Δ**196 mice but these also normalized by 1-month of age (Figure 14; the transgenic and wild type epidermis after 2weeks and 1 month are shown). For example, K5 and K15 were restricted to basal cells at all time points in both the wild type and Inv-Cldn6-C**Δ**196 epidermis (not shown), but K14, another basal compartment marker, occupied an expanded zone extending into the suprabasal compartment at birth and until epidermal thinning was achieved in the Inv-Cldn6-C**Δ**196 epidermis (Figure 14A). The suprabasal layers of the epidermis normally express K1 (Fuchs, 1994) and when in a state of hyperproliferation, K6 and K17 (Leigh et al., 1995;McGowan and Coulombe, 1998;McGowan and Coulombe, 1998). While neither K6 nor K17 was expressed

throughout the time analyzed (not shown), a broadened compartment of K1-positive cells, consistent with the increased number of suprabasal cell layers observed histologically, was seen early but normalized by 1-month of age in the Inv-Cldn6-CΔ196 transgenic epidermis (Figure 14B). Similarly the expression compartments of various structural proteins in the stratum corneum, including involucrin (Figure 14C), filaggrin and loricrin (not shown), were also expanded in the juvenile Inv-Cldn6-CΔ196 epidermis, and a tightly compacted stratum corneum was not observed in the transgenic epidermis until thinning comparable to that in the wild type was achieved.

In parallel with changes in epidermal markers, the thickened newborn and 1-week-old Inv-Cldn6-CΔ196 epidermal basal layer was devoid of Cldn1, which was clearly present in the wild type epidermis (not shown). However, after 2 weeks, sporadic Cldn1-positive basal cells were evident, and the frequency increased as thinning progressed until 1-month of age when Cldn1-positive cells completely occupied the basal and suprabasal compartments in both the transgenic and wild type mice (Figure 14D). The suprabasal specific Cldns, Cldn6 (Figure 14E), Cldn11, Cldn12 and Cldn18 (not shown) were seen in the expanded suprabasal region of the Inv-Cldn6-CΔ196 epidermis; each Cldn evaluated maintained a strictly membranous localization and normalized by 1-month to a zone comparable to wild type. Cldns that are not normally expressed in the epidermis (e.g. Cldn2, Cldn3 and Cldn5) (Turksen and Troy, 2002) were not observed in either the wild type or Inv-Cldn6-CΔ196 epidermis at any time evaluated (not shown). The data suggest that Inv-Cldn6-CΔ196 transgenic mice are born with differentiation abnormalities leading to slower epidermal maturation, but these are too mild to manifest in skin barrier defects or death, and are repaired between birth and 1-month of age.

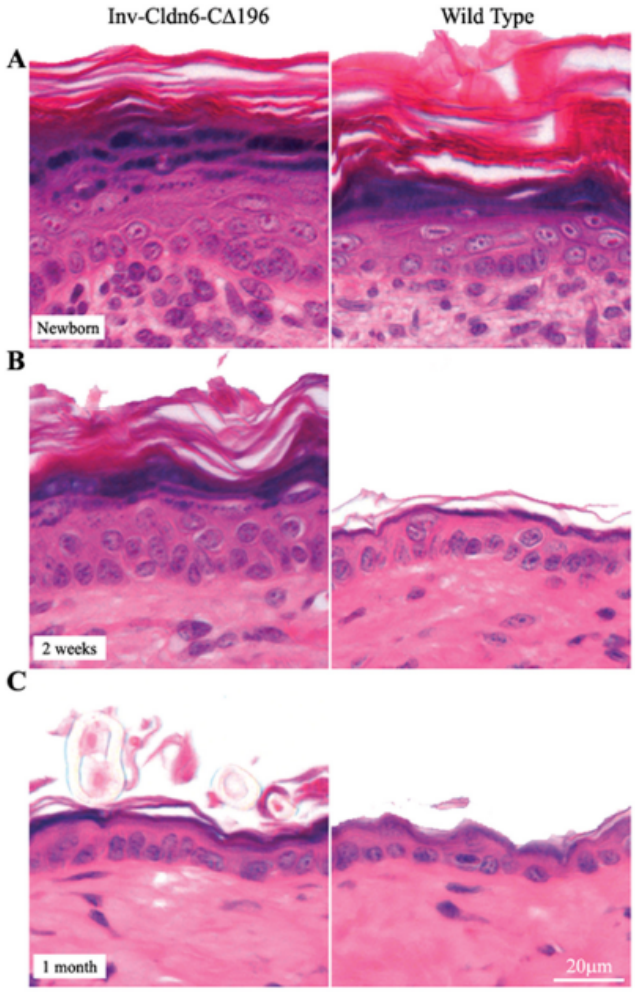

Figure 13. Histological Abnormalities in the Inv-Cldn6-C**Δ**196 Transgenic Epidermis. Histological analysis of newborn (A) Inv-Cldn6-C**Δ**196 transgenic mice as compared to the wild type revealed a moderately thicker epidermis in transgenic samples, with a thicker stratum corneum, a less-compacted granular layer and an overall expanded suprabasal compartment. At 2-weeks of age (B), the Inv-Cldn6-C**Δ**196 epidermis remained somewhat less mature and thicker than that of the wild type; however by 1-month of age (C) the transgenic and wild type samples were histologically indistinguishable.

Inv-Cldn6-CΔ196 Mice Experience Age-Related Perturbations in Epidermal Differentiation and Injury Repair Resulting in Chronic Dermatitis

Although by 1-month of age the Inv-Cldn6-CΔ196 epidermis was morphologically and biochemically indistinguishable from the wild type (Figure 13 and Figure 14), as early as 2-months (not shown) of age, the transgenic epidermis showed histological evidence of thickening that persisted, and was somewhat exacerbated, at 3 (Figure 15A) and 6 (Figure 15B) months. At the latter ages, an expanded stratum corneum, increased number of suprabasal/spinous cell layers and a less compacted granular layer were clearly present, and reminiscent of the 2-week-old Inv-Cldn6-CΔ196 epidermis (see above).

The histological results suggested that intrinsic changes occurred in the transgenic epidermis, a possibility supported by the perturbed expression of differentiation markers; because results were similar from 2- to 6-months of age, we report results from 3-month-old Inv- Cldn6-CΔ196 transgenic mice only (Figure 16). K14 was expressed throughout the basal and suprabasal compartments in the aging Inv-Cldn6-CΔ196 epidermis (Figure 16A), while K5 (not shown) and K15 (Figure 16B) remained restricted to basal cells as in the wild type epidermis. K1 (Figure 16C) was seen throughout an expanded suprabasal zone in the transgenic epidermis corresponding to the increased number of spinous cell layers; however, neither K6- (Figure 16D) nor K17- positive (Figure 16E) interfollicular epidermal cells were observed nor was there any up-regulation in the expression of Ki67 or CD3 (not shown), suggesting no detectable hyperproliferation or immune cell infiltration. The localization of involucrin (Figure 16F), loricrin (not shown) and filaggrin the (Figure 16G) suggested that there were also perturbations in terminal

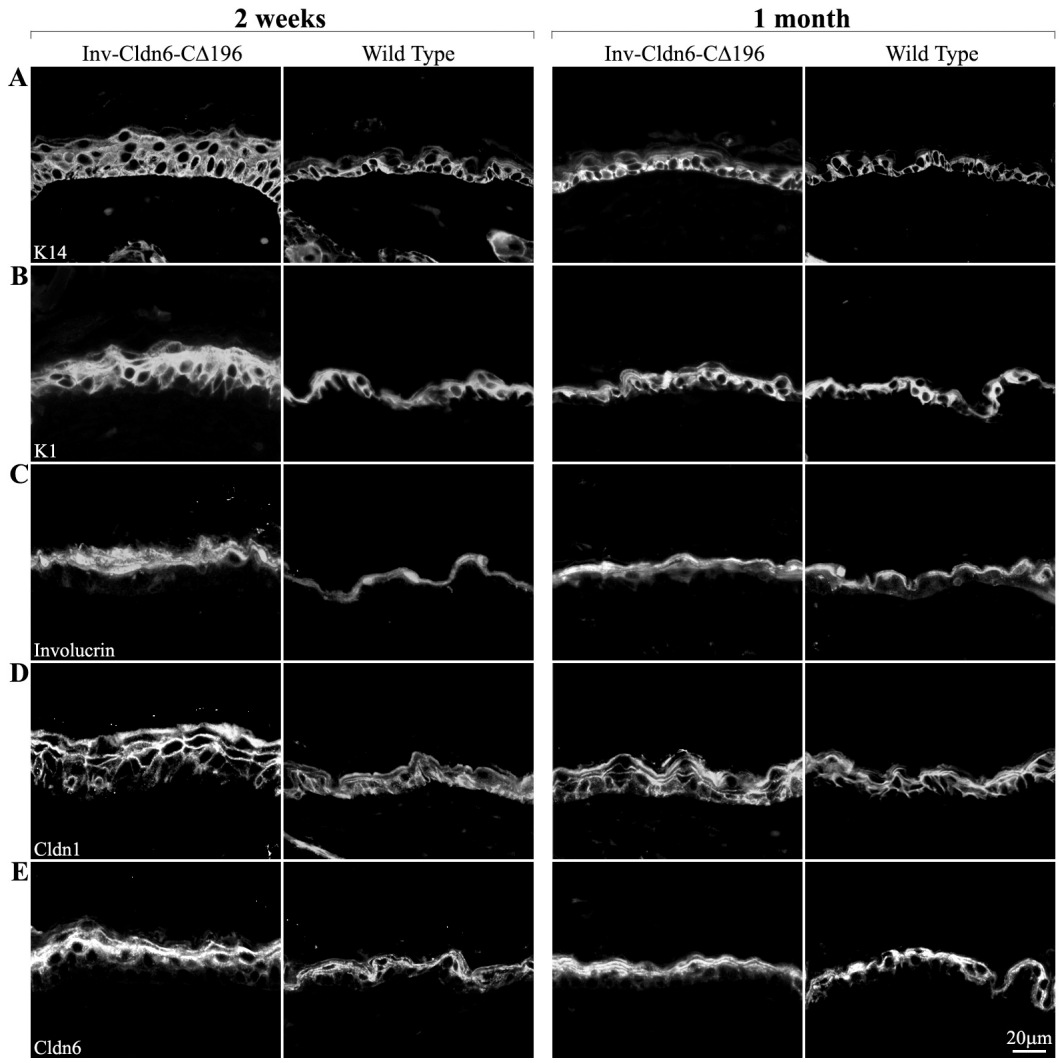

Figure 14. Perturbation of Markers of Epidermal Differentiation. Immunofluorescence was used to evaluate various markers of epidermal differentiation including K14 (A), K1 (B), involucrin (C), Cldn1 (D) and Cldn6 (E) in Inv-Cldn6-C**Δ**196 mice as compared to their age-matched wild type counterparts. In each case abnormalities were present in samples from 2-week old (left panel) transgenic mice that were normalized by 1-month of age (right panel). For example, the K14 expression zone was extended into the suprabasal compartment the Inv-Cldn6-C**Δ**196 epidermis until epidermal thinning was achieved. In addition, broadened K1 and involucrin expression compartments was seen early but normalized by 1-month of age. Sporadic Cldn1-positive basal cells were evident in the 2-week old transgenic epidermis; by 1-month of age Cldn1-positive cells occupied both the basal and suprabasal compartments reminiscent of the wild type. Cldn6, a suprabasal-specific Cldn, was observed in an expanded zone early on, and was normalized by 1-month of age.

differentiation in the aged Inv-Cldn6-CΔ196 epidermis, with each marker occupying an expanded zone of expression consistent with the abnormal morphological appearance of the stratum corneum described above.

Cldn expression was also modified with striking anomalies consistent with those we reported earlier in an acute irritant (12-O-tetradecanoylphorbol-13-acetate (TPA))-induced epidermal injury response (Arabzadeh et al., 2007). The number of Cldn1-positive basal and suprabasal cells was reduced (Figure 17A) and an expanded suprabasal zone of Cldn6- (Figure 17B), Cldn11- (Figure 17C), Cldn12- (Figure 17D) and Cldn18-positive cells (Figure 17E) was seen in the transgenic epidermis; concomitantly, a shift away from a strictly membrane localization was evident. Cldn2, Cldn3 and Cldn5 were not expressed in either wild type or transgenic animals (not shown).

Taken together, the data suggest an intrinsic propensity for injury and inefficient repair that increases with aging in the Inv-Cldn6-CΔ196 epidermis. Two lines of evidence further support this. First, histological and biochemical changes resulting from TPA treatment were seen earlier (4 hours versus 12 hours) and repaired more slowly (no repair versus normalization by 96 hours) in Inv-Cldn6-CΔ196 versus wild type epidermis when mice were tested at 1 month of age (data not shown). Second, obvious signs of dermatitis, especially in areas subjected to repetitive mechanical stress during grooming (e.g. the neck and behind the ears), were seen in the aging Inv-Cldn6-CΔ196 epidermis (Figure 18A). Histological evaluation of skin samples from these regions revealed a significantly thickened epidermis with an increased number of spinous/suprabasal cell layers and cellular disorganization; the upper differentiation layers were also abnormal, with parakeratosis, the prevalent appearance of nuclei, an

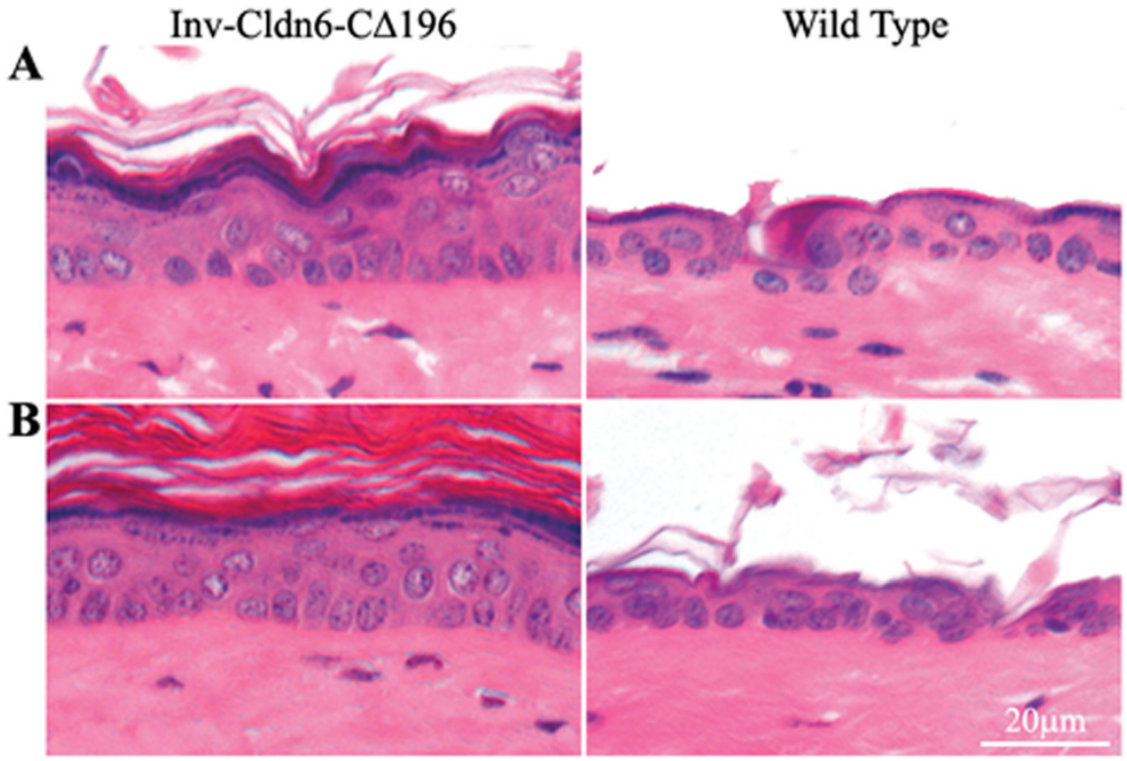

Figure 15. Histological Evidence of Epidermal Abnormalities in the Aging Inv-Cldn6-C**Δ**196 Transgenic Epidermis. Histological evidence of epidermal abnormalities emerged in aging Inv- Cldn6- C**Δ**196 transgenic mice after 3 (A) and 6 (B) months as compared to the wild type. These included an expanded stratum corneum, as well as an expanded suprabasal zone and a less compacted granular layer suggesting an intrinsic propensity for injury and inefficient repair that increases with aging in the Inv-Cldn6-C**Δ**196 epidermis.

improperly packed granular layer and a thicker stratum corneum (Figure 18B). In the dermatitis-affected areas, K14 (Figure 16H) and K5 (not shown) were expressed in an expanded zone far into the suprabasal compartment, and K15 expression was only sporadically expressed (Figure 16I). K1 was localized throughout the thickened suprabasal zone of the dermatitis-affected Inv-Cldn6- C**Δ**196 epidermis, and staining appeared punctate, suggesting a keratin filament bundling defect (Figure 16J). K6 was seen consistently throughout the basal and suprabasal layers of the dermatitis-affected epidermis (Figure 16K); K17-positive basal and suprabasal cells were somewhat more sporadically seen (Figure 16L). Expression compartments for involucrin (Figure 16M), loricrin (not shown) and filaggrin (Figure 16N) were also expanded, with an obvious packing defect reminiscent of the observed histological abnormalities of the stratum corneum. Abnormalities in Cldn1, Cldn6, Cldn11, Cldn12 and Cldn18 localization observed in the aged Inv-Cldn6-C**Δ**196 transgenic epidermis (see above) were strikingly exacerbated in the dermatitis-affected epidermis. Cldn1 was completely absent in the basal and lower suprabasal layers, but a strictly membranous localization was preserved in the Cldn1-positive upper suprabasal zone (Figure 17F). While the distribution of Cldn6 (Figure 17G), Cldn11 (Figure 17H), Cldn12 (Figure 17I) and Cldn18 (Figure 17J) corresponded to the expanded suprabasal compartment of the dermatitis-affected Inv-Cldn6-C**Δ**196 epidermis, immunostaining was less intense and membranous localization less evident in lower suprabasal layers.

The morphological and biochemical abnormalities described predict altered permeability barrier properties and potentially barrier dysfunction in areas of repeated insult of the aged Inv-Cldn6-C**Δ**196 transgenic epidermis. In keeping with this hypothesis

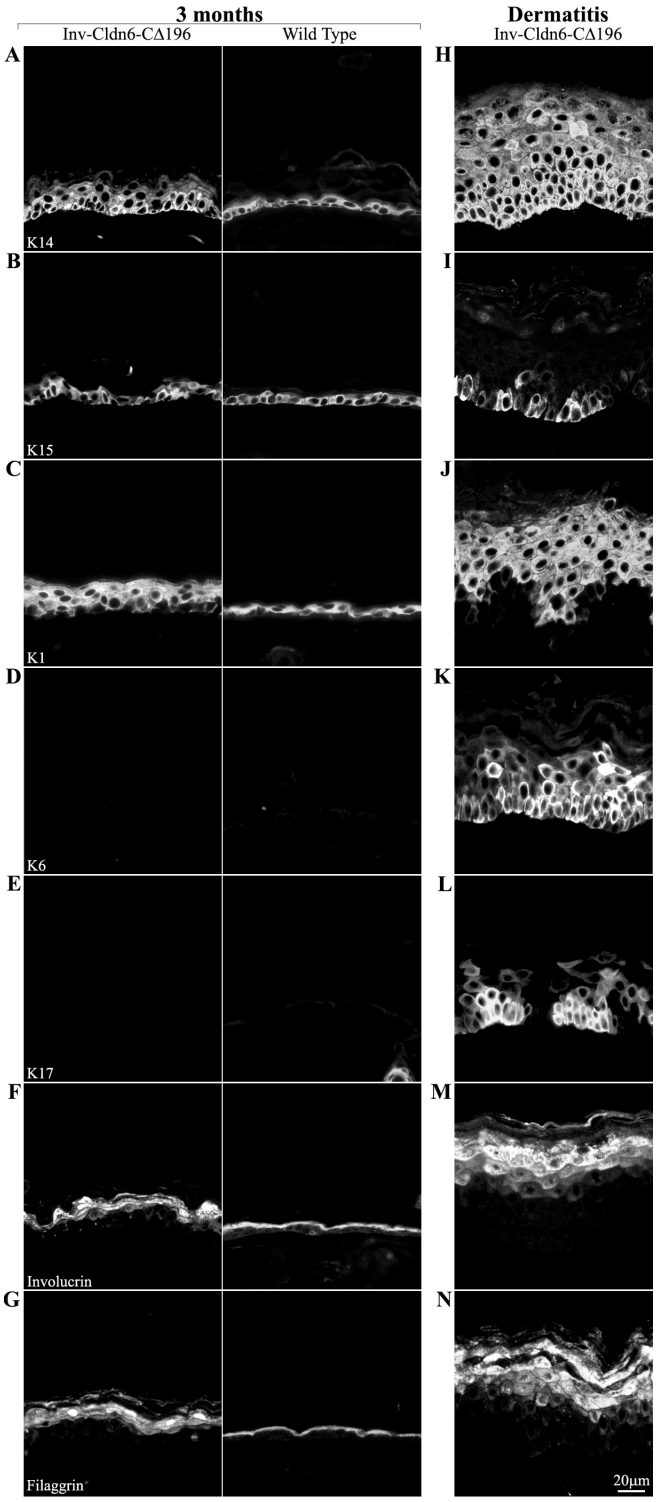

Figure 16. Aberrance in Markers of Epidermal Differentiation During Aging. Changes in the epidermal differentiation program of the aging Inv-Cldn6-CΔ196 transgenic epidermis were evaluated by immunofluorescence and compared to their age-matched wild type counterparts (samples from 3-month-old mice are shown – left panel); their expression/localization in dermatitis affected areas is also indicated (right panel). K14 was expressed throughout the basal and suprabasal compartments in the aging Inv-Cldn6-CΔ196 epidermis (A), while K15 remained restricted to basal cells (B). The zone of K1 expression was expanded in the 3-month-old transgenic epidermis (C); however, neither K6- (D) nor K17-positive (E) cells were observed. An expanded zone of expression, and improper packing, of involucrin (F) and filaggrin (G) suggested perturbations in terminal differentiation program of the aged Inv-Cldn6-CΔ196 epidermis. In the dermatitis-affected areas, K14 (H) expression was expanded far into the suprabasal compartment, while K15 was only sporadically observed (I). A punctate K1 localization was evident throughout the thickened suprabasal zone of the dermatitis-affected Inv-Cldn6-CΔ196 epidermis (J), and K6- (K) and K17-positive (L) basal and suprabasal cells were observed. In addition, involucrin (M) and filaggrin (N) expression compartments were also expanded, with obvious packing defects evident.

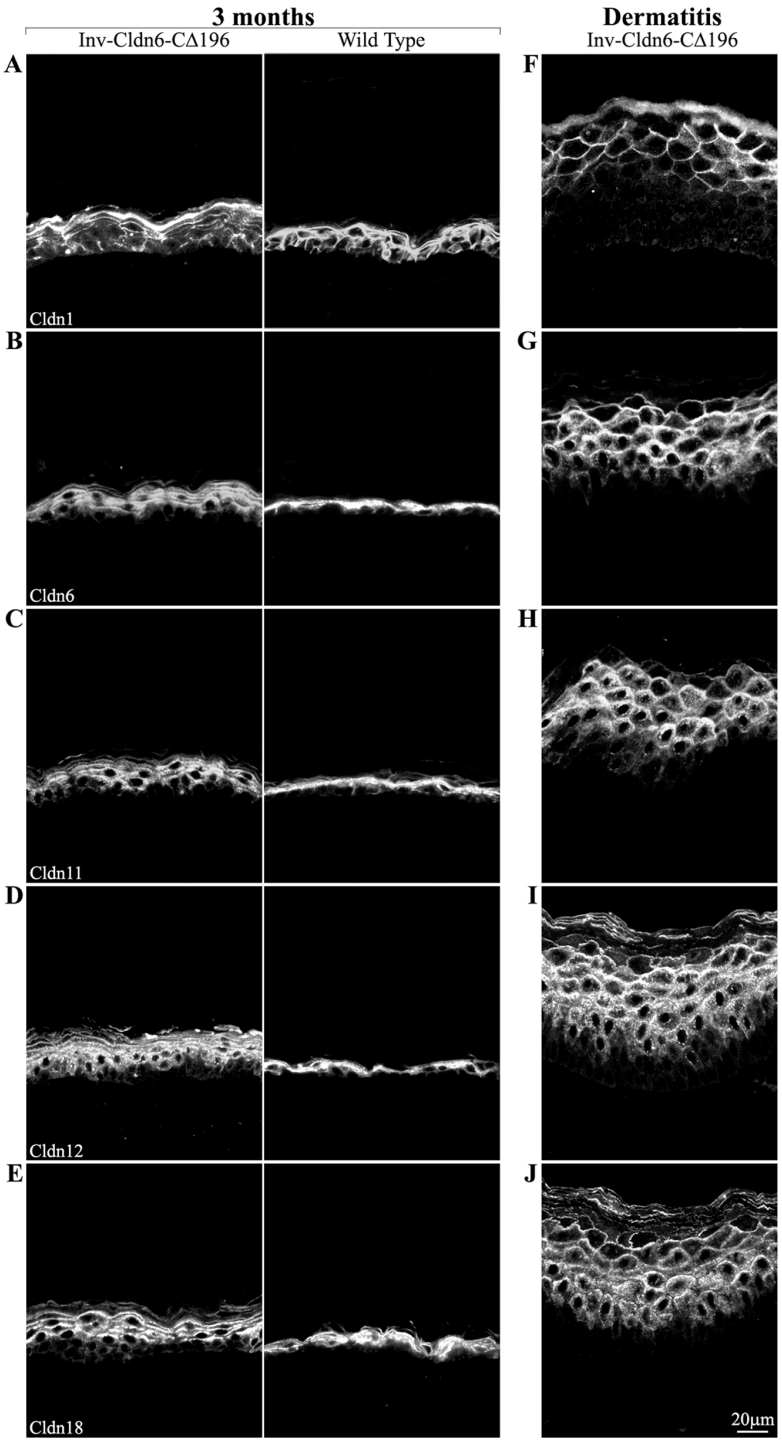

Figure 17. Evaluation of Cldns in the Aging Inv-Cldn6-C**Δ**196 Transgenic Epidermis. The expression/localization of Cldns was also perturbed in the aging Inv-Cldn6-C**Δ**196 transgenic epidermis (samples are from 3-month-old mice – left panel); the dermatitis-affected zones are also shown (right panel). Cldn1-positive basal and suprabasal cells were reduced in the aging Inv-Cldn6-C**Δ**196 epidermis (A). In addition, a shift away from a strictly membrane localization and an expanded zone of suprabasal Cldns was evident -Cldn6 (B), Cldn11 (C), Cldn12 (D) and Cldn18 (E). These abnormalities in were strikingly exacerbated in the dermatitis-affected epidermis. For instance, the basal and lower suprabasal layers were void of Cldn1 localization (F). In addition, Cldn6 (G), Cldn11 (H), Cldn12 (I) and Cldn18 (J) localization was observed throughout the expanded suprabasal zone with membranous localization less evident in the lower suprabasal layers.

TEWL measurements across the dermatitis-affected transgenic skin increased ~8-fold (DPM in the range of 790–830 versus 95–100 of normal adult skin) (Figure 18C).

DISCUSSION

In this study, we used transgenic mouse technology to expand on our structure-function approach to elucidating the role of the cytoplasmic tail of Cldn6 in epidermal development and the formation and maintenance of skin barrier integrity. We demonstrate that Inv-Cldn6-CΔ196 mice display epidermal differentiation abnormalities at birth that result in slower epidermal maturation but are apparently normalized by 1-month of age. However, with aging, intrinsic properties of the Inv-Cldn6-CΔ196 epidermis are manifested by a propensity for injury and inefficient repair, eventually resulting in chronic dermatitis especially in areas subjected to frequent insult via grooming. Changes in Cldn expression and localization suggest marked changes in the presence and function of TJs, as also evidenced by skin barrier dysfunction. Taken together, our data suggest that the overexpression of a Cldn tail truncation mutant in the suprabasal compartment of the mouse epidermis results in its delayed maturation, a propensity for injury, diminished repair and skin barrier deficiency reminiscent of the intrinsic aging process of human skin. It is well established that the formation of the epidermal permeability barrier (EPB) is developmentally regulated (Byrne and Hardman, 2005;Hardman et al., 1998;Hardman et al., 1999;Turksen and Troy, 2002) and that disruption or delay in its formation before birth has a critical role in the survival of the organism (Cartlidge, 2000;Elias, 2005;Mack et al., 2005;Williams and Feingold, 1998).

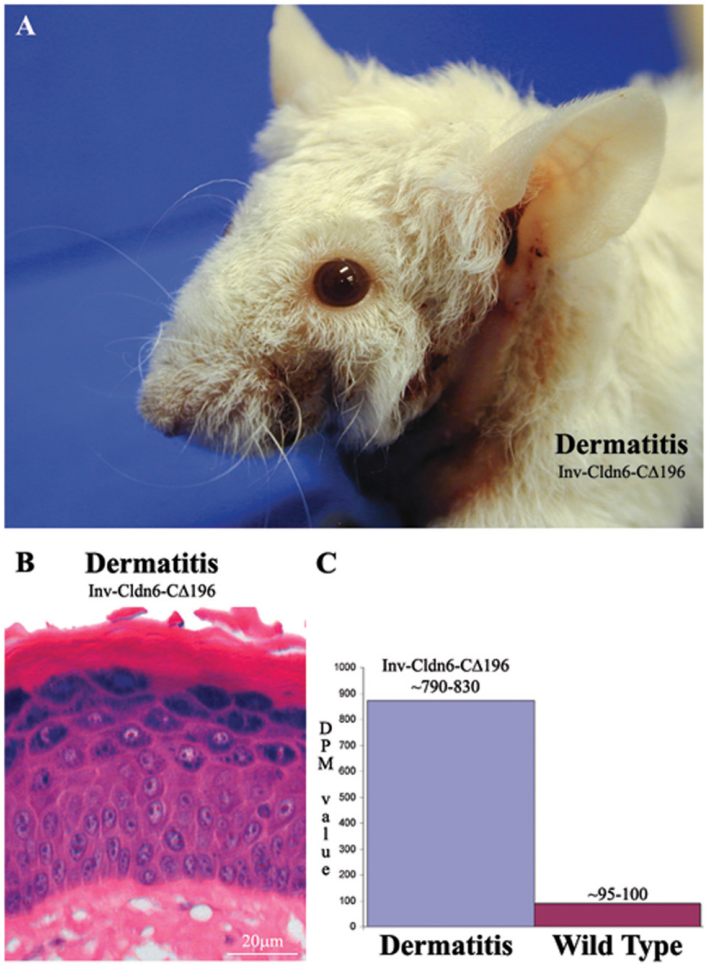

Figure 18. Aging-associated barrier dysfunction. Upon gross observation, obvious signs of dermatitis were evident in aging Inv- Cldn6-C**Δ**196 transgenic mice, especially in areas subjected to repetitive mechanical stress such as the neck and behind the ears (A). Histological evaluation revealed a significantly thickened epidermis with an increased number of spinous/suprabasal cell layers and cellular disorganization; the upper differentiation layers were also abnormal, with parakeratosis, the prevalent appearance of nuclei, an improperly packed granular layer and a thicker stratum corneum (B). TEWL measurements across the dermatitis-affected transgenic skin confirmed an approximately 8-fold increase in water loss and a barrier deficient phenotype (C).

Previously we reported that a perturbation of Cldn6 expression levels at its endogenous site of expression - the suprabasal layer of the epidermis - alters epidermal development and the formation of the EPB (Troy et al., 2005;Turksen and Troy, 2002). However, depending on whether normal or mutant forms of Cldn6 are expressed and at what levels, the severity of phenotypic anomalies varies markedly (Arabzadeh et al., 2006;Troy et al., 2005;Troy and Turksen, 2007;Turksen and Troy, 2002). For example, perinatal lethality resulting from a severely compromised epidermal differentiation program and dysfunctional EPB results when native Cldn6 is expressed at high levels (Turksen and Troy, 2002) while with lower levels of expression, less severe defects occur with the capacity for normalization of EPB function (Troy et al., 2005). In contrast, apparently normal prenatal epidermal development and formation of a functioning EPB, but an abnormal postnatal lifelong keratinocyte hyperproliferation leading to progressive thickening of the epidermis – but no dermatitis, results when a mutant form of Cldn6 lacking its entire tail domain (Inv-Cldn6-C**Δ**187) is expressed (Arabzadeh et al., 2006). On the other hand, high overexpression of a different mutant lacking only the C-terminal half of the tail domain of Cldn6 (Inv-Cldn6-C**Δ**196) results in a lethal barrier dysfunction with marked hyperproliferative squamous invaginations/cysts replacing hair follicles (Troy and Turksen, 2007). We now show that in Inv-Cldn6-C**Δ**196 mice with lower levels of transgene expression the abnormalities are much more subtle, and manifested by a slower than usual (i.e., 1 month versus 2 week) but otherwise apparently normal maturation of the epidermis from the multilayered structure seen at birth to the thinner 2–3 cell layer mature structure (Troy et al., 2005). These early postnatal changes in maturation are not accompanied by abnormalities in the formation of a functional skin

barrier. It is interesting to note that K6- and K17-positive suprabasal cells, which are normally not present in the mature epidermis except in situations of abnormal cell proliferation and differentiation (Leigh et al., 1995;McGowan and Coulombe, 1998;McGowan and Coulombe, 1998) are not detectable in the interfollicular epidermis of either the juvenile or aged Inv-Cldn6-CΔ196 transgenic samples, until patent dermatitis is evident (see below).

Phenotypic differences observed in the different transgenic mice appear to reflect different mechanistic consequences of expression of native or mutant Cldn6. Structure function details of different Cldns are only beginning to emerge, but the COOH-terminal tail varies considerably in length and is the region with the most sequence heterogeneity among Cldn isoforms, and in most cases contains a PDZ-binding motif that enables Cldns to directly interact with the TJ-associated MAGUKs (ZO1, ZO2, and ZO3) as well as with MUPP1 and PATJ (Assemat et al., 2008;Heiskala et al., 2001;Koval, 2006;Roh and Margolis, 2003). For at least some Cldns (e.g., 1 and 5), the cytoplasmic tail upstream of the PDZ-binding motif is thought to be required for targeting to the TJ complex (Ruffer and Gerke, 2004) and contributes to protein stability (Arabzadeh et al., 2006;Van Itallie et al., 2005). Post-translational modifications within the tail domain, including phosphorylation and palmitoylation, are also thought to regulate Cldn activities, including their targeting to the membrane and insertion into TJs (Simard et al., 2006) but to date most Cldns have not been subjected to exhaustive analysis. Thus, the similarities and differences between Inv-Cldn6-CΔ187 and Inv-Cldn6-CΔ196 mice are of interest. Our data suggest that the phenotype observed in the Inv-Cldn6-CΔ187 epidermis reflects protein instability and an unfolding protein response. On the other hand, the delayed

maturation and age-related deficit in wound healing and repair in the Inv-Cldn6-CΔ196 epidermis most likely involves an aberrant interaction with the cytoskeleton. Although to date no direct interaction between the cytoplasmic tail of Cldns and cytoskeletal molecules has been shown, the possibility of such direct interactions is not unlikely considering the known role of the actin cytoskeleton, for example, in cell shape and cell polarity (Ivanov et al., 2004;Ivanov, 2008;Miyoshi and Takai, 2008).

Despite the importance of the skin barrier to the survival of the mammalian species, the molecular mechanisms governing its formation and maintenance are not well-understood, nor are the changes that occur during the intrinsic aging process of the skin. In humans, aging-related changes in the skin are widespread and result from both intrinsic (gene mutations, hormonal changes, cellular metabolism) and extrinsic (UV exposure, chemicals, pollutants) factors. While extrinsic factors affect mainly the dermis, the epidermis is the main target of intrinsic skin aging processes where, especially in sun exposed skin, changes occur often with initial generalized epidermal thickening, indicative of epidermal hyperplasia and reduced capacity to repair, such that the epidermis becomes prone to a number of skin conditions including dermatitis, eczema and ulceration (Davies, 2008;Elias and Ghadially, 2002;Ghadially, 1998;Kligman, 1989;Makrantonaki and Zouboulis, 2007;Ward, 2005). Only recently has it been appreciated that abnormal skin barrier function might underlie these skin conditions (Barland et al., 2004) although no analysis of Cldn expression has been reported. The murine epidermis, however, does not normally undergo comparable aging-related changes; in fact, once the mouse epidermis is matured it maintains a homeostatic balance and undergoes morphological and biochemical changes only in response to injury or

disease. Thus, it was extremely interesting to note that in spite of the fact that Inv-Cldn6-CΔ196 mice achieve a normal-appearing epidermis by one-month of age (by morphology and biochemical markers), with aging they have a high propensity for epidermal injury and a diminished ability to repair lesions, leading eventually to severe dermatitis with associated changes in skin barrier function. It should be noted that this sensitivity to injury and inefficient repair were seen also in young mice subjected to acute injury by application of the irritant TPA (data not shown).

As already indicated, the precise mechanisms by which expression of low levels of Cldn6-CΔ196 in the suprabasal layers of the epidermis leads to age-related changes appearing to mimic those seen in humans are currently not known, but our observations suggest that changes in Cldn homeostasis leading to changes in the epidermal differentiation program play important roles. In this regard, not only changes elicited directly by changes in Cldn6 signaling but also changes imparted by alterations in the expression of other Cldns, notably Cldn1, must be considered. In the developing epidermis, Cldn1 is first restricted to the stratifying layers and matures to occupy the basal layer upon the completion of barrier formation at E17.5 (Troy and Turksen, 2007). However, in response to TPA-induced injury and loss of cell polarity seen in tumorigenesis, Cldn1 expression is downregulated in both the basal layer and immediate suprabasal layers of the epidermis (Arabzadeh et al., 2007;Arabzadeh et al., 2008) alterations seen also with aging of the Inv-Cldn6-CΔ196 epidermis. The drastic changes in TEWL that we observed in severely affected areas of the Inv-Cldn6-CΔ196 epidermis also appear to mimic those seen in the aging human epidermis subjected to stress or injury (Ghadially et al., 1995). Thus the data reported here suggest that the changes in

Cldn expression and localization in mice with low expression of Cldn6-CΔ196 in the epidermis lead to relatively subtle changes in the epidermal differentiation program and permeability in the young animal, but render them prone to injury and diminished repair that are exacerbated especially in areas subjected to repeated mechanical trauma, leading to chronic and increasingly severe dermatitis.

In summary, our data add to the evidence that Cldn6, and in particular the C-terminal half of the Cldn6 tail, contribute to the regulation of epidermal differentiation and skin barrier function throughout life. They also support the utility of the Inv-Cldn6-CΔ196 mouse model for studies aimed at understanding intrinsic changes in the aging epidermis and point towards a need to investigate Cldn expression profiles in the aging human epidermis.

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CHAPTER 3

Involucrin–Claudin-6 Tail Deletion Mutant (CΔ206) Transgenic Mice: A Model of Delayed Epidermal Permeability Barrier Formation and Repair

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AUTHOR CONTRIBUTIONS

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Adebola Enikanolaiye and Nathalie Lariviere were equally responsible for most experiments including sample collection and processing, immunohistochemistry, microscopy, PCR and Western blots. Adebola Enikanolaiye and Nathalie Lariviere also contributed to the re-write of the manuscript to include a disease focus and translational impact. Elif Atasoy and/or Azadeh Arabzadeh made the initial observations and performed the first replicates of immunohistochemistry. Tammy-Claire Troy wrote the initial manuscript, provided reagents and assisted with image acquisition and protocol optimization for all experiments. Kursad Turksen conceived and designed the experiments, contributed to data analysis and provided final approval of the manuscript.

SUMMARY

Preterm birth is a major global health problem that results in a large number of infant deaths, many of which are attributable to the complications of an immature epidermal permeability barrier (EPB), for which there is currently no effective therapeutic option. The mammalian EPB is formed during development and is essential for survival as it maintains thermoregulation and hydration, and provides a defense against infection. Using transgenic mouse technology, we have demonstrated the importance of claudin (Cldn)-containing tight junctions (TJs) in epidermal differentiation and, in particular, that epidermal suprabasal overexpression of Cldn6 results in an EPB-deficient phenotype that phenocopies the dysfunctional EPB of premature human infants. In this study, we used the same approach to target a Cldn6 tail deletion mutant to the epidermis of mice [Involucrin (Inv)-Cldn6-C**Δ**206 transgenic mice]. The Inv-Cldn6-C**Δ**206 transgenic mice displayed a developmental delay in EPB formation, as shown by the expression of keratins and Cldns, and by X-Gal penetration assays. Trans-epidermal water loss measurements and immunolocalization studies indicated that the epidermal differentiation program was also perturbed in postnatal Inv-Cldn6-C**Δ**206 transgenic mice resulting in a delayed maturation. Notably, however, expression/localization of epidermal differentiation and maturation markers, including Cldns, indicated that the transgenic epidermis matured and normalized by postnatal day 10, which is 3 days after the wild-type epidermis. Our results suggest that activation of the extracellular signal regulated kinase 1/2 (Erk1/2) pathway and Cldn1 phosphorylation are associated with the repair and maturation of the skin barrier processes. These studies provide additional support for the crucial role of Cldns in epidermal differentiation, maturation and the

formation of the EPB, and describe a novel animal model for evaluating postnatal epidermal maturation and therapies that may accelerate the process.

INTRODUCTION

The prevalence of preterm birth is widespread with very little understanding of its causes and no unambiguous epidemiological data for predicting its occurrence. Formed in weeks 30 to 33 of pregnancy (Wilson and Maibach, 1980) the protective epidermal permeability barrier (EPB) of the skin is essential for survival as it provides the first line of defense against infection, environmental insult, and the loss of heat and solutes (Baharestani, 2007;Gibson et al., 2006;Shwayder and Akland, 2005;Soll, 2008). In infants born before 32 weeks of pregnancy, severe EPB dysfunction may result in death or long-term complications (Pilling et al., 2008).

The EPB is formed in the later stages of epidermal terminal differentiation, and consists of tight junction (TJ) strands of adjacent cells that associate laterally (Brandner et al., 2002;Brandner, 2009;Langbein et al., 2002;Langbein et al., 2003;Schluter et al., 2007;Turksen and Troy, 2002) and function in sealing intracellular spaces for paracellular diffusion control (Farquhar and Palade, 1963). The selective permeability of the EPB is provided by a family of 23 highly conserved integral membrane proteins known as claudins (Cldns), a relatively recently identified component of TJs (Angelow et al., 2008;Chiba et al., 2008;Findley and Koval, 2009;Krause et al., 2008;Turksen and Troy, 2004;Van Itallie and Anderson, 2006). Heterogeneity within the Cldn family results from distinctly charged amino acid sequences within the first external loop; thus, the specific permeability properties of different epithelia are attributed to their different Cldn

compositions (Daugherty et al., 2007;Katoh and Katoh, 2003;Krause et al., 2008;Turksen and Troy, 2004). Recent studies have clearly demonstrated that Cldn-containing TJs are intricately involved in epidermal differentiation programs, and that TJ function, and thus barrier integrity, is modified in response to Cldn modulation (Arabzadeh et al., 2006;Furuse et al., 2002;Troy et al., 2005;Troy and Turksen, 2007;Turksen and Troy, 2002). For instance, Cldn1 knockout mice die shortly after birth owing to EPB dysfunction (Furuse et al., 2002). Inv-Cldn6 transgenic mice, in which the involucrin (Inv) promoter targets Cldn6 to the suprabasal layers of the epidermis, also suffer EPB abnormalities with a phenotype mimicking that seen in premature human babies, the severity/lethality of which is dependent upon the level of Cldn6 overexpression (Troy et al., 2005;Turksen and Troy, 2002). Inefficient membrane targeting of Cldn proteins and a highly proliferative epidermal phenotype – apparently as a result of the unfolded protein response pathway – were observed upon overexpression of a cytoplasmic tail-ablated Cldn6 (Inv-Cldn6-CΔ187) in mice (Arabzadeh et al., 2006). Furthermore, dependent on the level of overexpression, Inv-Cldn6-CΔ196 mice (with half the cytoplasmic tail ablated) (Troy and Turksen, 2007) displayed EPB dysfunction and an aging-related skin barrier defect resulting in an intrinsic propensity for injury, inefficient repair and chronic dermatitis. These data provide support for the importance of the cytoplasmic tail portion of Cldn molecules in epidermal differentiation and EPB function. Although relatively constant in length, sequences within the Cldn cytoplasmic tail are divergent but include a number of putative functional domains that are present in many family members. To continue to address functional domain activities, we again used the Inv promoter to target a Cldn6 cytoplasmic tail deletion mutant (Cldn6-CΔ206), to the suprabasal compartment

of the epidermis; this deletion encompasses the PDZ-binding domain and a putative protein kinase A (PKA) phosphorylation site. Inv-Cldn6-CΔ206 transgenic mice have a developmental delay in EPB formation, suffer trans-epidermal water loss (TEWL) at birth, and exhibit a perturbed epidermal maturation program manifested by a 3-day lag in the initiation of the normal epidermal thinning process (which occurs at day 10 in the Inv-Cldn6-CΔ206 transgenic mice versus day 7 in the wild-type epidermis). Our data suggest that this process stems from the remodeling of Cldn1 expression in the repairing Inv-Cldn6-CΔ206 transgenic epidermis, and that the first phase of repair requires shedding of phospoCldn1-expressing cells from the differentiated epidermal compartment where extracellular signal-regulated kinase 1/2 (Erk1/2) is activated. These results suggest that although Inv-Cldn6-CΔ206 transgenic mice suffer developmental delays in epidermogenesis, the epidermis undergoes epidermal maturation and repair after birth, normalizing by postnatal day 10, thus providing a model to elucidate the molecular mechanisms by which Cldns regulate the postnatal maturation of the epidermis. This model may also be useful to screen for agents that accelerate formation of a functional barrier, which could provide useful therapeutic options for improvement of the EPB in premature infants.

RESULTS

Generation and Phenotype of Inv-Cldn6-CΔ206 Transgenic Mice

We truncated the C-terminal cytoplasmic tail domain of Cldn6 after amino acid 206 (Cldn6-CΔ206), removing 14 amino acids to encompass a region including the PDZ binding domain (YV) and a putative PKA phosphorylation site (Fig. 19A,B). Cldn6-

CΔ206 cDNA was expressed in the suprabasal cells of the transgenic mouse epidermis, where TJs are localized, under the control of the 3.7 kb 5'-flanking element of the human Inv gene (IVL) (Fig. 19C). Founder Inv-Cldn6-CΔ206 transgenic mice (five females) were identified by PCR, and two lines were established with identical phenotypes. Newborn Inv-Cldn6-CΔ206 transgenic mice appeared grossly phenotypically comparable to the wild type. However, as was observed in our Inv-Cldn6, Inv-Cldn6-CΔ187 and Inv-Cldn6-CΔ196 mouse models, with time the Inv-Cldn6-CΔ206 transgenic mice were easily distinguishable from the wild type by coat appearance: smooth and shiny in the wild-type mice versus wiry and lackluster in the transgenic mice, a phenotype that was maintained throughout the life of the mouse (Fig. 19D). Since it does not appear to have any direct relevance to the delayed epidermal maturation and postnatal EPB formation reported here, coat characteristics have not been investigated further.

Although no gross phenotypic abnormalities were apparent at birth, daily dermal phase meter (DPM) measurements (Fig. 19E) demonstrated significant, albeit non-lethal, TEWL in neonatal Inv-Cldn6-CΔ206 transgenic mice. Compared with the wild-type mice (DPM in the range of 95-105), Inv-Cldn6-CΔ206 transgenic neonates expressed DPM values in the range of 175-185. However, DPM levels were already reduced in the transgenic mice at 2 days after birth (~125-135), and by 4 days after birth, DPM values reached wild-type levels (in the range of 100-106). Reverse transcriptase (RT)-PCR with primers spanning the junction of the Inv exon and the Cldn6 sequences in the transgene confirmed that the observed changes in TEWL leading to reversion to a normal or wild-type phenotype are not simply the result of decreased expression of the transgene (Fig.

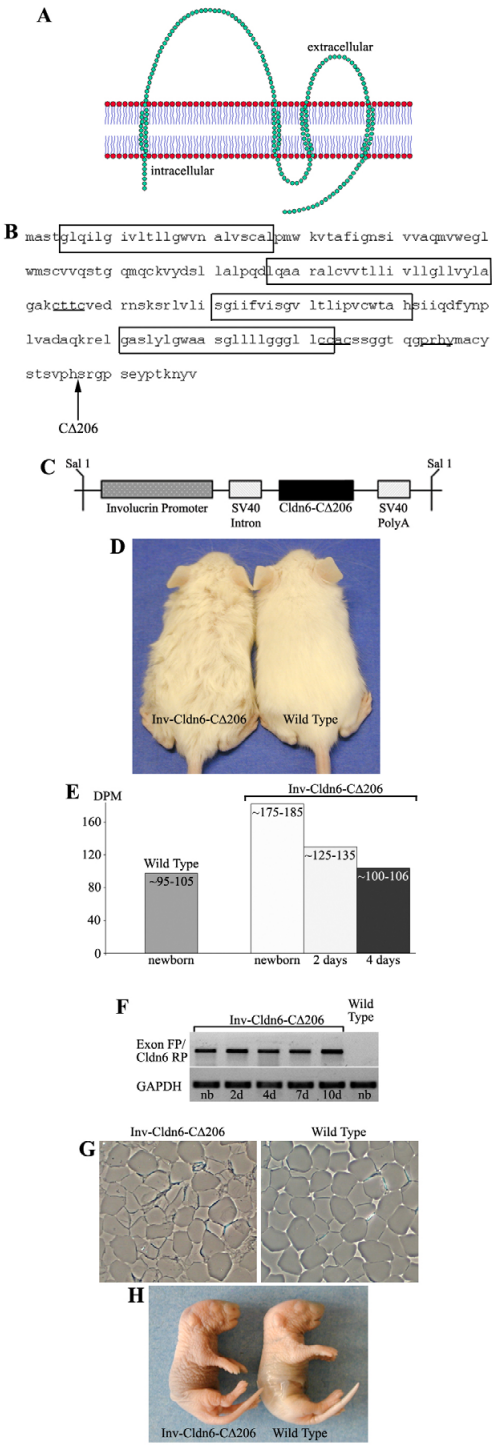

Figure. 19. Inv-Cldn6-CΔ206 Transgenic Mice. (A) The Inv-Cldn6-CΔ206 mutant was created by truncating the C-terminal cytoplasmic tail domain of Cldn6 after amino acid 206 (Cldn6-CΔ206). (B) The Cldn6 protein sequence is shown; transmembrane-spanning regions are encased within boxes, the CXXC motifs are underlined, and the deletion site is indicated with an arrow. (C) Inv-Cldn6-CΔ206 cDNA was expressed in the suprabasal cells of the transgenic mouse epidermis, where TJs are localized, under the control of the 3.7 kb 5'-flanking element of the human involucrin gene. (D) Inv-Cldn6-CΔ206 mice were easily identifiable by their wiry and lackluster coat, a phenotype that was maintained throughout life. (E) TEWL measurements demonstrated an approximately twofold increase in water loss for transgenic neonates, and daily measurements revealed a robust epidermal repair mechanism where normal hydration levels were achieved by 4 days after birth. (F) RT-PCR using primers spanning the Inv exon and Cldn6 confirmed that transgene expression was consistent and could therefore not be used to explain the changes in TEWL; GAPDH controls are shown. (G,H) Evaluation of cornified envelopes, which were characterized by a rigid shape and uniform size, comparable to that of the wild type (G), and the absence of X-Gal penetration (H) confirmed the presence of sufficient neonatal barrier function in Inv-Cldn6-CΔ206 mice.

19F, a GAPDH control is shown). Despite the higher TEWL in transgenic epidermis, the presence of substantial neonatal barrier function was confirmed not only by mouse viability but also by analysis of cornified envelope extracts from Inv-Cldn6-CΔ206 skin, which appeared relatively normal with a uniform size and rigid shape (Fig. 19G), and the absence of X-Gal (5- bromo-4-chloro-3-indolyl-β-D-galactopyranoside) penetration (Fig. 19H).

Barrier Formation and Epidermal Differentiation is Delayed in the Inv-Cldn6-CΔ206 Transgenic Epidermis During Development

Although non-lethal, the significantly higher TEWL observed in neonatal Inv-Cldn6-CΔ206 transgenic mice suggested aberrations in the epidermal differentiation program during development. X-Gal penetration assays confirmed that, at E15.5 (15.5 days post coitum), Inv-Cldn6-CΔ206 transgenic embryos were delayed in barrier formation as compared with their wild-type counterparts (Fig. 20A), a delay that was sustained at E17.5, when EPB function is normally achieved (Fig. 20B)(Hardman et al., 1998;Troy and Turksen, 2007) and at E18.5 (Fig. 20C, marked with arrows).

To complement these studies, we evaluated transverse histological sections of E15.5 and E17.5 embryo torsos in which the epidermal initiation sites, as well as the dorsal and ventral midlines, were included; because the data were consistent, we report results from E15.5 Inv-Cldn6-CΔ206 transgenic and wild-type mice only. At each site, a somewhat delayed/immature epidermal differentiation program was evident in the transgenic epidermis. For instance, in contrast to the well-established 3-4-cell layered stratum intermedium of the wild-type epidermis (Fig. 21A), the initiation site of the Inv-

Cldn6-CΔ206 transgenic epidermis possessed only 2-3 intermediate cell layers. Although the dorsal midline (Fig. 21B) of the developing wild-type epidermis was characterized by a 2–3-cell layer intermediate zone, that of the transgenic epidermis was thinner with only 1-2 layers of intermediate cells evident between the basal and periderm layers. Furthermore, the ventral midline (Fig. 21C) of the wild-type epidermis was characterized by two layers of intermediate cells, whereas that of the transgenic mice was thinner with only one defined intermediate cell layer together with some nascent infiltrating intermediate cells.

Consistent with the histological results, abnormalities in the expression of epidermal differentiation markers and Cldns were present in the developing Inv-Cldn6-C**Δ**206 embryo (Fig. 22, E15.5 epidermal initiation sites are shown). Although there was no apparent difference in the transgenic and wild-type epidermal basal compartments, as shown by the localization of K14 (Fig. 22A), K5 and K15 (not shown), K1 (Fig. 22B), which is associated with stratifying suprabasal cells (Fuchs, 1994) occupied a moderately thinner compartment in the transgenic epidermis corresponding with the thinner intermediate layer that was observed histologically. Cldn1 (Fig. 22C), Cldn6 (Fig. 22D), Cldn11 (Fig. 22E), Cldn12 (Fig. 22F) and Cldn18 (Fig. 22G), which at this developmental time point are all strictly localized to the epidermal suprabasal compartment (Troy et al., 2007) also occupied a comparatively thinner zone of expression in the developing Inv-Cldn6-C**Δ**206 transgenic epidermis. Similarly, the expression compartment of various structural proteins in the stratum corneum, including involucrin (Fig. 22H), filaggrin (Fig. 22I) and loricrin (Fig. 22J) was reduced in the E15.5 Inv-Cldn6-C**Δ**206 transgenic epidermis.

The Epidermis Matures Postnatally in the Inv-Cldn6-CΔ206 Transgenic Mice

In parallel with the delayed program of epidermal differentiation and EPB formation observed in the developing Inv-Cldn6-C**Δ**206 transgenic epidermis, postnatal TEWL measurements suggested that the Inv-Cldn6-C**Δ**206 transgenic epidermis underwent a robust epidermal maturation process after birth. Histological analyses of newborn (Fig. 23A), 2-day-old and 4-day-old skin samples (not shown) revealed that the Inv-Cldn6-C**Δ2**06 transgenic epidermis was comparable to the wild type. However, although by 1 week of age the wild-type epidermis had commenced the normal thinning pattern associated with epidermal maturation, the transgenic epidermis maintained an immature phenotype with many suprabasal cell layers, the prevalent appearance of nuclei in the upper differentiation layers, and a much less compacted granular layer (Fig. 23B). However, by 10 days after birth (Fig. 23C), the Inv-Cldn6-C**Δ**206 transgenic epidermis had thinned to be morphologically comparable to the wild-type epidermis. Samples from 1-month-old (Fig. 23D) and 3-month-old (not shown) transgenic mice were indistinguishable from wild-type samples. The histological results suggested that changes occurred in the transgenic epidermis reflecting approximately a 3-day lag in the normal epidermal maturation process, a possibility supported by the expression of epidermal differentiation markers and Cldns. Because results were similar from newborn to 7 days of age, normalized after 10 days, and were maintained throughout life, we report results

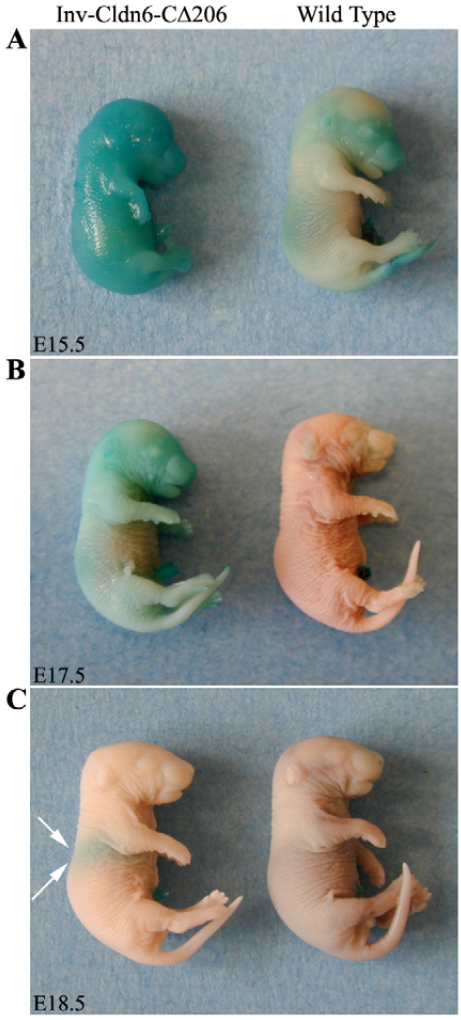

Figure 20. X-Gal Assays Demonstrate a Developmental Delay in EPB Formation for Inv-Cldn6-CΔ206 transgenic mice. (A-C) Using age-matched wild-type embryos as a control, X-gal penetration assays at E15.5 (A), E17.5 (B) and E18.5 (C) confirmed a developmental delay in EPB formation for Inv-Cldn6-CΔ206 transgenic mice. Even at E18.5 there was some evidence that barrier formation had not yet been completed in Inv-Cldn6-CΔ206 transgenic embyos (indicated with arrows).

only from 7- and 10-day-old Inv- Cldn6-CΔ206 transgenic mice compared with 7-day-old wild-type mice. K5 and K15 were restricted to basal cells at all time points in both the wild- type and Inv-Cldn6-Δ206 epidermis (not shown), but K14 occupied an expanded zone extending into the suprabasal compartment until epidermal maturation was achieved in the 10-day-old Inv-Cldn6-CΔ206 transgenic epidermis (Fig. 24A). K6 and K17, which are keratins associated with a hyperproliferative epidermis (Leigh et al., 1995;McGowan and Coulombe, 1998;McGowan and Coulombe, 1998) were not expressed throughout the time analyzed (not shown); however, a broadened K1 (Fig. 24B) expression compartment was seen in the transgenic epidermis early after birth, but normalized by 10 days of age. Similarly, the expression compartments for involucrin (Fig. 24C), filaggrin (Fig.24D) and loricrin (Fig. 24E) were also expanded, with an obvious packing defect reminiscent of the observed histological abnormalities of the stratum corneum, in the immature Inv-Cldn6-CΔ206 transgenic epidermis until thinning that was comparable to the wild-type epidermis was achieved.

In parallel with changes in epidermal markers, the suprabasal-specific Cldns, Cldn6 (Fig. 25A), Cldn11 (Fig. 25B), Cldn12 (Fig. 25C) and Cldn18 (Fig. 25D) were observed in the expanded suprabasal region of the immature Inv-Cldn6-CΔ206 transgenic epidermis, and immunostaining demonstrated a loss in membranous localization. Each of these Cldns was normalized, with a strictly membranous localization, upon epidermal maturation to a phenotype that was comparable to the wild type. Cldns not normally expressed in the epidermis (e.g. Cldn2, Cldn3 and Cldn5) (Turksen and Troy, 2002) were not observed in either the wild-type or Inv-Cldn6-CΔ206 transgenic epidermis at any time evaluated (not shown).

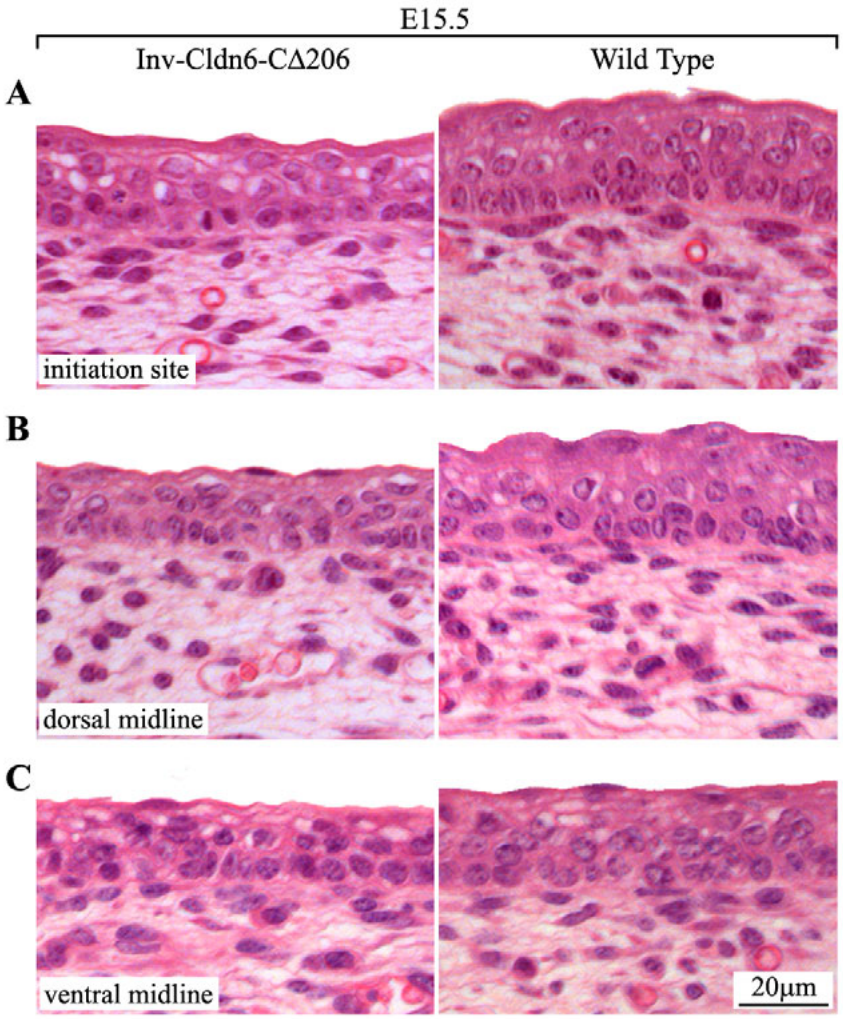

Figure 21. Histological evidence confirms EPB formation delays in transgenic mice. Evaluation of transverse histological sections at E15.5 confirmed a somewhat delayed epidermal differentiation in Inv-Cldn6-C**Δ**206 transgenic mice during development. (A) Transgenic epidermal initiation sites were characterized by 2-3 intermediate cell layers in contrast to the more mature 3–4-cell layered stratum intermedium of the wild type. (B,C) There were only 1-2 layers of intermediate cells at the dorsal midline (B) of the Inv-Cldn6-C**Δ**206 epidermis, whereas that of the wild type possessed 2-3 cell layers, and the transgenic ventral midline (C) was thinner than that of the wild type, with only one defined intermediate cell layer together with some infiltrating intermediate cells. Bar, 20**μ**m.

We also compared the expression of markers previously reported to be modulated during the epidermal maturation process in the newborn, 4-, 7- and 10-day-old transgenic mice compared with the wild-type epidermis. Changes in the overall surface pH of the epidermis contribute to the activation of enzymes involved in lipid processing for EPB function, and the plasma membrane Na+/H+ exchanger 1 (NHE1) has been implicated in this process (Behne et al., 2003;Fluhr et al., 2004;Fluhr et al., 2004;Hachem et al., 2005). Consistent with earlier reports (Behne et al., 2003), we found that NHE1 was localized in a 'punctate' pattern at the cell membrane of the basal and suprabasal compartments of the newborn wild-type epidermis, and as epidermal maturation progressed, NHE1 was gradually downregulated with localization restricted to the basal compartment by 7 days after birth (Fig. 26A-D, right column). By contrast, NHE1 was persistently upregulated and associated with both the basal and suprabasal cell compartments of the Inv-Cldn6-C**Δ**206 transgenic epidermis from birth to 7 days of age; downregulation and strict basal cell association was not observed until postnatal day 10 (Fig. 26A-D, left column). Immunoblot (Fig. 26E) and RT-PCR (Fig. 26F) analyses supported these findings. Similarly, aquaporins (AQPs), which are small integral membrane proteins that selectively transport water across cell membranes, and key lipid processing enzymes [**β**-glucocerebrosidase (**β**-celerase) and acid sphingomyelinase (**α**-SMase)] that are important in EPB homeostasis (Behne et al., 2003;Hara-Chikuma and Verkman, 2008) were also modulated in the Inv-Cldn6-C**Δ**206 transgenic epidermis versus the wild-type epidermis, as shown by immunoblot (Fig. 26E) and RT-PCR (Fig. 26F) analyses.

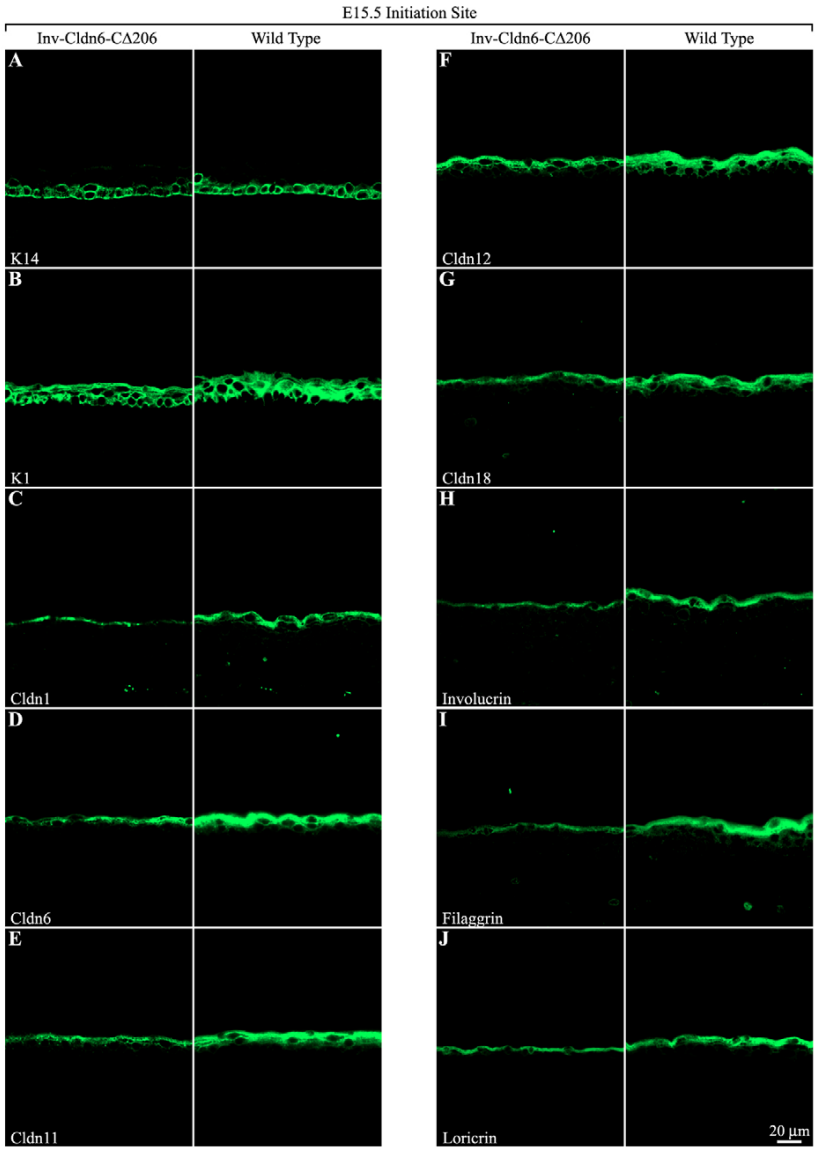

Figure 22. The localization of epidermal differentiation markers and Cldns is altered in the delayed EPB of the Inv-Cldn6-C**Δ**206 transgenic epidermis. Immunofluorescence was used to evaluate various markers of epidermal differentiation and Cldns during development in Inv-Cldn6-C**Δ**206 epidermal initiation sites compared with wild type sites (E15.5 is shown). Although there was no difference in K14 (A) expression (e.g. the basal compartment), markers of the epidermal suprabasal zone occupied a moderately thinner expression compartment in the developing Inv-Cldn6-C**Δ**206 transgenic epidermis as shown by the expression of K1 (B), Cldn1 (C), Cldn6 (D), Cldn11 (E), Cldn12 (F) and Cldn18 (G). In addition, involucrin (H), filaggrin (I) and loricrin (J) expression compartments were also reduced in the developing Inv-Cldn6-C**Δ**206 transgenic epidermis. Bar, 20**μ**m.

Changes in Cldn1, phosphoCldn1 and Erk1/2 Expression Profiles Delineate Repair and Maturation Processes in the Inv-Cldn6-CΔ206 Transgenic Epidermis

The relatively rapid postnatal normalization of TEWL in Inv-Cldn6-CΔ206 transgenic mice, together with the morphological and marker expression changes observed, suggested a robust maturational or repair process. Previous studies, including our own (see above), have demonstrated the importance of Cldn1 in epidermal morphogenesis, differentiation, and EPB formation and repair. In particular, the expression of Cldn1 is unique compared with other Cldns and undergoes a maturation switch, from a strictly suprabasal association to being localized to cell-cell borders in all the living layers of the epidermis, coinciding with the acquisition of barrier function during epidermogenesis (Troy et al., 2007). This normal expression profile is maintained throughout life except in response to acute injury and in tumorigenesis, where basal layer association is lost, and Cldn1-null epithelial cells are progressively more frequent in the lower suprabasal compartment (Arabzadeh et al., 2007;Arabzadeh et al., 2008). In comparison to the wild-type basal to suprabasal localization of Cldn1 (Fig. 27A-D, right column), Cldn1-null epidermal cells were observed in the basal and lower suprabasal layers of the Inv-Cldn6-CΔ206 transgenic epidermis from 4-7 days after birth; as anticipated based on normalization of TEWL and morphology, Cldn1 localization was normalized in samples from 10-day-old mice (Fig. 27A-D, left column).

Although no differences were detected in mRNA levels (Fig. 27F), immunoblotting confirmed decreased Cldn1 protein levels (Fig. 27E) in samples of 2-day-old Inv-Cldn6-CΔ206 transgenic epidermis. Given the evidence suggesting that the phosphorylation of different Cldns is involved in either the strengthening or weakening

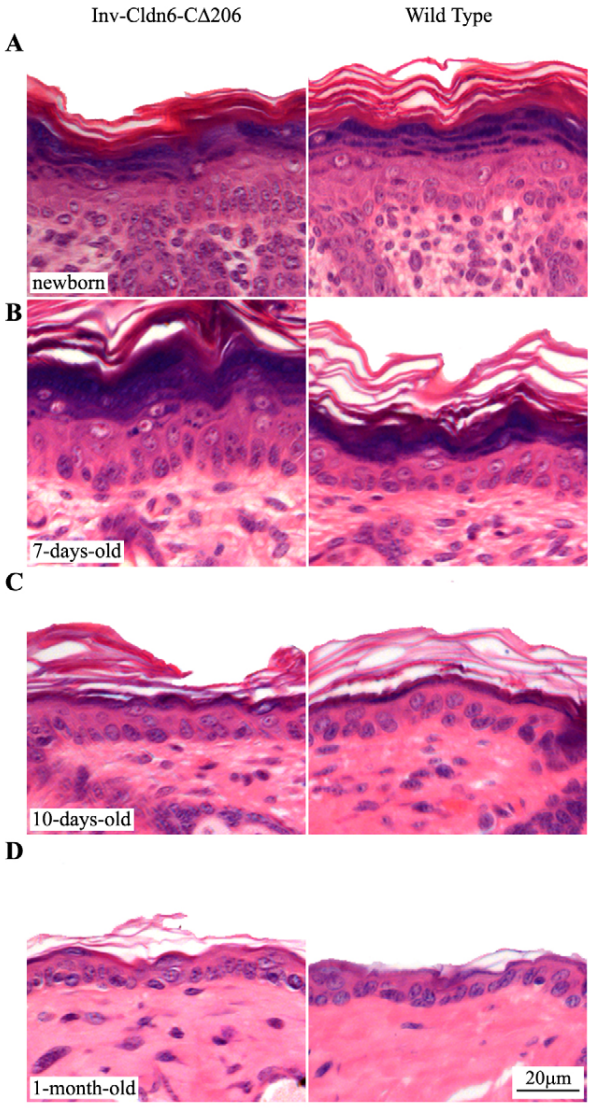

Figure 23. Histological Abnormalities in the Inv-Cldn6-CΔ206 Transgenic Epidermis after Birth. (A) Histological analyses of skin samples revealed that, at newborn stages, the Inv-Cldn6-CΔ206 epidermis was comparable to the wild type. (B) However, it was evident that, after 7 days, the transgenic epidermis did not undergo the normal process of maturation/thinning that was observed in the wild-type epidermis and was characterized by an increased number of suprabasal cell layers, the prevalent appearance of nuclei in the upper differentiation layers, and a much less compacted granular layer. However, representing a 3-day lag behind the wild type, the transgenic epidermis thinned to be morphologically comparable to the wild type by 10 days after birth (C), a phenotype that was maintained after 1 month (D) and throughout life. Bar, 20μm.

of TJs and in parallel barrier function (D'Souza et al., 2007;Findley and Koval, 2009;Ikari et al., 2008) we next asked whether expression of the phosphorylated form of Cldn1 (phosphoCldn1) was altered in transgenic versus wild-type epidermis. PhosphoCldn1 was not observed in the wild-type epidermis at any of the time points assayed (data not shown). However, phosphoCldn1 was localized to cell-cell borders in the upper suprabasal zone of the newborn Inv-Cldn6-CΔ206 transgenic epidermis until epidermal repair was achieved at postnatal day 10 (Fig. 28A-D). Concomitantly, expression of Erk1/2 followed the same distribution pattern of phosphoCldn1 (Fig. 28E-H) with a considerable amount of Erk1/2 localized to the cell membrane of differentiating cells in the newborn, 4-, 7- and 10-day-old Inv-Cldn6- CΔ206 transgenic epidermis.

DISCUSSION

In this study, we describe the generation of a novel animal model for evaluating developmental delays in EPB formation and the postnatal epidermal maturation processes that are analogous to those observed in the dysfunctional barrier phenotype of human premature babies. Using skin penetration assays and immunohistochemistry to evaluate the expression and localization of classical markers of epidermal differentiation and maturation, as well as the Cldns and signaling molecules that are involved in EPB formation, we demonstrated that Inv-Cldn6-CΔ206 transgenic mice suffered a developmental delay in epidermal differentiation and EPB formation leading to significant TEWL at birth, despite sufficient neonatal barrier formation (the presence of a

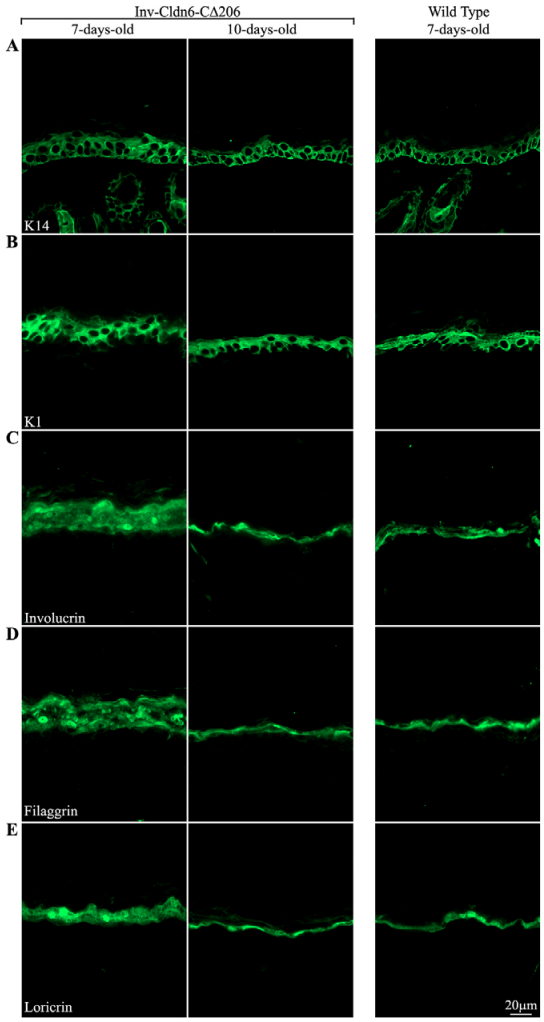

Figure 24: Perturbation of markers of epidermal differentiation are normalized by post natal day 10 in the Inv-Cldn6-C**Δ**206 transgenic epidermis. Changes in the epidermal differentiation program of the Inv-Cldn6-C**Δ**206 transgenic epidermis were evaluated by immunofluorescence and compared with their age-matched wild-type counterparts (samples from 7 and 10-day-old transgenic mice are shown and compared with the mature wild-type epidermis at 7 days). (A) In the transgenic epidermis, K14 occupied an expanded zone extending into the suprabasal compartment until epidermal maturation was achieved. (B) K1 was first observed in an expanded zone of expression, but normalized by 10 days of age in the Inv-Cldn6-C**Δ**206 transgenic epidermis. (C-E) Similarly, broadened expression compartments and obvious packing defects for involucrin (C), filaggrin (D) and loricrin (E) characterized the immature Inv-Cldn6-C**Δ**206 transgenic epidermis until thinning comparable to the wild type was achieved. Bar, 20**μ**m.

cornified envelope) and function (the absence of X-Gal penetration) for survival. Postnatal TEWL measurements, along with changes observed in the expression and localization of keratins and Cldns, suggested that the Inv-Cldn6-CΔ206 transgenic epidermis underwent a robust epidermal maturation process after birth to become indistinguishable from the wild type.

Although the molecular mechanisms underlying the delayed maturation and repair of the epidermis and EPB in these transgenic mice have not yet been delineated, our data indicate that the Inv-Cldn6-CΔ206 mice constitute an attractive model from a therapeutics point of view, i.e. for the identification of lead compounds for accelerated repair of the often life-threatening permeability barrier defects in premature human infants. Many studies have described the developmental formation of the EPB (Byrne and Hardman, 2005;Hardman et al., 1999;Turksen and Troy, 2002) and it is well recognized that a disruption or delay in its formation before birth may have severe consequences to the survival of the organism (Cartlidge, 2000;Elias, 2005;Mack et al., 2005;Williams and Feingold, 1998). We described previously that perturbations of Cldn6 expression levels in the suprabasal compartment of the epidermis – its endogenous site – result in epidermal differentiation abnormalities and EPB dysfunction (Troy et al., 2005;Turksen and Troy, 2002). However, depending on the level of expression, and whether normal or mutant forms of Cldn6 are expressed, the severity of the phenotype varies (Arabzadeh et al., 2006;Troy et al., 2005;Troy and Turksen, 2007;Turksen and Troy, 2002). For example, severe EPB dysfunction manifested in extreme TEWL and neonatal lethality occurs when native Cldn6 is expressed at high levels (Turksen and Troy, 2002) whereas lower levels of expression result in less severe EPB dysfunction and postnatal

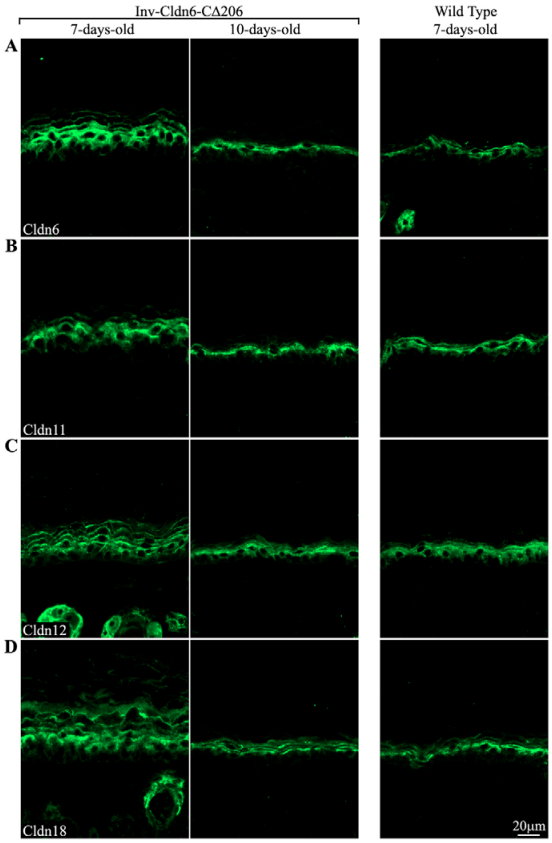

Figure 25. Evaluation of Cldns in the delayed EPB of Inv-Cldn6-C**Δ**206 transgenic mice. The expression / localization of Cldns was also perturbed in the immature Inv-Cldn6-C**Δ**206 epidermis (7- and 10-day-old transgenic samples are shown and compared with the mature wild type samples at 7 days). (A-D) Cldn6 (A), Cldn11 (B), Cldn12 (C) and Cldn18 (D) were observed in an expanded zone early on, demonstrating a loss in membranous localization, and were normalized by 10 days after birth. Bar, 20**μ**m.

normalization (Troy et al., 2005). Overexpression of a mutant form of Cldn6 lacking its entire tail domain (Inv-Cldn6-CΔ187 mice) does not appear to manifest in any prenatal epidermal developmental defects, but an abnormal, postnatal, lifelong epidermal hyperproliferation is observed (Arabzadeh et al., 2006). High overexpression of a different mutant lacking only the C-terminal half of the tail domain of Cldn6 (Inv-Cldn6-CΔ196 mice) (Troy and Turksen, 2007) results in a lethal barrier dysfunction with marked hyperproliferative squamous invaginations/cysts replacing hair follicles, while lower-level expression manifests in an aging-related skin barrier defect resulting in an intrinsic propensity for injury, inefficient repair and chronic dermatitis. We now show that transgenic mice expressing a mutant Cldn6 with a shorter tail deletion (removing the PDZ domain and a putative PKA phosphorylation site) possess a distinct developmental defect in epidermal differentiation resulting in EPB formation delays and that a robust repair response occurs for postnatal epidermal maturation. It is notable that formation of a skin barrier with functional TEWL characteristics that are indistinguishable from the wild type occurred more rapidly than, or prior to, complete morphological maturation of the epidermis in the postnatal Cldn6-CΔ206 mice, indicating an ability to disconnect aspects of the two processes, an observation that is interesting from a developmental standpoint but that may also be therapeutically important (see below). The mechanisms by which the expression of Cldn6-CΔ206 results in a developmental delay in EPB formation and postnatal epidermal maturation and repair are not yet known, but our observations support the need for Cldn homeostasis in a bona fide epidermal differentiation program and in epidermal repair. We have previously demonstrated that there is a defined Cldn expression profile in the epidermis and that changes in epidermal differentiation elicit

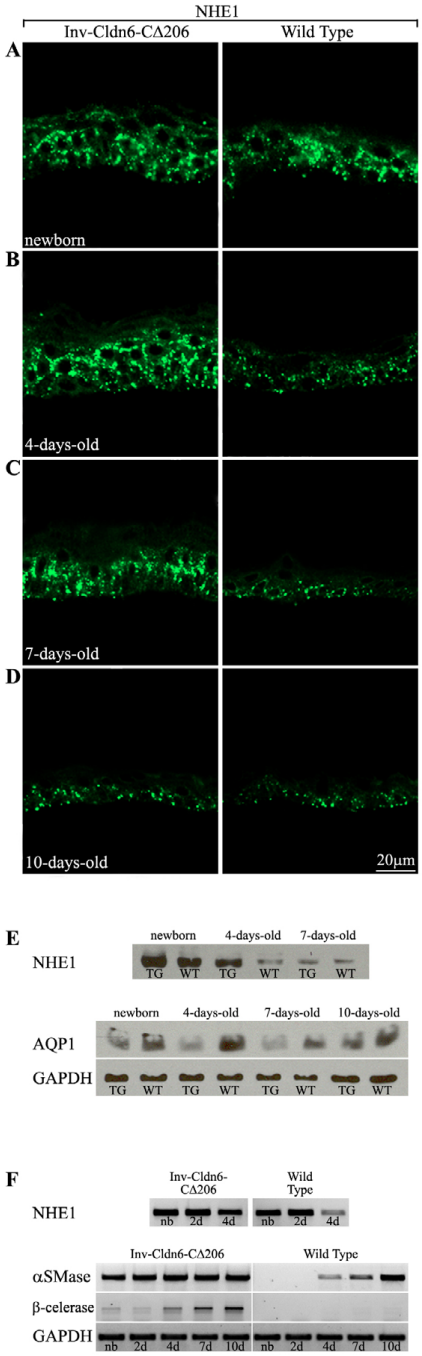

Figure 26. Modulation in NHE1 expression/ localization. (A-D) Localization of NHE1 was evaluated in skin samples from newborn (A), 4-day-old (B), 7-day- old (C) and 10-day-old (D) Inv-Cldn6-CΔ206 transgenic mice (left column) compared with wild-type mice (right column). In the Inv-Cldn6-CΔ206 transgenic epidermis, NHE1 was upregulated and associated with both the basal and suprabasal cell compartments from newborn to 7 days after birth; downregulation and strict basal cell association was not observed until postnatal day 10. Bar, 20μm. These data were supported by immunoblot (E) and RT-PCR (F) analyses. Modulations in mRNA or protein levels of AQP1, β-celerase and αSMase are also shown relative to GAPDH controls.

concomitant modifications in the Cldn expression profile and vice versa (Troy et al., 2005;Turksen and Troy, 2002;Turksen and Troy, 2004). Upon injury, or in response to differentiation abnormalities, the spatial expression of the suprabasal Cldns (Cldn6, Cldn11, Cldn12 and Cldn18) generally expands or shrinks to encompass all the cells of the perturbed suprabasal zone, with some concomitant loss in cell membrane association (Arabzadeh et al., 2006;Arabzadeh et al., 2007;Troy et al., 2005;Turksen and Troy, 2002). This was also true in the immature Inv-Cldn6-C**Δ2**06 epidermis: as the epidermis matured, by postnatal day 10, the localization and expression of the Cldns normalized to a strictly membranous association in a suprabasal zone that was comparable in thickness to that of the wild type. However, Cldn1 undergoes more dramatic alterations in response to epidermal homeostasis dysregulation. In the developing epidermis, Cldn1 is first restricted to the stratifying layers and matures to occupy the basal layer upon the completion of barrier formation at E17.5 (Troy and Turksen, 2007). However, in response to TPA (12-O-tetradecanoyl-phorbol-13-acetate)-induced injury and the loss of cell polarity that is seen in tumorigenesis, Cldn1 expression is downregulated in both the basal layer and immediate suprabasal layers of the epidermis (Arabzadeh et al., 2007). These changes are also observed in the intrinsic aging process of the Inv-Cldn6-C**Δ**196 transgenic epidermis and in the delayed epidermal maturation that we now report in Inv-Cldn6-C**Δ**206 transgenic mice. Notably, Cldn1 expression normalized with the normalization of epidermal differentiation markers and epidermal maturation (see below). Although Cldns demonstrate amino acid similarity among family members, the cytoplasmic tails of Cldns are divergent in sequence and possess a number of sites that provide clues about their structure-function relationships in epidermal differentiation,

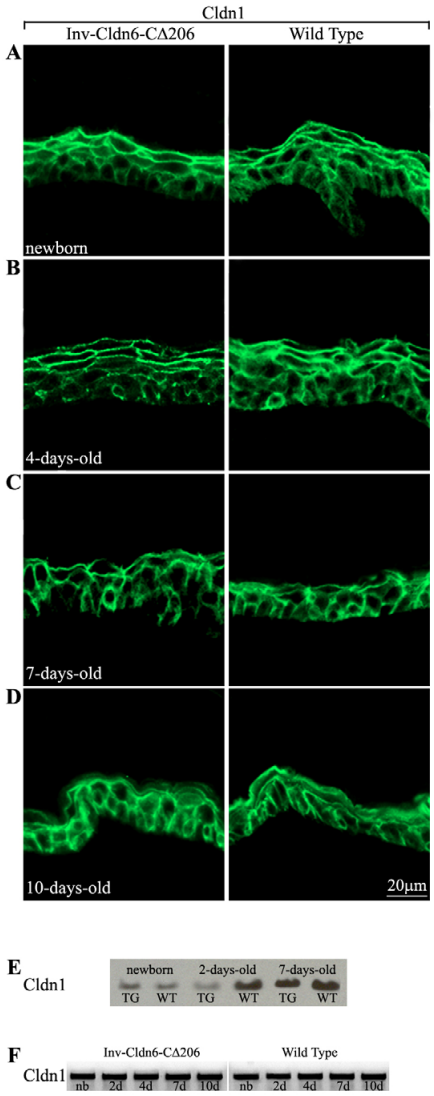

Figure 27. Changes in Cldn1 expression and localization in the Inv-Cldn6-C**Δ**206 transgenic epidermis. Unlike the normal wild type (right column) basal to suprabasal localization of Cldn1, the Inv-Cldn6-C**Δ**206 transgenic epidermis (left column) showed abnormalities. (A-C) Although localization was normal at newborn stages (A), Cldn1-null epidermal cells were observed in the basal and lower suprabasal layers from 4 days (B) to 7 days (C) after birth, and were normalized by 10 days after birth (D). Bar, 20**μ**m. (E-F) Immunoblotting confirmed the changes in Cldn1 protein level (E); whereas RT-PCR indicated that there were no differences at the mRNA level (F).

including a PDZ binding sequence (YV) and potential phosphorylation sites (Fujibe et al., 2004;Simard et al., 2006). Proteins with a PDZ domain such as the membrane associated guanylate kinase (MAGUK) family proteins [zonula occludens (ZO) 1, ZO2 and ZO3] (Itoh et al., 1999), as well as the recently identified multi-PDZ domain scaffolding proteins PATJ (protein associated to tight junctions) (Lemmers et al., 2002) and multi-PDZ domain protein 1 (MUPP-1) (Hamazaki, 2001), selectively recognize and bind to this sequence (Gonzalez-Mariscal et al., 2003). However, it seems likely that as yet unidentified novel molecules interact with Cldns in regulating gene expression and epidermal differentiation. Post-translational modifications within the tail domain, including phosphorylation and palmitoylation, are also thought to regulate Cldn activities, including their targeting to the membrane and their insertion into TJs to regulate paracellular permeability (Simard et al., 2006). Phosphorylation of a number of Cldns has been demonstrated to be required for their assembly into TJs [e.g. for Cldn1 and Cldn4 (Banan et al., 2005) and for Cldn16 (Ikari et al., 2008)] but, to date, most Cldns have not been subjected to exhaustive analysis. Our observation of the association of phosphoCldn1 with the differentiated layers of the immature Inv-Cldn6-C**Δ**206 transgenic epidermis points towards the potential role of Cldn1 phosphorylation in the process of epidermal maturation and EPB repair.

Although the precise sequence of events in the maturation of the skin barrier is not well understood, the notion that exposure to air after birth functions to initiate and accelerate the maturation and repair of the skin barrier has been suggested (Hanley et al., 1999;Williams and Feingold, 1998). The fetal and neonatal anomalies with spontaneous and apparently complete epidermal maturation and barrier repair that we observe in

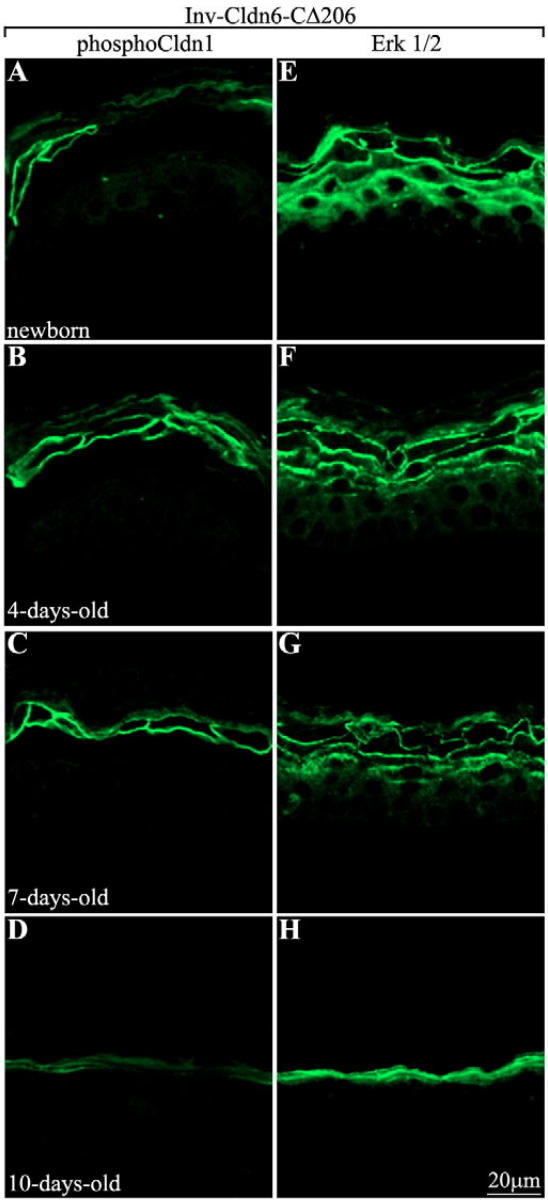

Figure 28. The localization of phosphoCldn1 and Erk1/2 in the epidermis of Inv-Cldn6-CΔ206 transgenic mice. (A-H) Skin samples from newborn (A,E), 4- day-old (B,F), 7-day-old (C,G) and 10 day-old (D,H) Inv-Cldn6-CΔ206 transgenic mice were used to evaluate the localization of phosphoCldn1 (left column) and Erk1/2 (right column). From newborn to 7 days of age, phosphoCldn1 was localized to cell-cell borders in the upper suprabasal zone of the Inv-Cldn6- CΔ206 transgenic epidermis. Correspondingly, Erk1/2 localization followed the same pattern with a considerable distribution at the cell membrane of differentiating cells. Bar 20μm.

postnatal Inv-Cldn6-CΔ206 transgenic mice support the view that exposure to air induces an intrinsic repair and maturation pathway. Other support for this hypothesis comes from a number of studies; for example, exposing embryonic immature epidermis to air (Williams and Feingold, 1998) lifting skin-equivalent cultures to the air-liquid interface (Fartasch and Ponec, 1994;Komuves et al., 1999) and inducing injury to the EPB by tape stripping (Ahn et al., 2001;Ahn et al., 1999) each result in a robust EPB repair and epidermal maturation response. Although collectively these studies do not provide a mechanism for the observed intrinsic repair/maturation process in the epidermis, our studies suggest that the first phase of repair is the shedding of defective differentiation layers, which we identified as phospoCldn1-expressing cells with co-expression of high levels of Erk1/2. Considering the important role of Erk1/2 in epithelial differentiation (Hobbs et al., 2004;Taupin and Podolsky, 1999;Yu et al., 2007) as well as in the regulation of Cldn expression (Lipschutz et al., 2005) our data support a role for Erk1/2 in the initiation or progression of the epidermal repair and maturation that was observed in Inv-Cldn6-CΔ206 transgenic mice. Concomitantly, initiation probably also involves the well-known phenomena of cross-talk between the differentiating and the basal compartments of the epidermis, which has been demonstrated to tightly regulate the epidermal differentiation program and is responsible for the normal maintenance program of the epidermis (Prowse et al., 1999;Troy et al., 2005) evidence for this comes from our reported observations regarding the remodeling of Cldn1 expression in the repairing Inv-Cldn6-CΔ206 transgenic epidermis. Further analyses are required to understand how 'air exposure' activates the Erk1/2 pathway and the observed Cldn1 phosphorylation process.

Although a variety of maternal and fetal diseases and conditions can lead to

premature birth, the reasons underlying the quite dramatic increases in premature birth, in not only underdeveloped but also developed countries, over the last decade are unknown (Darmstadt et al., 2008;Lange et al., 2009;Yeaney et al., 2009). Complications owing to compromised skin barrier function (e.g. poor temperature regulation and dehydration) in premature babies are among the primary causes of neonatal sepsis and mortality (Rutter, 1996;Saiman, 2006). Given the importance of skin barrier function in the health of premature babies and the fact that intrinsic risk factors of sepsis include compromised portals of entry for pathogens (Saiman, 2006) there is a surprisingly significant lack of successful approaches/strategies that have been designed specifically to accelerate the postnatal maturation of the epidermis (McIntire and Leveno, 2008;Shapiro-Mendoza et al., 2006;Shapiro-Mendoza et al., 2008;Tyson et al., 2008). Strategies for reducing the rate of sepsis are currently focused on limiting the spread of infection through aseptic clinical techniques and the use of antibiotics, which are increasingly becoming ineffective (Saiman, 2006). In addition, the topical application of relatively low-cost emollients, especially in underdeveloped countries, has proven to have some benefit, although whether this is from provision of a mechanical barrier or from induction of biological responses to some of the ingredients, or both, is not clear (Darmstadt et al., 2008). Understanding these mechanisms, as well as screening for potential novel therapeutics to accelerate postnatal epidermal maturation, have been hampered by the lack of suitable in vivo models. The capacity of the Inv-Cldn6-C**Δ**206 transgenic epidermis to undergo a postnatal repair response and acquire a mature epidermis by 10 days after birth makes this transgenic model an excellent tool for investigating not only the molecular changes taking place during this maturation period, but also for screening for novel therapeutics to

accelerate this process and improve the health of human premature infants.

METHODS

Generation of Inv-Cldn6-CΔ206 Transgenic Mice

Inv-Cldn6-CΔ206 mice were generated by truncating the cytoplasmic tail domain of Cldn6 after amino acid 206 (Cldn6-CΔ206) and then subcloning Cldn6-CΔ206 into the NotI site of the Inv cassette (H3700-pL2) by our previously utilized strategy (Arabzadeh et al., 2006;Troy and Turksen, 2002;Troy and Turksen, 2007). Purified DNA was injected into ova collected from superovulated CD1 mice at the Transgenic Mouse Facility of the Ottawa Hospital Research Institute (OHRI), as described previously (Arabzadeh et al., 2006;Troy and Turksen, 2002;Troy et al., 2007). The presence of the transgene was confirmed by PCR using genomic DNA and specific primers. Photography of age-matched wild-type and Inv-Cldn6-CΔ206 transgenic mice was performed using a Nikon Coolpix 950 digital camera (Nikon) and image processing was performed with Adobe Photoshop version 7.0 (Adobe Systems). All research was carried out in accordance with the principles and guidelines of the Canadian Council on Animal Care, and the policies of the OHRI Animal Care Committee.

RNA Isolation and RT-PCR

Skin samples dissected from the mid-dorsal region of transgenic and wild-type mice were frozen in liquid nitrogen, and then homogenized in Trizol (Invitrogen) reagent for total RNA isolation according to the instructions of the manufacturer. After DNase (Invitrogen) treatment, first-strand cDNA was synthesized using random hexamers

(Applied Biosystems) and 1 **μ**g of RNA. PCR analysis was then performed, as described previously (Troy et al., 2005a) using the following specific primers: Inv exon-Cldn6 [~350 bp; FP: 5'-CTGCCTCAGCCTTACTGTGAG-3' (KT323), RP: 5'-CCAACAGTGAGTCATACAC-3' (KT1526)], GAPDH [167 bp; FP: 5'-CAGTATGACTCCACTCACGG-3' (KT841), RP: 5' GTGAAGACACCAGTAGACTCC-3' (KT842)], NHE1 [498 bp; FP: 5'-GAGATCCACACACAGTTC-3' (KT1515), RP: 5'- TACTGTCAGGTAGTTGGTG-3' (KT1516)], **α**-SMase [567 bp; FP: 5'-AGACTGGAGAGGTCCTTA-3' (KT1537), RP: 5'-GTCCCAGTGTAGATCAGTAA-3' (KT1538)], **β**-celerase [512 bp; FP: 5'-TACTTTAGGAGAGACACACC-3'(KT1539),RP:5'- GGTAAGTGTGAATGGAGTAG -3' (KT1540)] and Cldn1 [644 bp; FP: 5'-AAAGAGCCATGGCCAACGC -3' (KT1315), RP: 5'- TCACACATAGTCTTTCCCACTAG -3' (KT1316)]. RT-PCR products, relative to a housekeeping control (GAPDH), were separated on ethidium bromide-containing agarose gels, visualized by ultraviolet light, and images were acquired using AlphaEaseFC software version 4.0 (Alpha Innotech Corporation).

Skin Permeability Assays

X-gal Penetration Assay

Freshly dissected Inv-Cldn6-C**Δ**206 transgenic embryos (E15.5, E17.5 and E18.5; the embryonic age was estimated based on the appearance of the vaginal plug at E0.5) and euthanized neonates, along with their age-matched wild-type counterparts, were rinsed in PBS and immersed in X-Gal reaction mix at pH 4.5 [100 mM NaPO4, 1.3 mM MgCl2, 3mM K3Fe(CN)6, 3 mM K4Fe(CN)6 and 1 mg/ml X-Gal], as described

previously (Hardman et al., 1998;Turksen and Troy, 2002;Turksen and Troy, 2003). Following an overnight room temperature incubation, specimens were fixed with formalin and images were acquired using a Nikon Coolpix 950 digital camera followed by processing with Adobe Photoshop version 7.0.

Trans-epidermal Water Loss (TEWL) Measurements

A dermal phase meter (DPM) (Nova Technology Corporation) was used to measure the dorsal and ventral impedance/TEWL of Inv-Cldn6-CΔ206 transgenic and wild-type mice, as described previously (Troy et al., 2005;Turksen and Troy, 2002) at various postnatal time points from birth to the emergence of coat hairs, at which time measurements are no longer feasible owing to improper contact between the meter probe and the skin. Higher DPM values measured over time translate to reduced barrier integrity, illustrated digitally by EDWINA (Nova Technology Corporation) and Excel (Microsoft) software programs.

Cornified Envelope Extracts

Purified cornified envelope extracts were prepared from CD1 and Inv-Cldn6-CΔ206 transgenic mice by immersion of dorsal skin samples into hot extraction buffer (0.1M Tris-HCl, pH 8.5, 2% SDS, 20 mM dithiothreitol, 5 mM EDTA), followed by a 15- minute incubation at 95°C and gentle centrifugation, as described previously(Hohl et al., 1991;Troy et al., 2005;Turksen and Troy, 2002).

Sample collection, Histology and Immunolocalization

Sample Preparation

Freshly dissected skin samples (~1 cm) and whole embryos were collected from wildtype and Inv-Cldn6-CΔ206 transgenic mice at various embryonic and postnatal time points (E15.5, E17.5, E18.5, newborn, and 2 days, 4 days, 6 days, 7 days, 8 days, 10 days, 12 days, 2 weeks, 3 weeks, 1 month and 3 months of age).

Paraffin Sections and Histology

Following an overnight fixation in Bouin's solution (75% saturated picric acid, 20% formaldehyde, 5% glacial acetic acid), skin samples were dehydrated by a graded series of ethanol washes (from 30% to 100%) and embedded in paraffin. Sections (5 μm) were mounted onto Superfrost/Plus slides (Fisher Scientific), and were dewaxed using toluene and rehydrated in a reverse series of ethanol washes to water. Following antigen unmasking and washes in PBS, sections were either stained with hematoxylin and eosin (H&E), as described previously (Troy et al., 2005) or used for immunohistochemistry (see below).

Immunohistochemistry

Non-specific antibody binding (10% goat serum, 0.8% BSA, 1% gelatin in PBS) was blocked, followed by incubation for 1 hour in antibodies that were diluted appropriately with incubation buffer (1% goat serum, 0.8% BSA, 1% gelatin in PBS) (Troy and Turksen, 2005). Antibodies against the following antigens were evaluated: K15 (1:100; rabbit #UC55), K5 (1:100; rabbit #5054), K14 (1:100; rabbit #199), K1

(1:100; rabbit #UC81), K6 (1:200; BabCO), K17 (1:500; a gift from Dr Pierre Coulombe, Johns Hopkins University School of Medicine, Baltimore, MD), involucrin (1:100; BabCO), filaggrin (1:100; BabCO), loricrin (1:100; rabbit #UC84), Cldn1 (6:100; Zymed Laboratories), Cldn2 (1:200; Zymed Laboratories), Cldn3 (1:50; Zymed Laboratories), Cldn5 (1:100; Zymed Laboratories), Cldn6 (1:100; chicken #3677), Cldn11 (1:100; chicken #3680), Cldn12 (1:100; chicken #5186), Cldn18 (1:100; rabbit #A9953), NHE1 [3:100; Chemicon-AB3031 (incubation was overnight at 4°C)], phosphoCldn1 [1:100; a custom antibody generated from rabbit #2827 against the tail domain of the mouse phosphoCldn1 sequence (Ac-C-Ahx-PYPKP[pT]PSSGKDY-amide), 21st Century Biochemicals] and Erk1/2 (1:100; StressMarq Biosciences Inc.). After incubations in wash buffer (0.8% BSA, 1% gelatin in PBS), FITC-conjugated secondary antibodies against mouse, rabbit and chicken (1:50; Jackson ImmunoResearch) were diluted in incubation buffer and used for 1-hour incubations at room temperature. Following the final washes, skin samples were mounted with Mowiol 4-88 (Calbiochem), containing 2.5% 1,4 diazobicyclo-[2,2,2]-octane (DABCO; Sigma), for observation with a Zeiss Axioplan 2 fluorescence microscope equipped with an AxioCam camera and Axio Vision 2.05 software (Carl Zeiss). Digital photography was presented with Adobe Photoshop version 7.0.

Protein Isolation and Immunoblotting

Freshly dissected back skin samples (0.4 grams) were homogenized in SDS extraction buffer [62.5 mM Tris, pH 6.8, 25% glycerol, 2% SDS and 2% β−mercaptoethanol, with pepstatin A and a complete mini protease inhibitor cocktail

(Roche Diagnostics) tablet] followed by high-speed centrifugation. The supernatant containing the proteins was collected and assayed for protein concentration. Proteins were incubated at room temperature for 30 minutes in sample reducing buffer (62.5 mM Tris, pH 6.8, 6 M urea, 25% glycerol, 2% SDS, 0.1% Bromophenol Blue and 2% β-mercaptoethanol), boiled for 5 minutes (samples were not boiled for analysis of NHE1), and centrifuged at high speed for 10 minutes before 5-50 μg samples were separated on 7.5-12% SDS-PAGE gels, transferred to nitrocellulose and incubated in blocking buffer [5% skimmed milk in TBS/0.1% Tween-20 (TBS-T)] for 1 hour at room temperature. Appropriately diluted (5% skimmed milk in TBS-T) antibodies were used for overnight incubations at 4°C. The following antibodies were used: NHE1 (91 kDa, 1:1000; Chemicon), AQP1 (28 kDa, 1:1000; Alpha Diagnostic), Cldn1 (23 kDa, 1:1000; Zymed Laboratories) and GAPDH (36 kDa, 1:10,000; Abcam). After washing in TBS-T, blots were incubated for 1 hour at room temperature in HRP-conjugated secondary antibodies against rabbit or mouse (1:20,000; Amersham Biosciences), then diluted in 5% skimmed milk/TBS-T. Following washes in TBS-T, the Immobilon Western blotting detection system (Millipore) was used for detection, and expression levels were normalized to a housekeeping control (GAPDH) and visualized on Kodak BioMaxXAR film (Kodak). Films were digitally scanned and images were processed with Adobe Photoshop version 7.0.

TRANSLATIONAL IMPACT

Clinical Issue

An estimated 28% of the 4 million annual neonatal deaths are because of preterm

birth. It is likely that many of these deaths result from an immature permeability barrier, leading to dehydration and infection with associated septic shock. The statistics published by the March of Dimes indicate a 36% increase in premature births in America in the last 25 years, and the cause is not understood. This is an alarming, and emotionally and ethically complex, situation that adds significant pressure to pediatric medicine. The absence of appropriate disease models in which to delineate the basis of barrier dysfunction and test new therapies limits progress in the field.

Results

Here, the authors characterize a mouse model that mimics a debilitating, nonfatal form of a dysfunctional epidermal permeability barrier, similar to that seen in human premature babies. The tight junction-associated proteins, claudins, play a crucial role in establishing the epidermal permeability barrier. One theory is that claudin-containing tight junctions act as command centers in which the claudin extracellular loops influence selective permeability, while their cytoplasmic tail domains send signals to other receptors to coordinate downstream effectors, such as the cytoskeleton. The transgenic mouse reported here contains a short claudin-6 tail truncation. This mutation results in a delayed and defective epidermal permeability barrier that, surprisingly, is repaired within 2-4 days after birth. The repair process was associated with remodeling of the claudin-1 expression domains that eliminated the defective differentiated epidermal cells and replaced them with apparently 'normal' and terminally differentiated epidermal cells. The trigger for repair appears to be exposure to air. The potential signaling pathways involved include the sphingosine-1-phosphate (S1P) pathway and preliminary observations

implicate Erk1/2 downstream of the S1P-receptor 2. The relevance of this model for understanding human disease is confirmed by the authors' observations of similar features in specimens from premature babies, including dramatic delays in barrier formation and dysregulation of claudin expression, notably claudin-1.

Implications and Future Directions

Clinical outcomes for premature babies have been improved by using incubators that carefully control heat and humidity, but currently there are no therapeutic approaches for accelerating repair of the premature epidermal permeability barrier. The observation that activation of S1P and S1P-receptor 2 induce permeability repair processes suggests the therapeutic potential of selective receptor agonists. A few agonists have been described and this model is a viable model to test them. In summary, we think it is important to have made a model that mimics a repairable human epidermal permeability barrier, to have documented important cellular mechanisms of that repair, and to have begun delineation of the underlying molecular mechanisms, which are also pointing towards potential new therapies.

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CHAPTER 4 - GENERAL DISCUSSION I

One of the most fundamental roles of the skin is its barrier function. It is essential for the prevention of excessive loss of water and solutes and to protect the body from external assaults such as the invasion of pathogens, penetration of harmful substances and UV irradiation. In the findings presented in this thesis, we have shown that Cldn6-based TJs are important for this barrier function and that the Cldn6 tail plays an important in epidermal differentiation, maturation and repair.

Claudin 6 Regulates Epidermal Development Through its Cytoplasmic Tail

Cldn6, one of the earliest factors expressed as ESCs become committed to an epithelial cell fate, has been shown to affect epidermal differentiation and barrier function (Troy et al., 2005;Turksen and Troy, 2002). Since these reports, complementary *in vitro* studies have shown that the expression of Cldn6 in mouse ESCs induces epithelial differentiation (Sugimoto et al., 2013). Furthermore, Cldn6 induction led to the expression of other TJ molecules including Cldn7, occludin and ZO-1. Conversely, the expression of these proteins is lost upon attenuation of Cldn6 by siRNA knockdown.

From a mechanistic point of view, our studies support the hypothesis that Cldn6 mediates its function in epidermal homeostasis through its cytoplasmic tail. Transgenic mice that express a truncation of the entire Cldn6 tail show histological and biochemical defects in epidermal differentiation and epidermal hyperproliferation leading to a thickened epidermis, a phenotype that persisted throughout life. In addition, the protein-unfolding pathway was activated and a number of Cldns were improperly targeted to the membrane (Arabzadeh et al., 2006). We speculate that the truncation in this mouse model might compromise the function of a nearby conserved Cysteine residue (C184), which

has been shown to undergo a palmitoylation event that is implicated in TJ localization of other Cldns (Van Itallie et al., 2005).

Additional studies presented here detail the impact of more minimal Cldn6 tail truncations on the epidermis when expressed in the suprabasal layer. The first truncation mutants carrying deletions of the last twenty-three AAs mice resulted in dermatitis and defects in signaling (Troy et al., 2009). The second truncation mutant, carrying deletions of the last thirteen AAs including the PDZ-binding domain and a putative protein kinase A (PKA) phosphorylation site, caused dysfunction and delays in postnatal maturation of the epidermis. This repair was facilitated by the shedding of phosphoCldn1 in layers where extracellular signal-regulated kinase 1/2 (ERK1/2) activity is activated (Enikanolaiye et al., 2010).

Although, in both models, the mice were born with a barrier sufficient for survival, the process of epidermal maturation, evidenced by epidermal thinning, was delayed. The observation that epidermal maturation occurred in a shorter time in the more minimal truncation mouse model suggests that the last 13 AAs are harbor determinants critical for epidermal maturation. Some of the role for post-translation modification in Cldn-mediated TJ function have been shown (Findley and Koval, 2009). For example, phosphorylation at residue T202 is required for Cldn1 incorporation into TJs and to promote barrier function (French et al., 2009;Fujibe et al., 2004). Cldn7 has been shown to interact with the epithelial cell adhesion molecule (EpCAM) (Ladwein et al., 2005) and the EpCAM-Cldn7 complex is enriched at glycolipid-enriched membranes, a domain also enriched with phosphorylated Cldn7. Cldn7 has been shown to be phosphorylated at S204 and S206 within its cytoplasmic tail (Hoggard et al., 2013;Tatum et al., 2007) and

this tail is indispensible for interaction with EpCAM. This suggests that Cldn7 phosphorylation influences its ability to interact with EpCAM and this in turn affects other cellular processes such as proliferation and motility (Nubel et al., 2009). With respect to Cldn6, human embryonic stem cell (hESC) studies have mapped its phosphorylation at resides S201, T202 and S203, all of which are found within the last few AAs of the Cldn6 tail (Phanstiel et al., 2011;Rigbolt et al., 2011;Van Hoof et al., 2009). Furthermore, phosphorylation of Cldn6 is increased in the wake of the induction of differentiation (Rigbolt et al., 2011;Van Hoof et al., 2009). Therefore phosphorylation events within this region of the tail may affect TJ structure and Cldn-dependent signaling involved in proliferation and differentiation.

Although we speculate that phosphorylation of the Cldn tail may be important for its interactions with other proteins, it is possible that these effects may not be direct. Cldn4 has been shown to interact with both the Eph receptor kinase EphA2 (Tanaka et al., 2005a) and its partner Ephrin B1 (Tanaka et al., 2005b). EphA2 directly phosphorylates the cytoplasmic tail of Cldn4 at Y208 within the PDZ binding motif and this event attenuates the interaction between Cldn4 and ZO-1, leading to loss of Cldn4 at TJ and increased barrier leakiness (Tanaka et al., 2005a;Tanaka et al., 2005b). The interaction between Cldn4 and with EphA2 however, occurs at the Cldn4 extracellular domain independently from the tail. In this example, phosphorylation of the Cldn4 tail affects its interaction with ZO-1 in an indirect manner.

A role for Cldn-based TJs in regulating paracellular transport has been recognized for decades. However, there is an increasing body of evidence that shows that they are also important in mediating the transportation of signals from the membrane to the

cytoplasm and nucleus in order to regulate cellular proliferation and differentiation. One theory is that Cldn-based TJs function as command centers where the extracellular loops form trans- and cis-cellular homo- and heterophilic interactions to regulate paracellular permeability while the cytoplasmic tail sends signals to other parts of the cell. A specific example of this is the ability of the TJ molecule ZO1 to interact both with Cldns at the membrane and with the transcription factor ZONAB within the cytoplasm. ZONAB is necessary for normal proliferation and sequestration by ZO-1 inhibits its transcriptional activity (Balda and Matter, 2000;Balda et al., 2003). The accumulation of ZONAB within the cytoplasm (both by its overexpression or knockdown of ZO1), leads to increased proliferation (Georgiadis et al., 2010). Another example can be found in the EpCAM-Cldn7 and the EphA2-EphrinB1/Cldn4 interactions mentioned above. The EpCAM-Cldn7 complex was shown to promote tumorigenicity and tumor growth (Nubel et al., 2009) and EpCAM knockout mice show defects in intestinal barrier function, loss of expression of various Cldns as well as disarranged TJ fibrils (Lei et al., 2012). Consistent with this, EpCAM has been shown to be upregulated in cancer and implicated in stem cell signaling (Munz et al., 2009) and it is likely that its interaction with Cldn7 is implicated in these processes. The Eph receptors and their Ephrin ligands are involved in a number of cell signaling pathways that are active both in development and cancers reviewed in (Kandouz, 2012;Klein, 2012). The interaction of Ephrin/Eph with Cldns might explain for example, the role of Ephrin B in septation within the tracheoesophageal foregut of the developing mouse embryo (Dravis and Henkemeyer, 2011) or its role in the junction breakdown that precedes metastasis in tumors that overexpress Eph receptors. With respect to Cldn6 and its ability to interact with cytoplasmic or nuclear

proteins thereby promoting proliferation or differentiation, recent studies have confirmed the interaction between Cldn6 and ZO-1 in keratinocytes. In addition, a novel interaction between Cldn6 and the transcription factor FIZ1 (fms-like receptor tyrosine kinase 3-Interacting Zinc finger) was also identified (Lariviere et al., 2014). These studies show that, in keratinocytes, FIZ1 operates as a mitogenic factor that promotes proliferation. FIZ1 was initially identified as part of a protein complex that regulates photoreceptor-specific genes at the transcriptional level (Mali et al., 2007;Mali et al., 2008;Mitton et al., 2003). It is therefore conceivable that it could play similar regulatory roles in keratinocytes thereby affecting TJ formation. In addition, initial analysis of the epidermis of a FIZ1 knock out mouse model suggests that loss of FIZ1 affects epidermal differentiation and maturation (Figure 46-47), a phenotype that is repaired by 7 days of age (Figure 48). In addition, Cldn6 has also been shown to interact with the actin-associated protein, Filamin (Figure 49), providing the first direct link between a Cldn and the cytoskeleton.

Claudin 6 Truncation Mutants are Novel Mouse Models for Barrier Dysfunction

The necessity of transgenic and knockout mouse models with barrier defects for the advancement of our understanding of the regulatory pathways that underline barrier acquisition and maintenance cannot be overstated. Although there are a number of model systems that have been developed to study barrier function, very few of these are suitable for the study of barrier acquisition in early life of preterm and newborn infants and for aging in late life.

Although the skin barrier is fully formed by birth, premature infants have a poorly

developed barrier. The skin of premature babies undergoes postnatal barrier maturation within 2-4 weeks of birth; for neonates younger than 25 weeks, this may take up to 5-7 weeks (Kali et al., 2013). During this time, the high TEWL can lead to multiple complications such as neonatal sepsis and eventually mortality. Current therapies are limited to maintaining a humidified environment and the application of petrolatum-based topical emollients until a competent barrier is formed (Darmstadt et al., 2008;Telofski et al., 2012). More effective therapy would entail stimulation of the normal development of the barrier, either in utero or post-natally. Studies in the fetal rat where some steroids were able to stimulate while other able to inhibit, barrier formation suggest that such acceleration is possible (Hanley et al., 1999;Williams and Feingold, 1998). This however was not the case in humans where the administration of steroids had no impact on barrier formation (Jain et al., 2000). In fact, there is a lack of successful strategies for accelerating the postnatal maturation of the epidermis in humans (McIntire and Leveno, 2008;Shapiro-Mendoza et al., 2006;Shapiro-Mendoza et al., 2008). The study of the relevant mechanisms as well as screening for new therapeutics has been impeded by a lack of suitable experimental models. The dramatic increases in premature birth rates around the world necessitates the development of such models for a better understanding of epidermal barrier development and function, with the end goal of helping improve the current management of these fragile infants (Darmstadt et al., 2008;Lang and Iams, 2009;Yeaney et al., 2009).

The Inv-Cldn6-CΔ206 mouse model presented in this study mimics the delayed and reparable skin barrier found in preterm infants. A good deal of information can be obtained by analyzing these mice and trying to understand both the molecular causes of

the delay as well as the mechanistic solutions to effect the repair. The interpretation and clinical application of findings from models as the Inv-Cldn6-CΔ206 mouse can help to improve the efficacy of treatments of people with delayed barrier formation.

Aging of the skin can be caused by intrinsic and extrinsic factors. The former constitutes a subset of inevitable biological changes while the latter is caused by environmental factors such as UV, chemical and pollutants. One such intrinsic factor is the complex set of changes that occur within the stem cell and committed progenitor compartments of the epidermis (Bell and Van Zant, 2004;Chien and Karsenty, 2005;Gazit et al., 2008;Miura et al., 2004). These intrinsic factors can lead to hyperplasia and reduced repair capability leading to dermatitis, eczema and other conditions. Also, reduced repair and/or regeneration capacity is thought to contribute to the diminished integrity of various aging tissues (Bell and Van Zant, 2004;Chien and Karsenty, 2005;Gazit et al., 2008;Ghadially, 2012;Miura et al., 2004). The natural aging-related changes can also be further accentuated by the environmental stresses described above (Ghadially et al., 1995;Ghadially, 1998;Goukassian and Gilchrest, 2004;Hashizume, 2004).

Although we know that aging skin has a structurally poor SC, reduced differentiation and barrier function as well as poor healing capabilities, there is a paucity of data on biological and molecular pathways that affect barrier function in aging skin (Barland et al., 2004;Ghadially, 1998). The aging skin therefore provides an excellent model system to investigate the molecular basis of cellular aging in general and the consequences of inefficient epidermal repair. Because most mouse models focus on the effects of photo-aging and dermal structural changes associated with aging skin, there are

few suitable models with which to address these questions. Furthermore, there is little or no data on the expression profile, localization and function of Cldn-based TJs in aging skin. This lack of progress in dissecting the deregulated biological pathways that drive the aging process can be addressed using our transgenic mouse model. The transgenic model, in which we overexpress a thirteen AA Cldn6 tail deletion mutant in the suprabasal compartment of the epidermis (Inv-Cldn6-CΔ196 mice), is characterized by a marked and accelerated aging-related phenotype that phenocopies the epidermal changes seen in human aging (Troy et al., 2009). In more severe forms these changes can lead to chronic dermatitis and other painful skin conditions in humans. In addition, there are changes in the expression and/or localization of epidermal differentiation markers and Cldns leading to severe barrier dysfunction. These changes are reproducible, robust and more accelerated than what is observed in "normal" wild type aging. These observations suggest that our Inv-Cldn6-CΔ196 transgenic mice provide a novel and unique model with which to evaluate and dissect the biological and molecular basis of aging-related skin changes.

The Inv-Cldn6-CΔ196 transgenic mice are also sensitized to injury, evident even at 1 month of age. As the mice age, they show such a reduced ability to repair that they develop dermatitis lesions in areas of natural mechanical stress from grooming. This is not unlike the reduced ability of aging human epidermis to repair, suggesting that this mouse model might be applicable to study injury response in the context of aging. Sensitization to injury is an area often unexplored in the various aging mouse models. Elucidation of the pathways that regulate the intrinsic aging process of the skin may not only provide new insight into the mechanisms governing normal cellular aging processes

in general but also has the potential to help identify new diagnostic tools and therapeutic approaches that can be used to prevent or cure aging-related skin conditions.

In addition to preterm infants and the elderly, poor barrier function is also implicated in a myriad of skin diseases found in the general population. These include various forms of dermatitis, psoriasis and cancer (Elder, 2010;Elder et al., 2010;Kezic et al., 2014;Ramos-e-Silva and Jacques, 2012). These skin diseases are characterized by impaired skin barrier function, altered proliferation/differentiation of the epidermis and/or infiltration of inflammatory cells, and altered expression patterns of TJ proteins. In addition, there are numerous reports that document modulation in Cldn expression profiles in a number of these pathophysiological conditions including cancers (Burgel et al., 2002;D'Souza et al., 2005;Kucharzik et al., 2001;Swisshelm et al., 2005;Wolburg et al., 2003). Although some of these skin diseases are rarely life threatening, recent findings show that disruption of barrier function is implicated in the pathogenesis of a number of other more serious conditions such as inflammatory bowel disease, asthma and Atopic Dermatitis (AD). AD, for example a chronic or relapsing inflammatory skin disease highly prevalent in industrialized countries, often precedes other conditions such as asthma and allergic disorders (Kezic et al., 2014).

Future Directions

There are still many unanswered questions regarding Cldns and their role in epidermal homeostasis. Although new insights were obtained from overexpression studies such as these, the value of loss of Cldn function studies cannot be overstated. Future studies would entail investigation of Cldn6 loss-of-function in epidermal

development. Although global loss of Cldn6 resulted in mice that are viable and fertile with no obvious phenotypic abnormalities, the role of Cldn6 in the epidermis was not fully analyzed (Anderson et al., 2008). In order to look at the effects of Cldn6 in the epidermis specifically, the Cre-lox system with an epidermal-specific Cre such as the K5- or K14-Cre could be employed. In order to better dissect the finer details of the molecular pathways responsible for the knock out phenotype, loss of Cldn6 could be effected *in vitro* using the gene-editing tool CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) (Garneau et al., 2010;Horvath and Barrangou, 2010;Mali et al., 2013). We and others have shown that modulations in levels of one Cldn is often accompanied by modulations in expression of other Cldns suggesting that there is functional compensation within the Cldn family (Arabzadeh et al., 2006;Turksen and Troy, 2002). Given that a number of other Cldns are expressed in the epidermis and that the expression of other Cldns were up-regulated in the Cldn6 knockout mice, double or triple homozygous null mice may have to be generated and assessed to fully understand the role of Cldns in the epidermis.

Claudins are phosphorylated and palmitoylated and we have hypothesized that such post-translational modifications within the Cldn6 tail could prove relevant to epidermal function. In order to address these questions definitively, the Cldn6 phosphorylation events in the epidermis would have to be confirmed using a suitable *in vitro* system using epidermal cells such as HaCaT cells (Boukamp et al., 1988) or, ideally, *in vivo*. The specific sequences could then be mutated and the effects on epidermal development analyzed using appropriate mouse models. Specifically, the effects of these mutations on TJ assembly, structure and function as well as on

proliferation/differentiation could be assessed. Subsequently, identification of the relevant kinases and phosphatases could be of diagnostic or therapeutic value.

The importance of potential Cldn6 interactions with signaling and actin-associated scaffolding proteins has been discussed. Future studies along these lines would entail mapping of the entire protein-protein interactome at TJs using methods such as proximity-dependent biotin identification (BioID) (Roux et al., 2012;Roux et al., 2013;Roux, 2013) or affinity purification followed by mass spectrometry (AP-MS) (Armean et al., 2013;Gingras et al., 2007;Lambert et al., 2014). Upon validation of novel Cldn interactions, specific sites of interactions as well as the specific residues that mediate these interactions would need to be identified and mapped. It may also be informative to determine if any potential post-translation modifications could regulate such protein interactions. The nature of Cldn interactions within the TJs will provide insights into TJ behavior and barrier function.

Taken together, the findings of these studies provide new insights into the role of the Cldn6 tail in epidermal development and barrier function. They have also provided informative mouse models with which to study barrier dysfunction associated with preterm birth, aging skin and skin diseases in general with the end goal of furthering the development of diagnostic tools and therapeutics for the treatment of pathophysiological conditions caused or exacerbated by barrier dysfunction.

CHAPTER 5-GENERAL INTRODUCTION II

Pathologies Associated With the Mammalian Endoderm

The three primary germ layers of the mammalian body are the ectoderm, mesoderm and endoderm. Each germ layer contributes to distinct lineages-the ectoderm forms the nervous system, the epidermis and its associated appendages while the mesoderm gives rise to the vertebrae of the axial skeleton, muscles and the dermis, among other tissues (Figure 2). The endoderm is the innermost germ layer of the metazoan embryo and gives rise to the epithelial lining of the digestive and respiratory systems as well as associated organs such as the thyroid, lungs, liver, kidney, gall bladder and pancreas (Figure 29). These endodermally-derived organs are important for many essential processes such as gas exchange, digestion and nutrient absorption, glucose homeostasis, detoxification and blood clotting, therefore perturbations in endodermal function underlie a large number of diseases. These include diseases involving the lungs, liver, pancreas, biliary system and gastrointestinal (GI) tract such as cystic fibrosis, chronic hepatitis, diabetes, biliary atresia and celiac disease respectively. Although these conditions affect millions of people worldwide (Spence and Wells, 2007), little is known about how endoderm cells are patterned along the anterior-posterior (A-P) axis or how this positional identity is maintained throughout development to serve as a blueprint for endoderm organogenesis. The study of endoderm is therefore necessary for understanding the genetic basis for many of these diseases and for the discovery of molecular diagnostics with which to detect the reasons underlying these pathologies. In addition, it is also important for the advancement of regenerative medicine, which explores the possibility of culturing endoderm organs for use in transplantation-based therapies (Spence and Wells, 2007).

ENDODERM DEVELOPMENT

Gastrulation and Endoderm Specification

The mouse blastocyst before implantation consists of the trophectoderm (TE) and the inner cell mass (ICM) (Figure 30). The ICM gives rise to the epiblast and the overlying primitive endoderm. The primitive endoderm is the precursor for the visceral endoderm, which underlies the epiblast, and the parietal endoderm, which underlies the yolksac. The parietal and visceral endoderm, along with the TE, contributes to extra-embryonic structures such as the yolk sac (Kadokawa et al., 1987;Lawson et al., 1986;Tam and Loebel, 2007). The epiblast, on the other hand, gives rise to the embryo proper.

Gastrulation is the process by which the three primary germ layers of the embryo are formed (Figure 30). Gastrulation begins when totipotent cells from the posterior epiblast divide, and rearrange to form the primitive streak. In mouse, endoderm and mesoderm precursor cells undergo an epithelial to mesenchymal transition and migrate through the primitive streak (Kinder et al., 1999;Lawson et al., 1991;Tam and Beddington, 1987). As the prospective definitive endoderm cells migrate, they do not displace cells from the underlying visceral endoderm as was previously believed (Lawson et al., 1991;Tam and Beddington, 1992) but instead become intercalated with the visceral endoderm, with a significant portion of the visceral endoderm remaining in the definitive endoderm (Kwon et al., 2008). These migratory cells go on to form the mesoderm and definitive endoderm of the embryo while the remaining epiblast cells that do not migrate through the streak form the ectoderm (Figure 30).

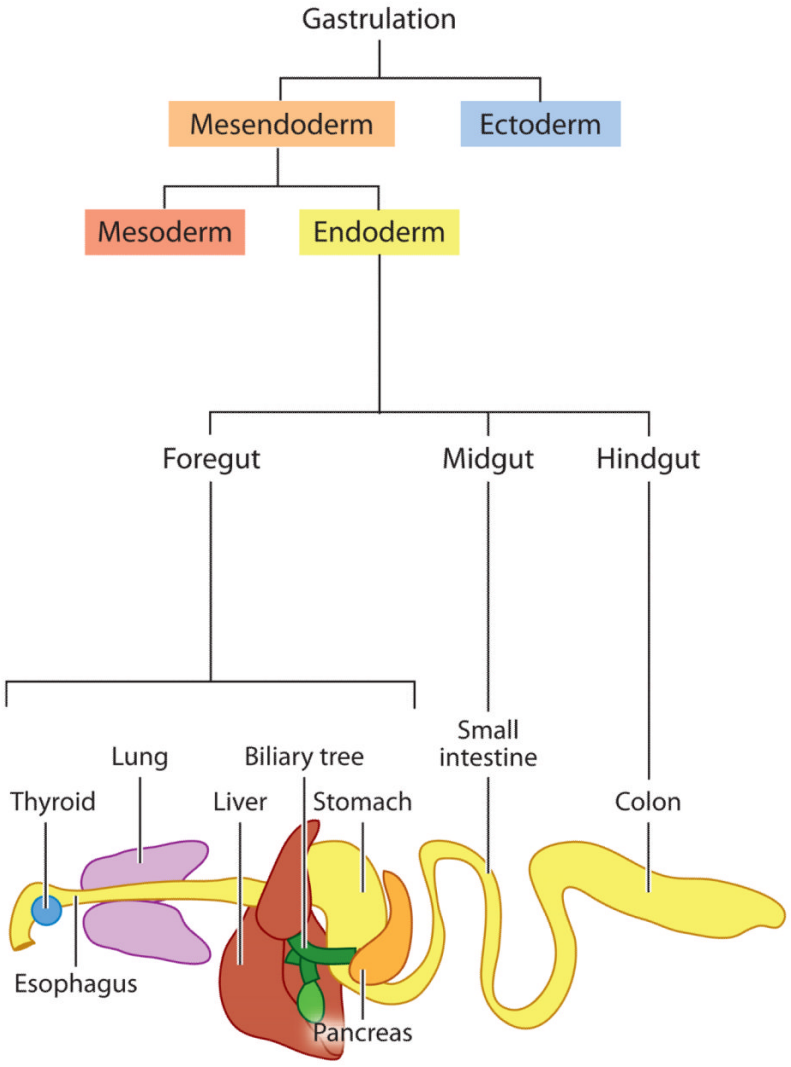

Figure 29: Endoderm Derivatives. Adapted from Zorn and Wells, 2009.

Lineage tracing studies in zebrafish and *C. elegans*, suggest that at least some endoderm and mesoderm cells arise from a common progenitor pool of "mesendodermal" cells; reviewed in (Rodaway and Patient, 2001). It has been suggested that this may hold true in mouse as well (Lawson and Pedersen, 1987) as mouse embryonic stem (ES) cells can be differentiated to express both endodermal and mesodermal markers (Kubo et al., 2004;Tada et al., 2005). However, such a population has yet to be identified *in vivo*. More recent evidence suggests that if there is such a common progenitor pool, its contribution to the definitive endoderm is minimal (Tzouanacou et al., 2009). In addition, recent 3D-time lapse imaging suggest that endoderm cells are specified even before migration through the primitive streak as evidenced by *Sox17* and *Foxa2* expression (Viotti et al., 2014). In general, it is believed that endoderm lineage is increasingly more specified as gastrulation proceeds (Lawson et al., 1991).

Formation of the Primitive Gut Tube

After gastrulation, the endoderm is a ~500-cell sheet of pseudo-stratified epithelium overlying the embryonic mesoderm (Grosse et al., 2011). The definitive endoderm undergoes a series of morphogenetic events that lead to the sheet being pushed inside of the developing embryo to form the primitive gut tube (Franklin et al., 2008;Lawson et al., 1986;Lawson and Pedersen, 1987;Tam et al., 2004;Tremblay and Zaret, 2005). The development of the primitive gut continues with the formation of two

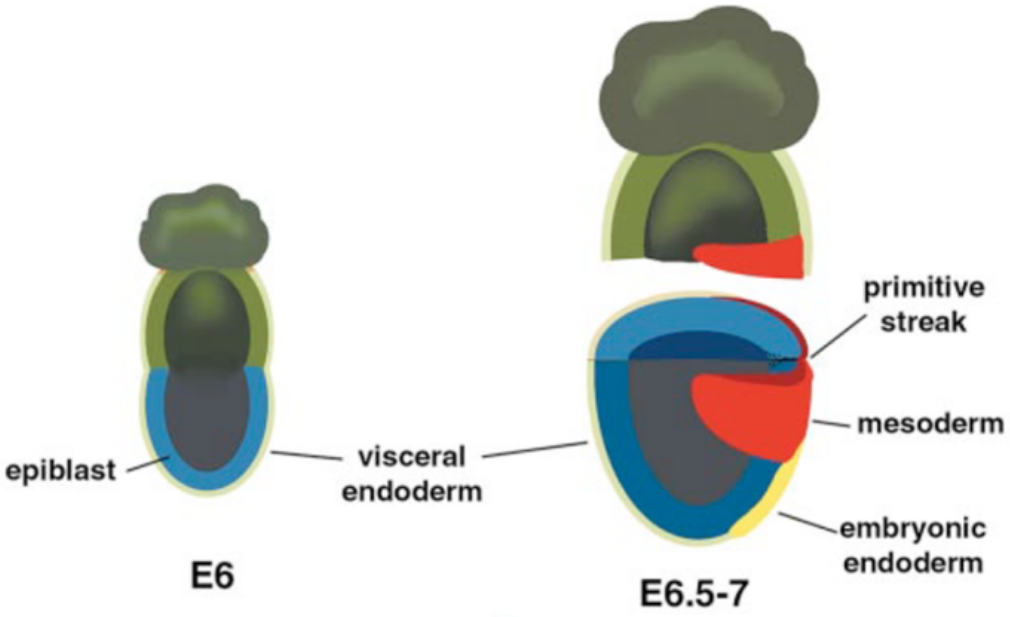

Figure 30. Gastrulation. Gastrulation begins at E6.5, when a population of cells from the epiblast of the inner cell mass are recruited through the primitive streak to form either the mesoderm of the definitive endoderm. Adapted with permission from Wells and Melton, 1999.

intestinal invaginations at the anterior and posterior ends of the embryo to form two intestinal pockets, the anterior intestinal portal (AIP) and the caudal intestinal portal (CIP), respectively. The foregut invagination begins as a depression beneath the neural fold, which then deepens and extends rostrally. The opening then moves posteriorly to close up as the foregut develops. Six-eight hours later, the hindgut pockets begins in a similar fashion, however its opening closes anteriorly. As the two pockets elongate and close, the lateral walls of the embryonic gut between the invaginations fold ventrally cumulating in the meeting of the tissue folds at the yolk stalk to form the gut tube. The closure of the gut tube is also linked to the change in posture of the embryo from the lordose to a fetal position. The internalization of the endoderm is accomplished by the inversion of the arrangement of the germ layer derivatives so that the ectoderm is rotated to the outside and the endoderm rolls into the inside of the embryo (Franklin et al., 2008;Lawson et al., 1986;Lawson and Pedersen, 1987;Tam et al., 2004;Tremblay and Zaret, 2005) (Figure 31).

Endodermal Patterning

Formation of the endoderm is coupled with its specification along its A-P axis such that the first cells that emerge from the streak populate the foregut endoderm while cells that emerge later go on to populate the mid-and hindgut endoderm (Franklin et al., 2008;Lawson et al., 1986;Lawson and Pedersen, 1987;Tam et al., 2004;Tam and Loebel, 2007;Zorn and Wells, 2009). As the embryo develops, broad gene expression patterns within the fore-, mid- and hindgut become more defined into specific domains that will give rise to specific organs. The beginnings of visceral organs are generated from these

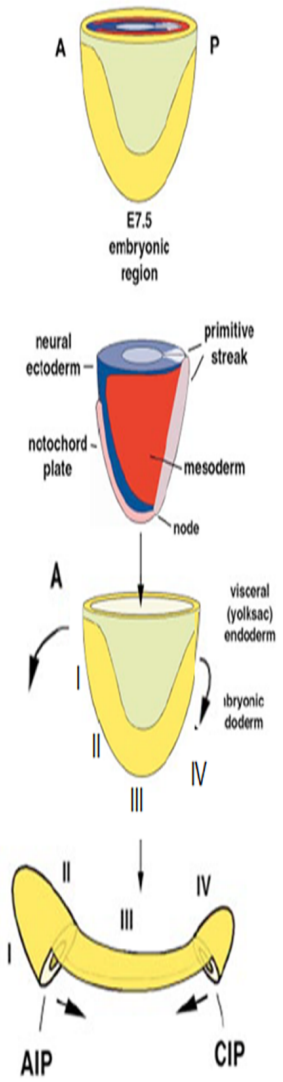

Figure 31. Gut Tube Formation. The three germ layers of the post-gastrulation embryo are indicated as follows: endoderm (yellow), mesoderm (red) and ectoderm (blue). The endoderm exists as a sheet of cells covering the outside of the embryo. Cell fated to become endoderm migrate from the primitive streak, intercalating with and displacing the visceral endoderm. I-IV represent regions of the E7.5 embryo that fate map to I-IV of the E8.5 gut. The posterior migration of the anterior intestinal portal (AIP) and anterior migration of the caudal intestinal portal (CIP) concurrently with embryonic turning leads to closure of the gut tube. Adapted with permission from Wells and Melton, 1999.

specific regions of the gut as outgrowths by swelling, budding and coiling of endodermal epithelium intermingled with the surrounding mesenchyme. Together the tissue proliferates and then differentiates into functional organs (Figure 32). The ventral foregut gives rise to the liver, ventral pancreas and lungs, the dorsal foregut and the anterior region of the midgut gives rise to the dorsal pancreas, stomach, esophagus and duodenum. The mid and hindgut forms the small and large intestines respectively (Lawson et al., 1986;Lawson et al., 1991;McLin et al., 2009;Seifert et al., 2008;Sherwood et al., 2009;Spence et al., 2009;Wells and Melton, 1999;Zorn and Wells, 2007;Zorn and Wells, 2009) (Figure 29).

REGULATION OF ENDODERMAL DEVELOPMENT

The regulation of endoderm development is controlled by transcription factors, which regulate gene expression, and by cell non-autonomous signaling factors emanating from the adjacent mesoderm (Bort et al., 2004;Grapin-Botton and Melton, 2000;Kumar and Melton, 2003;Molotkov et al., 2005;Serls et al., 2005;Stainier, 2002;Tremblay and Zaret, 2005;Wells and Melton, 2000). Some of the critical families of signaling molecules and transcription factors essential for endoderm formation or patterning will be discussed below.

Inductive Mesodermal Cues

The endoderm cannot develop normally without signals from the overlying mesoderm and sometimes ectoderm (Haffen et al., 1983;Haffen et al., 1987;Le Douarin, 1965;Mizuno and Yasugi, 1990). *In vitro* studies show that cultures of primitive foregut

endoderm cannot differentiate without co-culture with mesodermal tissues (Koike and Yasugi, 1999) and reciprocal communication between the endoderm and the mesoderm is paramount to proper endodermal patterning. Relevant signaling pathways from the overlying mesoderm include transforming growth factor beta (TGF)-β)/Nodal, Wnt, bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and retinoic acid (RA). Studies in *Xenopus*, zebrafish and mice suggest that these pathways in endoderm development are also evolutionary conserved. Although the specific details of how these pathways interact are beyond the scope of this thesis, TGF-β/Nodal pathway is at the top of this molecular hierarchy as it has been shown to regulate expression of a number of downstream transcription factors such as mix-paired homeobox (Mix1), GATA, SRY-related HMG box 2 (Sox) and forkhead box (Fox) genes.

Signaling Pathways Important For Endodermal Development

NODAL: Nodal ligands are members of the TGF-β family of secreted growth factors. In mouse there is a single Nodal gene. Endodermal regionalization occurs concurrently with endoderm specification and is mediated initially at least in part by Nodal signaling. Nodal signaling pathways have also been shown to play critical roles in specification of the anterior primitive streak, from which the definitive endoderm arises (Ang and Rossant, 1994;Schier, 2003;Shen, 2007;Weinstein et al., 1994;Yamamoto et al., 2001). It has also been postulated that in *Xenopus*, fish, mouse and humans high levels of Nodal signaling support endoderm development while low levels support mesoderm development (Clements et al., 1999;D'Amour et al., 2005;Green and Smith, 1990;Shen, 2007;Zorn and Wells, 2007). Nodal in turn promotes the expression of a complex

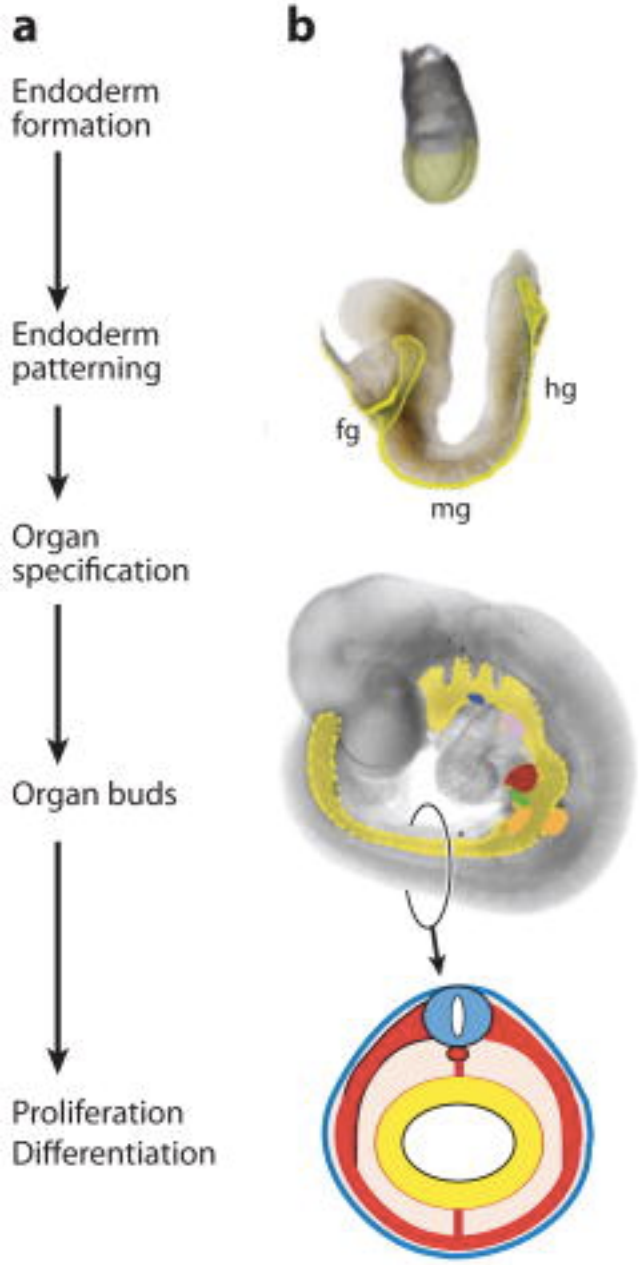

Figure 32: Overview of Endoderm Formation. The major events of endoderm formation are listed in chronological order with images of the mouse embryo at E7.5 (top), E8.5 (middle) and E9.5 (bottom). A schematic of a cross section of the E9.5 embryo shows arrangement of the germ layers with the endoderm lining the gut tube (*yellow*), surrounded by mesoderm (*red*), and ectoderm (*blue)*. Fg; foregut, mg; midgut, hg; hindgut. Adapted with permission from Zorn and Wells, 2009.

transcriptional network that gives regional identity to the endoderm and the other effectors of Nodal signaling play important roles in endoderm specification. These include the Nodal co-receptor and target gene *crypto*, the downstream effector *Smad2* and its binding partner *FoxH1* as well as Nodal target genes *Eomesodermin (Eomes)* and *Foxa2*. The loss of any of these factors results in embryos with gastrulation and/or endoderm specification defects (Ding et al., 1998;Hoodless et al., 2001;Russ et al., 2000;Tremblay et al., 2000;Waldrip et al., 1998).

Wnt/β-Catenin: The canonical Wnt ligand, Wnt 3, and its downstream effector β−catenin have also been shown to be necessary for gastrulation (Huelsken et al., 2000;Liu et al., 1999) as $Wnt3^{-/-}$ and $\beta-catenin^{-/-}$ embryos arrest during early gastrulation. This early lethality precludes analysis of Wnt function in the endoderm. Loss of β−catenin in the node, notochord, and in the anterior primitive streak leads to lack of definitive endoderm formation and the presence of ectopic cardiac cells in the posterior embryo. In addition, ES cells lacking β−catenin are less able to contribute to the endoderm. Instead, they form cardiac clusters in the endoderm germ layer suggesting that β−catenin is involved in an endoderm vs. mesoderm cell fate decision (Lickert et al., 2002). Conversely, ES cell lines carrying a mutation in the adenomatous polyposis coli (APC) gene, which results in elevated Wnt signaling, show an increased ability to form endoderm *in vivo* (Kielman et al., 2002). Studies in frog and mouse show that the Wnt/β-catenin signaling pathway regulates Sox17 expression for both visceral endoderm pattering and definitive endoderm formation (Engert et al., 2013;Sinner et al., 2004). In frog and zebrafish, Wnt/β−catenin signaling is also required for posteriorization events underlying hindgut development (Goessling et al., 2008;McLin et al., 2007). Conversely,

Wnt antagonists such as Cerberus, Dickkopf and Frzb, expressed in the foregut endoderm, are necessary for the formation of foregut derivatives such as the liver and pancreas (Li et al., 2008). Furthermore the Wnt effectors Tcf4 and Tcf1 are important for gut tube formation (Gregorieff et al., 2004) as disruption of both genes resulted in severe defects in hindgut formation and loss of expression of endodermal markers. Taken together, these data underscore the multiple roles played by canonical Wnt signaling in endoderm formation and patterning along the A-P axis.

Fibroblast Growth Factor: In mouse and chick, mesodermal FGF4 signaling plays a role in posteriorizing the developing gut. For example, FGF promotes expression of *Cdx* in the hindgut endoderm and represses the expression of anterior genes such as *Hhex* and *Foxa2* (Dessimoz et al., 2006;Haremaki et al., 2003;Wells and Melton, 2000). FGF4 effects on the endoderm become more restricted to the mid and hindgut where increasing levels of signaling induces more posterior fates. Later, FGF signaling also plays a role in induction of endoderm organ primordial. For example, FGF2 is necessary for liver and lung formation (Deutsch et al., 2001;Gualdi et al., 1996;Jung et al., 1999;Serls et al., 2005) while FGF10 is necessary for formation of the pancreas, caecum, lungs and stomach glands (Bhushan et al., 2001;Nyeng et al., 2007;Sekine et al., 1999).

Bone Morphogenetic Proteins: In zebrafish, *Xenopus* and chick, BMP signaling promotes posterior endoderm development (Tiso et al., 2002;Wills et al., 2008). BMP4 is necessary for liver induction whereas it appears that the pancreas is formed independent of BMPs (Rossi et al., 2001;Spagnoli and Brivanlou, 2008).

Retinoic Acid: RA signaling is important for maintaining the foregut/hindgut boundary (Escriva et al., 2002;Kumar and Melton, 2003;Schubert et al., 2005;Stafford

and Prince, 2002;Stafford et al., 2004;Stafford et al., 2006). RA is produced by the mesoderm and increasing levels of RA activity induces posterior identity. In the mouse, RA is also required for pancreas formation and for patterning the endoderm of the branchial arches (Huang et al., 1998;Huang et al., 2002;Martin et al., 2005;Matt et al., 2003;Molotkov et al., 2005).

Transcriptional Regulation of Endodermal Development

In general, regulation of gene expression can be attributed to a system comprised of three main components; reviewed in (Dermitzakis and Clark, 2002;Levo and Segal, 2014;Lynch, 2006;Weake and Workman, 2010). First, is the core promoter and RNA polymerase II (Pol II), a large protein complex that is usually recruited to a consensus "TATA" box sequence upstream of the transcriptional start site (TSS) (although there are many examples of promoters that do not carry this "TATA" box). Second are regulatory DNA sequences both within the proximal promoters of target genes, as well as distal regulatory enhancer elements. The third component is the transcription factors, which are protein adaptor molecules that can detect regulatory sequences in proximal or distal DNA and nucleate the assembly of protein complexes that are necessary for the control of gene expression, either as activators or repressors. Regarding endoderm formation, there are several families of transcription factors that bear further discussion, as elaborated below.

Transcription Factors Important For Endodermal Development

Sox17: Sox 17 is a member of the *Sry-related HMG box factor (Sox)* family of transcription factors. Studies in *Xenopus* and zebrafish show that *Sox17* is necessary and sufficient for endoderm specification. Deletion of *Sox17* in the mouse results in a failure in gastrulation, turning defects and posterior truncations due to defective mid- and hindgut development (Kanai-Azuma et al., 2002;Viotti et al., 2012). The prospective foregut initially develops properly, until increased apoptosis leads to a reduction in definitive endoderm in the foregut, while the mid- and hindgut tissues fail to expand. This is accompanied with replacement of the definitive endoderm in the lateral region of the embryonic gut, by cells that resemble visceral endoderm suggesting that *Sox17* is involved in the specification of the posterior definitive endoderm. In addition, although *Sox17*-null ES cells can contribute to ectodermal and mesodermal tissues, few of them populate the foregut endoderm and they are completely excluded from the mid- and hindgut endoderm (Kanai-Azuma et al., 2002;Niakan et al., 2010).

GATA 4/5/6: While GATA4, -5, and -6 are important for cardiac and gut development (Laverriere et al., 1994), GATA4 and GATA6 are also involved in visceral endoderm specification (Kuo et al., 1997;Molkentin et al., 1997;Morrisey et al., 1998). GATA4 null embryos gastrulate but arrest between E8.5 and E9 and have ventral defects in heart and develop a malformed AIP and therefore do not form a foregut (Kuo et al., 1997;Molkentin et al., 1997;Watt et al., 2007). GATA6 null embryos die at E7.5 mainly due to improper development of the visceral endoderm (Morrisey et al., 1998). GATA 4/5/6 also have roles in liver and lung development (Morrisey et al., 1998;Zhao et al., 2005).

Mixl1: Mixl1 belongs to a family of paired homeodomain transcription factors that is expressed in the primitive streak by the time the endoderm is formed. Mixl1 is activated by Nodal signaling via Foxh1/Smad DNA-binding sites on its promoter. Although embryos that lack Mixl1 do not have an overt endoderm specification defect, they do have a thickened anterior primitive streak. In addition, mutant *Mixl1*$^{-/-}$ ES cells contribute to all mouse endoderm tissue except for the hindgut (Hart et al., 2002). This suggests that Mixl1 may play a role in late gastrulation.

Two other important transcription factors that are of particular relevance to this thesis are *Cdx2*, a member of the *ParaHox* gene family as well as *Foxa2*, a member of the *Forkhead* box gene family. The role of these two transcription factors in endoderm formation and patterning will be discussed in further detail. Although, it is unclear how all the effects of the aforementioned molecular pathways are integrated, the interactions of these pathways promotes the expression of a complex transcriptional network that gives regional identity to the endoderm and patterns it into molecularly distinct A-P domains (Figure 33).

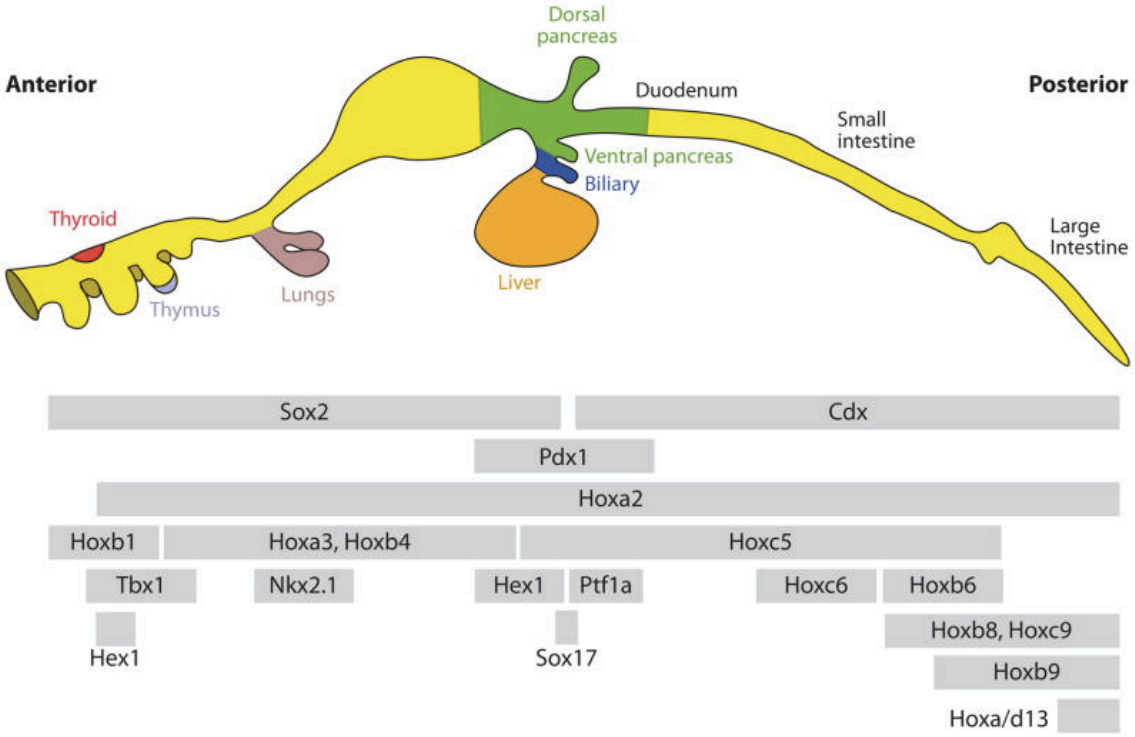

Figure 33. Molecular Heterogeneity in Developing Gut Tube. A schematic representation of the E9.5 gut tube showing the regionalization of transcription factors along its length. Regions of organ formation are delineated by the restrictive domains of expression of these transcription factors. Adapted with permission from Zorn and Wells, 2009.

FORKHEAD BOX A (FOXA) Genes

The *Forkhead box (*Fox) family of transcription factors is a family of evolutionarily conserved genes named after the *Drosophila melanogaster* gene fork-head (*fkh*). Mutations in the Drosophila gene *fkh* gene cause defects in head fold involution, resulting in a characteristic spiked head appearance (Weigel et al., 1989). *Fkh* is also required for terminal segment differentiation necessary for gut invagination (Lai et al., 1991;Weigel and Jackle, 1990). Fox genes harbor a 110-amino acid conserved fork-head winged-helix DNA binding domain (DBD) (Carlsson and Mahlapuu, 2002). More than 170 Fox genes have been identified in species ranging from yeast to humans, and are divided into 19 subfamilies (*Foxa* to *Foxs*) based on their sequence conservation (Tuteja and Kaestner, 2007;Tuteja and Kaestner, 2007).

The vertebrate Foxa subfamily of Fox transcription factors is the most closely related to the *Drosophila forkhead*. Foxa proteins were first identified as transcription factors required for liver-specific gene expression, and occupy promoters such as that of the *transthyretin* (Ttr), α1-*antitrypsin* (Serpina1) and *albumin* (Alb1) genes in liver nuclear extracts (Herbst et al., 1991;Lai et al., 1991). The proteins were initially named hepatocyte nuclear factor-3 (HNF-3) α, β and γ until the nomenclature of the vertebrate forkhead box genes was standardized (Kaestner, 2000). In mouse, this family is now referred to *Foxa1*, *Foxa2* and *Foxa3*.

Expression of Mouse *Foxa* Genes

All three mouse *Foxa* genes are expressed during gastrulation (Ang et al., 1993;Monaghan et al., 1993;Sasaki and Hogan, 1993). *Foxa2* is the first to be expressed

in the anterior primitive streak and the node at E6.5. Mesodermal and endodermal cells migrating from the node at this time point also express *Foxa2*. By E7.5 its expression is maintained in the notochord and throughout the definitive endoderm. *Foxa2* is present in the definitive endoderm posterior to the oral plate and in the endoderm derived structures; this expression persists through development and adulthood (Kaestner et al., 1993;Kaestner et al., 1994;Lai et al., 1991;Monaghan et al., 1993;Sasaki and Hogan, 1993;Sasaki and Hogan, 1994). *Foxa2* is also present in the ventral neural plate and maintained in the floor plate, as well as in the midbrain and diencephalon (Besnard et al., 2004;Monaghan et al., 1993;Sasaki and Hogan, 1994). *Foxa2* is also weakly expressed in the developing skeleton.

Foxa1 expression parallels *Foxa2* with a few exceptions. Notably, *Foxa1* expression in the respiratory and gastrointestinal tracts is more pronounced than that of *Foxa2*. Unlike *Foxa2*, *Foxa1* is also expressed in the renal pelvis, ureters, bladder and male reproductive organs (Besnard et al., 2004). *Foxa3* expression is significantly different from the other *Foxa* genes (Monaghan et al., 1993). It is first expressed at E8.5 from the midgut-foregut boundary to the hindgut. Expression in this region and its resulting organs persists throughout development into adulthood. It is not expressed at any time in the primitive streak, axial mesoderm or neural structures. *Foxa3* is also the most highly expressed family member in the liver, and is the only *Foxa* gene expressed in the long bones (Monaghan et al., 1993).

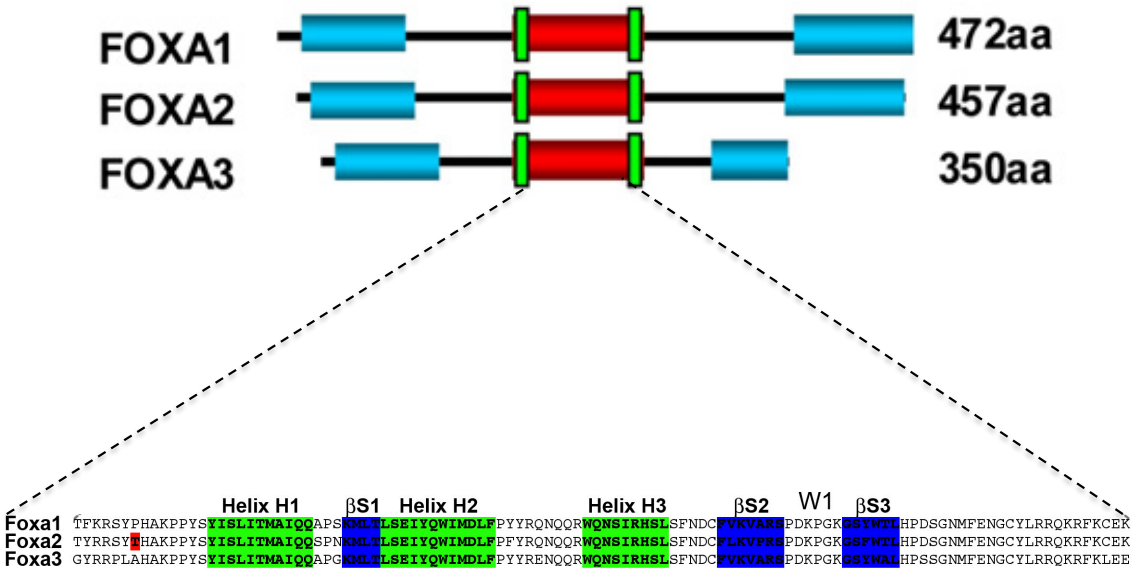

Figure 34. Mouse Foxa Genes. Foxa1/2/3 are highly homologous proteins that consist of an N- and C-terminal transactivation domains (blue rectangle) and a central *forkhead* domain (red rectangle). Within the forkhead domain there is 95% sequence homology between the three family members, while the flanking region shows less similarity. The forkhead domain has three α-helices (Helix H1-3) and three β-strands (βS1-3). Foxa proteins interact with DNA in three ways: the major grove interacts with Helix H3; the DNA backbone interacts with wing 1, which contains β-strands 2 and 3 along with an intermediary loop; and the minor groove interacts with wing 2. Only Foxa2 undergoes undergoes phosphorylation at T156 (Red). Adapted with permission from Friedman and Kaestner, 2006.

Structure and Function of *Foxa* Genes: Regulation of Gene Expression

Fox proteins typically contain a central DBD comprised of three-α-helices arranged in a helix-loop-helix fold flanked by two wings that are similar to the DBD of linker proteins H1 and H5, although they lack the amino acids necessary for nucleosomal DNA compaction (Figure 34). The DBD is highly conserved and the *forkhead* domains of Foxa are 95% identical (Clark et al., 1993;Overdier et al., 1994). Regions outside of the DBD are bound by core histones H3 and H4 and function in chromatin opening.

The importance of the structural similarity between the fork head domain and the H1 is underscored by the discovery that Foxa proteins are able to play a role in chromatin remodeling by accessing highly compacted chromatin without requiring remodeling by the SWI/SNF complex (Cirillo et al., 2002;Holmqvist et al., 2005). Rather, the winged helix HI-like domain in Foxa proteins could disrupt DNA–histone contacts, thereby creating accessible transcription factor-binding sites. In the absence of Foxa, other transcription factors are not able to access their binding sites. Foxa proteins therefore are critical for the recruitment of secondary transcription factors. It has been suggested that before the endoderm can undergo tissue-specific differentiation, it must first become 'competent' to respond to inductive signals and accessible to other transcription factors necessary for organ differentiation. It is postulated that Foxa proteins are responsible for this competence and are the first transcription factors to bind regulatory regions (Cirillo et al., 2002). For this reason, Foxa proteins have been termed 'pioneer factors'.

Foxa proteins also regulate gene expression by acting as "classical" transcription factors. Indeed, Foxa proteins share some homology outside of the forkhead box and these regions have been shown to act as transcriptional activators (Pani et al., 1992;Qian

and Costa, 1995). Furthermore, sequence-specific DNA binding activity and interaction between Foxa and the transcriptional machinery has been demonstrated. In addition Foxa2 targets have been identified through electrophoretic shift mobility assays (EMSA), chromatin immunoprecipitation (ChIP), transcriptional reporter assays and differential gene expression in Foxa null mouse models; reviewed in (Friedman and Kaestner, 2006). In this manner, Foxa is able to regulate diverse genes involved in a number of different processes.

Foxa Mouse Models: Role of Foxa2 In Endodermal Development

Foxa1 and Foxa3 are not necessary for early mouse development as embryos lacking either factor are viable and initial endoderm specification is unperturbed. Foxa1 mutant mice show only post-natal defects related to glucose homeostasis and respiratory cell maturation in the lungs (Besnard et al., 2005;Gao et al., 2005;Kaestner et al., 1999;Shih et al., 1999). Loss of *Foxa3* results in a mild phenotype related to glucose metabolism; this is speculated to be due to compensation by the other Foxa members as suggested by their up-regulation in this mouse model (Shen et al., 2001).

Conversely, Foxa2 null mutant embryos die at E10 due to defects in all three germ layers. Specifically the neural tube and somites are abnormal and the node, notochord and the gut tube fail to form (Dufort et al., 1998;Monaghan et al., 1993;Weinstein et al., 1994;Zaret, 1999). In chimeric studies, these abnormalities can be rescued when Foxa2 ES cells populate the definitive embryo in Foxa2 null chimeras, illustrating that Foxa2 acts cell autonomously (Dufort et al., 1998). Although the endoderm is specified in these mutants, the foregut and midgut are severely disrupted

while the hindgut is less affected (Dufort et al., 1998). This suggests that other as yet unknown factors cooperate with Foxa2 to regulate posterior endoderm patterning.

The severity of the Foxa2 phenotype necessitated the generation of conditional null mutants (Lee et al., 2005). The results of these and other studies are summarized in Table 4. Regarding the endoderm and its derived organs, studies using a hepatocyte-specific Cre transgene dependent on expression of the *Albumin* promoter and the *α-fetoprotein* enhancer showed that Foxa2 is necessary for the induction of the hepatic transcriptional program in gluconeogenesis (Sund et al., 2000;Zhang et al., 2005). Loss of *Foxa2* in the lung results in defects in pulmonary maturation upon birth (Wan et al., 2004), while deletion in the endoderm using a *Foxa3*-Cre result in mice that do not survive past the first week of life due to severe hypoglycemia (Lee et al., 2005). It should however be noted that the restricted expression of the Foxa3-Cre transgene results in considerable residual *Foxa2* expression thereby preventing a thorough analysis of *Foxa2* function in the endoderm.

The *Foxa* genes have been shown to be redundant in function, necessitating the generation of compound mutants. Compound mice homozygous for a Foxa1 null allele and an endoderm-specific Foxa2 allele do not form a liver and die by E9.5-10.5 (Lee et al., 2005) while compound mice homozygous for a Foxa1 null allele and a lung-specific conditional Foxa2 allele show abnormal differentiation of the lung epithelial tissue (Besnard et al., 2004). Taken together, these and other models show that, although Foxa1 and Foxa3 may be indispensible for endoderm formation, Foxa2 by itself or in conjunction with Foxa1 is necessary for the formation of the endoderm and its associated organs. However the molecular mechanisms by which Foxa2 regulates endoderm

formation are poorly understood.

CAUDAL TYPE HOMEOBOX (CDX) GENES

The transcription factor families involved in A-P patterning in mammals are the *Hox* and *ParaHox* genes. Although Hox factors are known to be important for mesoderm and neuroectoderm patterning, they appear to be less involved in endoderm AP patterning (Aubin et al., 1997;Boulet and Capecchi, 1996;Manley and Capecchi, 1995;Warot et al., 1997;Zacchetti et al., 2007). It is now well established that *Cdx2*, a member of the *ParaHox* gene family, is a critical factor in posterior endoderm patterning.

Vertebrate *Cdx* genes encode a family of hexapeptide homeodomain transcription factors related to the *Drosphila* gene *Caudal (Cad)*. *Cad* is a homeobox-containing gene analogous to vertebrate *Hox* in the manner in which it regulates the A-P axis development in *Drosophila* (Dearolf et al., 1989;Macdonald and Struhl, 1986;Mlodzik et al., 1985;Mlodzik and Gehring, 1987;Mlodzik et al., 1990). In mouse, there are three *Cdx* genes; *Cdx1*, *Cdx2* and *Cdx4* (Duprey et al., 1988;Gamer and Wright, 1993;James and Kazenwadel, 1991). *Cdx2* is linked with *Pdx1* and *Gsh2* on chromosome 5 (Chawengsaksophak and Beck, 1996), *Cdx1* is on chromosome 18 (Duprey et al., 1988) and *Cdx4* is X-linked (Horn and Ashworth, 1995).

Expression of Mouse *Cdx* Genes

All three *Cdx* genes are expressed in overlapping domains during early embryogenesis at the posterior primitive streak, however, only *Cdx1* and *Cdx2* are expressed in developing endoderm and intestinal tract. *Cdx1* expression is first observed

in the ectoderm and mesoderm of the primitive streak at E7.5; in the ectoderm it is expressed in the future hindbrain and spinal cord and in the mesoderm it is expressed in the somites, the proximal developing limb buds and the mesonerphros (Meyer and Gruss, 1993). At E12, it is turned off in the neural tube, somites and limb buds, but retained in the developing endoderm and persists in the adult intestine (Duprey et al., 1988;Hierholzer and Kemler, 2009;Meyer and Gruss, 1993;Silberg et al., 2000). *Cdx2* has an earlier onset of expression in the extra-embryonic trophectoderm at E3.5. Its expression in the embryo proper is reported to begin in the posterior gut, neural plate and tail bud at E8.5. By E12.5, it becomes limited to the epithelium of the posterior gut tube, with expression persisting throughout life in the mature intestine (Beck et al., 1995;Chawengsaksophak et al., 2004). *Cdx4* has a more restricted expression domain, beginning at E7.0 in the allantois and the primitive streak and briefly in the posterior hindgut, neurectoderm, lateral plate and presomitic mesoderm and becomes extinguished around E10.0 (Gamer and Wright, 1993) (Figure 35).

Structure of the Mouse *Cdx* Genes

There are four structural domains within each of the three Cdx members (Figure 36) with little sequence conservation among members outside of their homeodomains. The first three domains include an amino terminal signal peptide involved in processing and sub-cellular trafficking, a hexapeptide motif located just upstream of the homeodomain, as well as a poorly defined transactivation domain which encompasses the first two conserved regions (Lynch et al., 2003;Rings et al., 2001;Taylor et al., 1997;Taylor et al., 1997;Trinh et al., 1999).

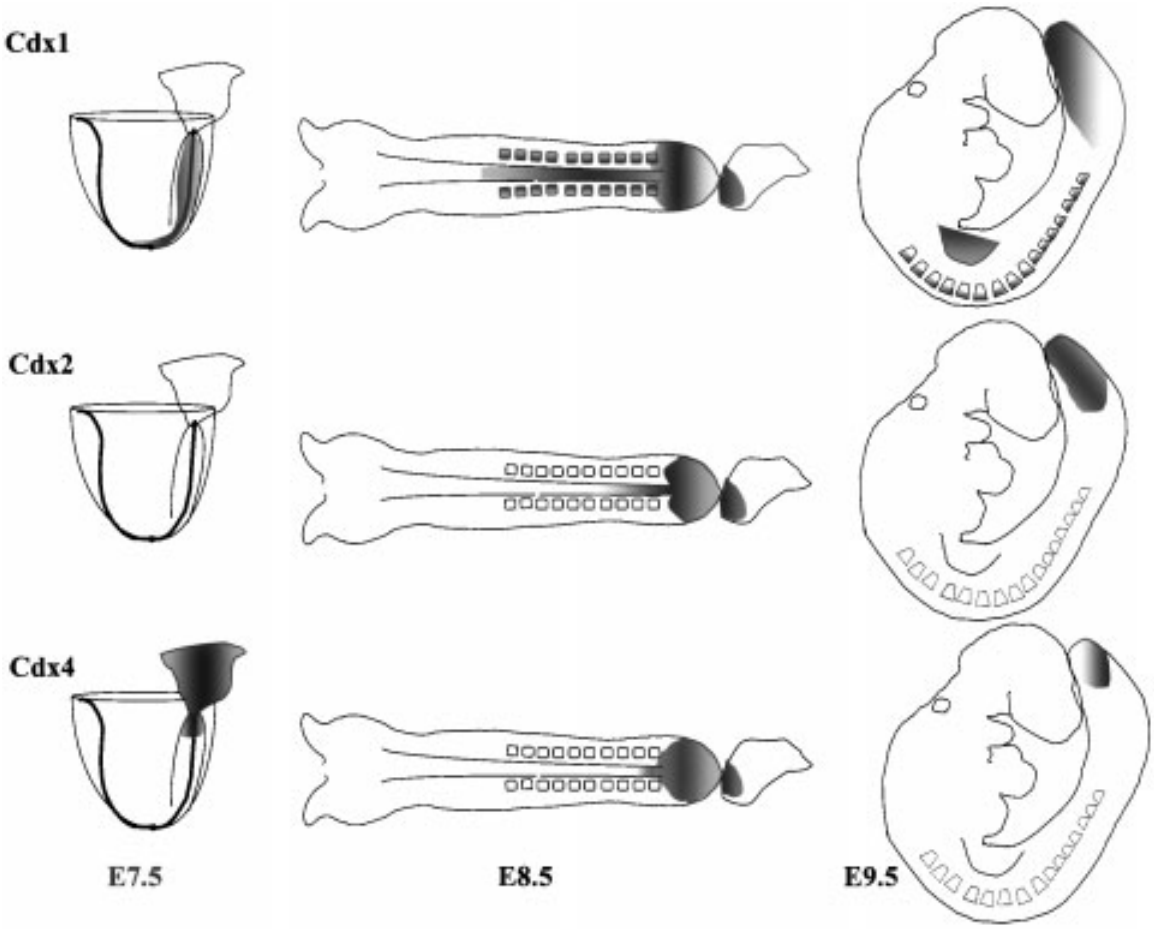

Figure 35: Cdx Expression at E7.5-E9.5. Relative gene expression within the embryo is denoted by shading for each gene. Adapted with permission from Lohnes, 2003.

The highly conserved DNA binding homeodomain is the largest domain (Gamer and Wright, 1993), while the carboxy terminus has indetermined function (Lynch et al., 2003).

Cdx Mouse Models: Cdx2 Regulates A-P Patterning of the Endoderm

Cdx mouse models have provided critical insight into their function, and are summarized in Table 5. *Cdx1*$^{-/-}$ mice are viable and fertile but show malformations and anterior homeotic transformations of the cervical and upper thoracic vertebrae. For example, the second cervical vertebrae C2, frequently acquires characteristics of the first cervical vertebra, C1 (Figure 37) (Subramanian et al., 1995). *Cdx1*$^{-/-}$ mice have no defects with respect to the endoderm and intestine. Cdx4 null mice are viable and fertile due to functional overlap with other Cdx members (van Nes et al., 2006). *Cdx2*$^{-/-}$ mice are peri-implantation lethal, due to a requirement for Cdx2 in the trophectoderm critical for implantation. Cdx2 heterozygotes, although viable and fertile, exhibit anterior homeotic shifts in the cervical and thoracic vertebrae of the axial skeleton as well as a defect in axial elongation (Chawengsaksophak et al., 1997). These mutants also exhibit an intestinal phenotype in the peri-cecal region of the intestinal tract, which are devoid of Cdx2 expression and exhibit epithelial tissue similar to more anterior regions of the gastrointestinal tract including the esophagus, stomach and small intestines. These observations suggest that Cdx2 plays a role in posterior patterning of the GI tract

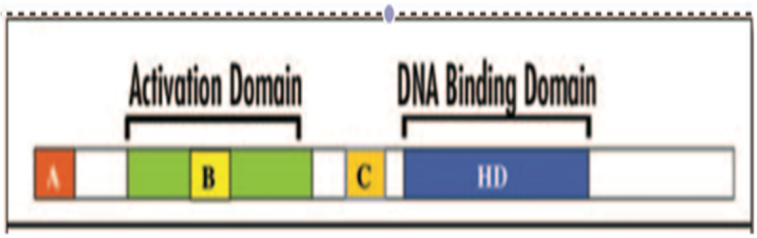

Figure 36. Schematic Representation of Conserved Protein Domains Among Cdx Family Members. The homeodomain, HD, is the largest and most conserved. The other domains A, B and C are poorly characterized, Adapted with permission from Guo et al, 2004.

(Beck et al., 1999;Chawengsaksophak et al., 1997;Tamai et al., 1999). Conversely, overexpression of Cdx1 or Cdx2 under the control of stomach or esophageal specific promoters results in conversion of the stomach epithelium to that of the small intestine (Kong et al., 2011;Mutoh et al., 2002;Mutoh et al., 2004a;Mutoh et al., 2004b;Mutoh et al., 2009;Silberg et al., 2000).

The early embryonic lethality of Cdx2 null mice necessitated the generation of Cdx2 conditional mutants using the Cre-loxP system (Gu et al., 1994;Sauer, 1998). Using this approach, it was found that deletion at E5.5 of the *Cdx2* floxed allele using an *Actin*-Cre results in severe truncation of the embryo (Savory et al., 2009a) and death at E11. This finding is consistent with the axial truncation seen in Cdx2 homozygous null embryos generated by tetraploid aggregation (Chawengsaksophak et al., 2004). Deletion of Cdx2 using the endodermal-specific *Foxa3-Cre* resulted in anterior transformation of the small intestine to an esophageal identity (Gao et al, 2009), suggesting that Cdx2 antagonizes a foregut differentiation program. Ablation of *Cdx2* at later time points using a *villin*-Cre resulted in transformation of the intestine to a partial stomach-like phenotype (Grainger et al., 2010) exemplified by an increase in expression of stomach markers concomitant with a decrease in expression of intestinal markers (Figure 38). Finally, mice that are null for all three *Cdx* genes do not survive beyond E10.5 as axial elongation stops abruptly at the post-occipital level. The progenitor cells for the trunk and tail are not maintained and the embryos are severely truncated (Savory et al., 2009;van Rooijen et al., 2012).

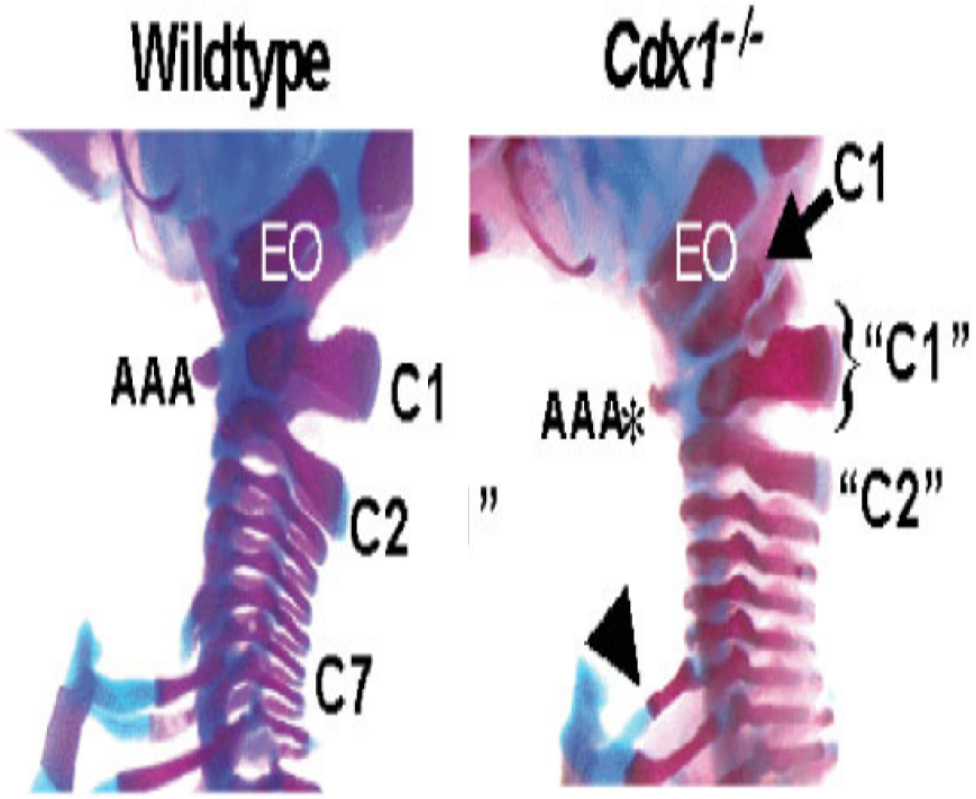

Figure 37: Anterior homeotic transformation of vertebral column in Cdx1-null mice. Lateral view of the cervical region shows anterior transformation of "C2" to "C1" identity as evidenced by ectopic anterior arch (*) and wider neural arches (bracket) on "C2". Partial transformation to an occipital identity is indicated by the arrow. "EO" exoccipital, "AAA", "C" cervical, anterior arch of the atlas, arrow head Adapted with permission from Lohnes, 2003.

A role for *Cdx* genes in the endoderm and intestine has been suggested in other models. In zebrafish, *Cdx1b* (equivalent to *Cdx2*) has been shown to regulate intestinal proliferation and differentiation (Chen et al., 2009;Flores et al., 2008), while knockdown of all *Cdx* genes in *Xenopus* results in mis-regulation of midgut and hindgut genes and loss of intestinal identity (Faas and Isaacs, 2009). Taken together, these studies suggest that Cdx2 is important for the establishment of posterior identity in the endoderm and for the maintenance of the intestine. However, our understanding of the molecular mechanisms directed by *Cdx* in these events is limited. In particular, very few *Cdx* targets in the endoderm have been identified.

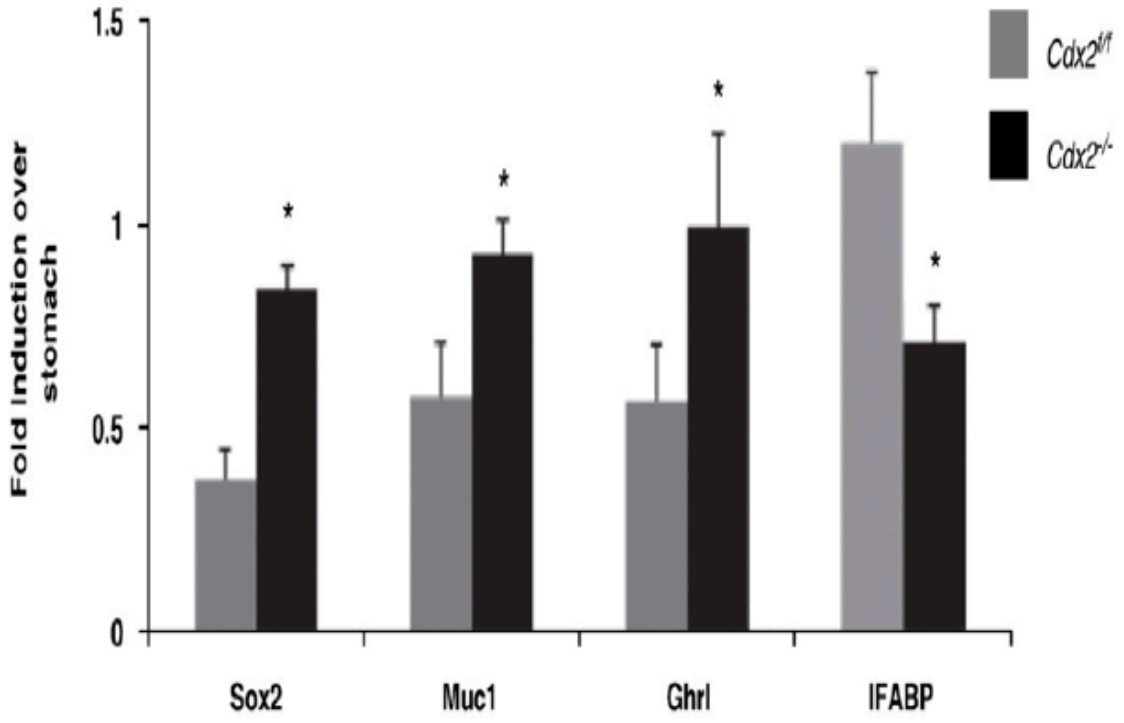

Figure 38: Semi-Quantitative RT-PCR of Intestinal Tissue of $Cdx2^{-/-}$ Conditional Mouse Model. Anterior homeotic shift of intestine to stomach upon loss of Cdx2 at E13.5 is evidenced by gain of stomach markers *Sox2*, *Muc1 and Ghrl* and loss of intestinal marker *ifabp.* $P<0.05$. Adapted with permission from Grainger et al, 2010.

RATIONALE II

Formation and patterning of the mammalian endoderm is a complex process that is not well understood. *Cdx2* has been shown to be important for the establishment of posterior identity in the endoderm and for the maintenance of endoderm-derived organs however very few *Cdx* regulated targets in the endoderm have been identified and the interplay between *Cdx* and its targets is even more poorly understood. In order to determine Cdx specific-targets in the mouse endoderm, the *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (Cdx1/2 DKO) mouse model system was used (Savory et al., 2009). The conditional CRE-ER^{T} system allows us to circumvent the early lethality of Cdx2 null mice and to temporally regulate Cdx2 loss-of-function to identify early-response genes. To determine genes that were differentially expressed in Cdx1/2 DKO endoderm, we took a transcriptome gene profiling approach (Figure 39). Cdx2 expression was ablated at E7.5 for 36h only to preserve enough endoderm tissue for analysis and to enrich for proximal Cdx targets. Caudal tissue from Cdx1/2 DKO and *Cdx1*$^{-/-}$*Cdx2*$^{fl/fl}$ (control) embryos were then subjected to fluorescence activated cell sorting (FACS) using the endoderm-specific surface marker *EpCAM* (Sherwood et al., 2007). RNA was extracted from the sorted cells and then amplified to increase the amount of RNA available for microarray analysis (Singh et al., 2005;Wang et al., 2000). To ensure that variability in transcript abundance was not introduced during the amplification (Nygaard and Hovig, 2006), we performed semi-quantitative RT-PCR to compare the relative transcript abundance of genes before and after amplification (data not shown).

Three replicate sample sets of amplified RNA of control and Cdx-null cells were then processed by Affymetrix Genechip technology. The data was analyzed using Partek

Genomics Suite 6.5 software. Principle Component Analysis and other quality control metrics suggested that there was a modest separation between control and Cdx1/2 DKO samples and that the overall intensity of signals obtained from the Affymetrix chips were generally low (data not shown). However, the presence of previously known Cdx2 targets *fabp2/ifabp and Dll1* in a list of genes down-regulated in the mutant versus control endoderm, suggested that there might be some validity to the microarray (Table 6). Further validation of down-regulated genes was carried out by qRT-PCR (Figure 50). To identify bona-fide *Cdx2* targets, we used bioinformatic algorithms to assess the presence of Cdx response elements (CDREs) in the promoter regions of these deregulated targets. Based on these analyses, the Foxa family member *Foxa2* emerged as a promising candidate for a *Cdx2* target gene within the endoderm.

Given that *Foxa2* was down-regulated in the Cdx1/2 null endoderm and that both Foxa2 and Cdx2 null mice have defects with respect to endoderm patterning and development, I **hypothesize** that *Foxa2* is a Cdx2 target and that together they regulate a subset of genes that are important for the proper development of the mammalian endoderm. In order to pursue this hypothesis, my main **objective** is to determine whether *Foxa2* is a *Cdx2* target and to elucidate the potential mechanism by which *Cdx2* regulates *Foxa2* and the development of the endoderm.

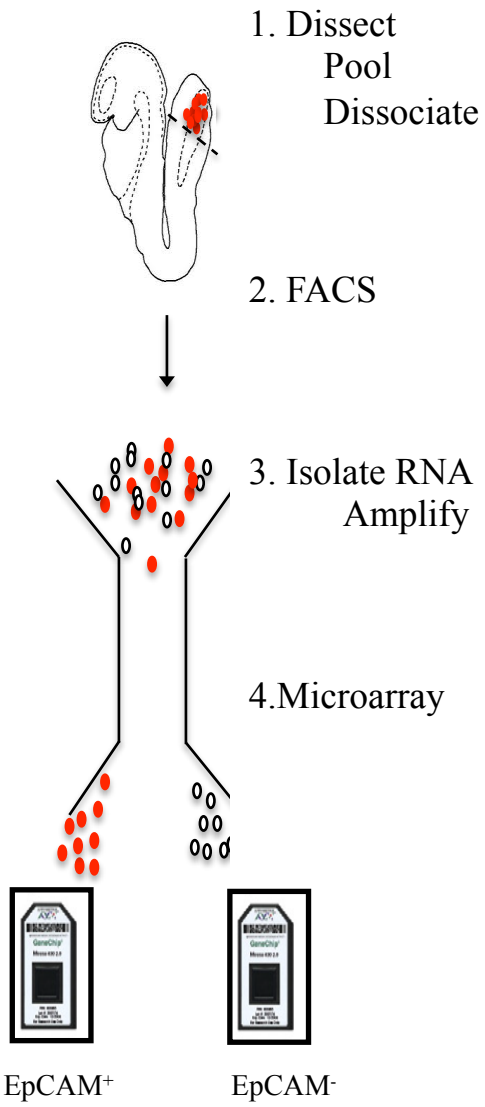

Figure 39: Overview of Experimental Plan for Isolation of Endoderm for Microarray Analysis. Caudal ends of *Cdx1*$^{-/-}$*Cdx2*$^{-/-}$ (DKO) and *Cdx1*$^{-/-}$*Cdx*$^{fl/fl}$ (control) E9.5 embryos were dissected and analyzed by Fluorescence activated cell sorting (FACS) using endoderm-specific surface marker, *epithelial cell adhesion marker* (*EpCAM*). Amplified RNA samples from these sorted cells were then prepared for microarray analysis. Replicate sample sets of control and DKO amplified RNA that showed comparable relative transcript amplification were assessed by Affymetrix Genechip technology.

CHAPTER 6

Foxa2 and *Cdx2* Cooperate to Regulate A Subset of Endodermal Genes.

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Key words. Endoderm; Transcription; Co-regulation; *Cdx2*; *Foxa2*; Gastrulation

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AUTHOR CONTRIBUTIONS

This manuscript is to be submitted to the journal "Molecular and Cellular Biology".

Adebola Enikanolaiye designed and carried out experiments, wrote manuscript and made figures. Joanne G.A. Savory generated the $Cdx2^{f/f}$ mice, the Cyp26a1 luciferase constructs and provided useful comments towards preparation of the manuscript. David Lohnes helped to design experiments, write the manuscript and provided approval of manuscript for submission.

ABSTRACT

The mammalian *Cdx* genes *Cdx1* and *Cdx2* encode homeodomain transcription factors that function in anterior-posterior patterning of the endoderm and the intestine, and are required for intestinal homeostasis in the adult. However, the molecular mechanisms underlying these functions are largely unknown, and few direct Cdx target genes within the endoderm have been identified. We show that endodermal expression of the *Foxa* family of transcription factors is modulated in Cdx1-Cdx2 double null embryos. Chromatin immunoprecipitation and *in vitro* reporter assays show that the *Foxa2* promoter is occupied, and regulated, by Cdx2, suggesting that *Foxa2* is a Cdx target gene. We also show by co-immunoprecipitation that Cdx2 and Foxa2 physically interact *in vitro* and that they synergistically regulate the expression of several common target genes. Furthermore, complementation assays revealed genetic interactions between *Cdx2* and *Foxa2*. In addition, the expression of genes important for endoderm function are further reduced in the absence of both *Foxa2* and *Cdx2,* when compared to lack of *Foxa2* or *Cdx2* alone, indicating that *Cdx2* and *Foxa2* cooperate to regulate patterning of the endoderm. We propose that Cdx2 regulates *Foxa2* expression with subsequent Cdx2-Foxa2 co-regulation of a subset of genes implicated in endoderm development.

INTRODUCTION

The mammalian definitive endoderm gives rise to the epithelial lining of the digestive and respiratory systems as well as associated organs such as the thyroid, lungs, liver, kidney, gall bladder and pancreas (Wells and Melton, 1999). The endoderm is formed during gastrulation, a process that begins at embryonic day (E) 6.5 in the mouse, when a population of pluripotent $Oct4^{+}$ epiblast cells undergo an epithelial to mesenchymal transition (EMT) and migrate through the primitive streak (Han et al., 2010;Tam and Behringer, 1997). These migratory cells go on to form the mesoderm and definitive endoderm while the remaining epiblast cells form the ectoderm. As the definitive endoderm cells emerge from the streak, they displace, intercalate and are incorporated into the existing visceral endoderm (Beddington and Robertson, 1999;Kwon et al., 2008;Lawson et al., 1986;Lawson and Pedersen, 1987;Tam and Beddington, 1992;Tam and Loebel, 2007). By late gastrulation at E7.5, the definitive murine endoderm is a single layer overlying the embryonic mesoderm (Wells and Melton, 1999).

The timing of cell emergence from the primitive streak determines the final anterior-posterior (A-P) orientation of prospective definitive endoderm, with cells that emerge first populating foregut endoderm and those exiting later populating the mid- and hindgut endoderm (Lawson and Pedersen, 1987). This patterning occurs as a result of molecular heterogeneity and regionalized expression of transcription factors along the A-P axis of the embryo.

It is now well established that *Cdx2*, a member of the *ParaHox* gene family, is a critical transcription factor in the patterning of the endoderm and specification of the intestine after gut tube formation. Vertebrate *Cdx* genes encode a family of homeodomain

transcription factors related to the *Drosphila* gene *Caudal.* In mouse there are three *Cdx* genes, *Cdx1*, *Cdx2* and *Cdx4*. All three genes are expressed during gastrulation stages in the caudal embryo; however, only *Cdx1* and *Cdx2* are expressed in developing endoderm and in epithelium of the adult intestinal tract throughout life. *Cdx1* expression is first observed in the ectoderm and mesoderm of the primitive streak at E7.5, with additional expression at the base of the forelimb buds at E9.5. By E12, it is extinguished in the neural tube, somites and limb buds, but retained in the developing endoderm and later in the adult intestine. *Cdx2* has an earlier onset of expression at E3.5 in the extraembryonic trophectoderm; however, its expression in the embryo proper begins at E8.5 in the posterior gut, neural plate and tail bud. By E12.5, its expression becomes limited to the epithelium of the posterior gut tube, with expression persisting throughout life in the intestinal epithelium. *Cdx4* expression begins at E7.5 in the allantois, the posterior tip of the primitive streak and then the paraxial mesoderm. It is also expressed in the neuroectoderm before being extinguished at E10.5 (Beck et al., 1995;Gamer and Wright, 1993;Lohnes, 2003;Meyer and Gruss, 1993;Strumpf et al., 2005).

Cdx1$^{-/-}$ mice have no overt intestinal defects (Bonhomme et al., 2003) but exhibit vertebral homeoses in the anterior axial skeleton (Subramanian et al., 1995). *Cdx2*$^{-/-}$ mice are embryonic lethal due to implantation defects however, *Cdx2* heterozygotes, conditional mutants and Cdx2 mutants derived by tetraploid aggregation mice show a number of defects, including anterior homeotic shifts in the axial skeleton and intestinal patterning defects, as well as defects in axial elongation (Beck et al., 1999;Chawengsaksophak et al., 1997;Chawengsaksophak et al., 2004;Savory et al., 2009;Savory et al., 2011;van den Akker et al., 2002). *Cdx2* also plays a crucial role in the

patterning of the definitive endoderm as loss of *Cdx2* in the early definitive endoderm results in anterior transformation of the small intestine to an esophageal character (Gao et al., 2009). Ablation of *Cdx2* at later time points results in a less severe transformation of the small intestinal epithelium to a partial stomach-like epithelium (Grainger et al., 2010).

A role for *Cdx* genes in the endoderm and intestine has also been suggested from work in other vertebrate model systems. For example, zebrafish *Cdx1b* (functionally equivalent to mouse *Cdx2*) has been shown to regulate intestinal proliferation and differentiation (Chen et al., 2009;Flores et al., 2008). In *Xenopus*, knockdown of all *Cdx* genes results in deregulation of expression of midgut and hindgut genes leading to loss of intestinal identity and disruption of gut coiling (Faas and Isaacs, 2009). Together, these studies suggest that *Cdx2* is important for the establishment of posterior identity in the endoderm and for the maintenance of intestinal epithelium. However, our understanding of the role *Cdx* plays in endoderm specification and patterning is limited. In particular, only a few Cdx-regulated genes have been identified in the endoderm.

Foxa2, a member of the *Forkhead* gene family of transcription factors, has been shown to be important for endoderm development. *Fox* genes are characterized by the presence of a Forkhead DNA binding domain (Weigel et al., 1989;Weigel and Jackle, 1990;Weigel et al., 1990). There are 3 members of the mammalian Foxa gene family, namely *Foxa1*, *Foxa2* and *Foxa3*. The Foxa proteins have been shown to play a role in chromatin remodeling by interacting with nucleosomal core histones to displace them and allow recruitment of other transcription factors to promoter and/or enhancer sites; they are therefore thought to function as 'pioneer factors' (Cirillo et al., 1998;Cirillo and Zaret, 1999;Cirillo et al., 2002;Holmqvist et al., 2005).

Foxa2 is expressed throughout the definitive endoderm and is required for gut formation. *Foxa1* expression parallels that of *Foxa2* but expression of *Foxa1* in the respiratory and gastrointestinal tract is more pronounced than that of *Foxa2*. *Foxa3* expression is more restricted and only detected at the hindgut to the midgut/foregut boundary (Ang et al., 1993;Ang and Rossant, 1994;Besnard et al., 2004;Dufort et al., 1998;Kaestner et al., 1993;Kaestner et al., 1994;Monaghan et al., 1993;Sasaki and Hogan, 1993).

Of the three *Foxa* genes, only *Foxa2* has been shown to be critical for proper embryonic development. Foxa1 mutants show only post-natal defects while loss of *Foxa3* results in a mild phenotype, possibly due to compensation by the other two Foxa members (Kaestner et al., 1998;Kaestner et al., 1999;Shen et al., 2001;Shih et al., 1999). Foxa2 mutant embryos die at E10 due to severe defects in structures related to all three germ layers including abnormalities in the node, notochord, neural tube and somites and failure to form the gut tube (Ang and Rossant, 1994;Dufort et al., 1998;Weinstein et al., 1994). Although the endoderm is specified in these mice, the foregut and midgut are severely disrupted while the hindgut is less affected (Dufort et al., 1998). This suggests that other as yet unidentified factors may cooperate with Foxa2 to regulate posterior endoderm patterning. In addition, *Foxa2* null cells are unable to integrate into the definitive endoderm due to a cell-autonomous defect impacting cell polarity and epithelialization (Burtscher and Lickert, 2009). Later in development, *Foxa2* has been shown to be important for the formation of endoderm-derived organs such as the liver, pancreas and lungs (Besnard et al., 2005;Sund et al., 2000;Sund et al., 2001;Wan et al., 2005;Zhang et al., 2005). Collectively, these mouse models show that *Foxa2* is necessary

for the formation of the endoderm and its associated organs. The molecular mechanisms by which *Foxa2* regulates endoderm formation, and the means by which *Foxa2* itself is transcriptionally regulated, are not well known. In this study, we show that *Foxa2* is regulated by Cdx2 and that Foxa2 and Cdx2 converge on common target genes in the definitive endoderm.

MATERIALS AND METHODS

Mice

The $Cdx1^{-/-}Cdx2^{f/f}$ (hereafter referred to as Cdx1/2 DKO), littermate controls $Cdx1^{-/-}Cdx2^{+/+}$, $Foxa2^{f/f}$ and Actin-Cre-ER^{T} mice have been previously described (Santagati et al., 2005;Savory et al., 2011;Sund et al., 2000). $Cdx1^{-/-}Cdx2^{+/-}Foxa2^{+/-}$ mutants were generated by crossing $Cdx1^{-/-}Cdx2^{f/f}$ Actin-CRE ER^{T} mice with $Foxa2^{f/f}$ mice. $Cdx1^{-/-}Cdx2^{+/-}Foxa2^{+/+}$ and $Cdx1^{-/-}Cdx2^{+/+}Foxa2^{+/-}$ littermates were used as controls. Animals were mated overnight and noon on the day of detection of a vaginal plug was counted as E0.5. Deletion of Cdx2 or Foxa2 was effected by administration of a 2 mg single dose of tamoxifen at the specified times.

Fluorescence Activated Cell Sorting (FACS)

Embryos were aseptically dissected in PBS at E9.5 and tissue caudal to the hind limb bud were collected, pooled and treated with 1 mg/ml of dispase/collagenase for 5-10 min at 37 °C. The cells were then resuspended in flow cytometry staining buffer (eBioscience 00-4222) containing 10% heat inactivated goat serum and 2mM EDTA and pelleted. Dissociated cells were then resuspended and incubated for 20 min in a 1:10 dilution of

Epithelial Cell Adhesion Molecule (EpCAM) primary antibody (Developmental Studies Hybridoma Bank) followed by incubation in 1:1000 dilution of a texas-red goat-anti-mouse secondary antibody (Santa Cruz sc-2781). Cells were then washed in staining buffer, filtered through a 30 μm mesh filter (Partek) and EpCAM-positive cells isolated by FACS with a MoFlo cytometer (Dakocytomation).

Whole mount In-Situ Hybridization

Whole mount in situ hybridization for *Cyp26a1* was performed as previously described (Savory et al., 2009).

Semi quantitative Reverse Transcriptase polymerase chain reaction (RT-PCR)

Semi-quantitative RT-PCR was performed as previously described (Savory et al., 2009). Briefly, RNA was extracted from the caudal ends of E9.5 embryos using Trizol (Invitrogen) and used to generate cDNA by standard means. cDNA was then amplified by PCR using GoTaq (Promega) and mouse primers as indicated in Table 7. PCR products were resolved by gel electrophoresis on a 2% agarose gel, quantified by scanning densitometry and normalized to β−actin. In each experiment, a minimum of three independent biological samples were analyzed; error bars represent standard deviation of the mean.

Identification of Cdx Response Elements (CDRE) and Chromatin Immunoprecipitation (ChIP) Assays

Putative CDREs were identified from genomic sequences using TRANSFAC (Matys et al., 2006). ChIP assays were performed against regions harboring potential CDREs as previously described using chromatin from E9.5 embryos and an antibody against Cdx2 (Pilon et al., 2006). Rabbit IgG (Santa Cruz) was used as a negative control. Primers used for ChIP are indicated in Table 7.

Cell Based Expression Assays

The Cdx2 and Foxa2 expression vectors have been previously described (Sasaki and Hogan, 1994;Savory et al., 2009). To generate luciferase reporter constructs, 0.8-1 kb sequences carrying putative Cdx response elements (CDRE) of the promoters of *Foxa2* and *Eomes* were PCR-amplified from mouse genomic DNA and subcloned into pXP2 harboring a basal promoter (Nordeen, 1988;Savory et al., 2009). Putative CDREs were mutagenized using QuikChange Site-Directed mutagenesis system (Stratagene) according to the manufacturers instructions. The luciferase vectors from the *Cyp26a1* promoter have been previously described (Savory et al., 2009). Transfections were carried out in P19 cells using the calcium phosphate precipitation method. DNA mixes were made up of 1 μg luciferase reporter, varying amounts of expression vectors for Cdx2 and/or Foxa2, 0.2 μg of β-galactosidase expression vector and empty expression vector to a total of 2 μg of DNA per transfection. Cells were harvested 48 h post-transfection and lysates processed for analysis using the Promega Luciferase Assay System according to the manufacturers instructions using a Synergy H1 Hybrid Multi-Mode Microplate Reader

(Biotek). Transfection efficiency was normalized using β-galactosidase activity assessed by the chlorophenolred-β-D-galactopyranoside system as previously described (Houle et al, 2000; Savory et al., 2009). Transfections were performed in triplicate and error bars represent the standard deviation from the mean.

Co-Immunoprecipitation (Co-IP) and Western blotting

Cos7 cells were transfected with 20 μg of Cdx2 and/or Foxa2 expression vectors using the calcium phosphate precipitation method. Protein was harvested in lysis buffer (20 mM Tris HCl, 25 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton, 10% Glycerol, 1 mM DTT and cocktail of Protease Inhibitors) and sonicated for 30 seconds at 10% output using a Branson sonifier 450. Lysates were centrifuged and supernatant pre-cleared by an overnight incubation with protein A/G beads (Santa Cruz). Antibodies for Cdx2 (Pilon et al, 2006) or Foxa2 (Santa Cruz sc-6554) were incubated with protein A/G beads overnight and then added to pre-cleared lysates. Immunoprecipitation was carried out for 2 h at 4 °C using pre-immune IgG (Santa Cruz) as negative controls. Immunoprecipiates were washed in RIPA buffer and complexes processed for western blot as previously described using anti-Foxa2, anti-Cdx2 or anti-FLAG M2 (Sigma) antibody (Savory et al., 2009).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA assays were performed as previously described (Houle et al., 2000;Pilon et al., 2006) using nuclear extracts from COS-7 cells transfected with FLAG-tagged Cdx2 (Grainger et al., 2013) as a source of protein. End-labeled double stranded primers

corresponding to putative CDREs from the *Foxa2* promoter, or primers in which the putative CDREs had been mutated, were used; sequences are indicated in Table 7. Primers corresponding to the CDRE from the *Hoxb8* locus were used as a positive control (Charite et al., 1998). A supershift was achieved using 1 μg of Cdx2 antibody (Savory et al., 2009).

Whole Mount Immunhistochemistry

Whole mount immunohistochemistry was carried out on E9.5 embryos as previously described (Qiu et al., 1997) using antibodies against Cdx2 (Savory et al., 2009) or EpCAM (Developmental Studies Hybridoma Bank).

RESULTS

Loss of Cdx2 impacts *Foxa* gene expression

Cdx2 has been shown to play a role in specification and patterning of the mammalian endoderm (Gao et al., 2009;Grainger et al., 2010) however its specific targets relevant to these events are largely unknown. To identify potential targets, we undertook gene expression studies using our previously described Cdx1/2 DKO mice (Savory et al., 2009). Preliminary analysis suggested that *Foxa2*, the forkhead box transcription factor was down regulated in the Cdx1/2 DKO embryos (unpublished data). To investigate this further, we isolated RNA from EpCAM positive tissue of the E9.5 caudal embryos, comparing Cdx1/2 DKO and control mice; EpCAM marks both definitive and visceral endoderm between E9.5 and E12.5 (Sherwood et al., 2007).

We have previously found that deletion of Cdx2 at E5.5 results in severe truncation of the caudal embryo and loss of posterior endoderm (Savory et al., 2009). In

order to effect sufficient loss of Cdx2 expression while still preserving the caudal endodermal tissue, mice were treated with tamoxifen at E8.0 and harvested 36 h later. Whole mount immunohistochemistry analysis for Cdx2 and EpCAM following such treatment showed efficient ablation of Cdx2 in the caudal embryo and retention of EpCAM expression throughout the remaining endoderm (Figure 40A).

We subsequently isolated EpCAM-positive endodermal cells from the caudal Cdx1/2 DKO and control embryos by fluorescence activated cell sorting (FACS) (Figure 51). Semi-quantitative RT-PCR indicated that EPCAM positive cells were enriched for the endoderm markers EPCAM and Intestinal Fatty Acid Binding Protein (IFABP) and depleted for neuro-ectodermal markers Paired box 6 (Pax6) and ASCL1/Mash1 as well as the mesodermal marker Brachyury/T (Figure 52). Semi-quantitative RT-PCR analysis further showed that, although *Foxa1* and *Foxa3* expression were relatively unaffected by loss of Cdx, *Foxa2* levels were significantly down regulated (Figure 40B).

Foxa2 is a Cdx2 Target

The loss of *Foxa2* expression in the Cdx1/2 DKO mouse endoderm suggests that *Foxa2* might be a Cdx2 target. Consistent with this, studies in zebrafish suggest that *Foxa2* is regulated by the cdx2 ortholog, cdx1b (Cheng et al, 2008). In order to further investigate if *Foxa2* was a Cdx2 target in mouse, we used the binding site algorithm TRANSFAC (Matys et al, 2006) to scan 10kb 5' of the *Foxa2* transcriptional start site, which revealed multiple potential CDREs (Figure 41A). Chromatin Immunoprecipitation analysis using chromatin from E9.5 embryo revealed enrichment of Cdx2 occupation of the *Foxa2* promoter at CDRE A, B, and C but not D (Figure 41B). EMSA analysis

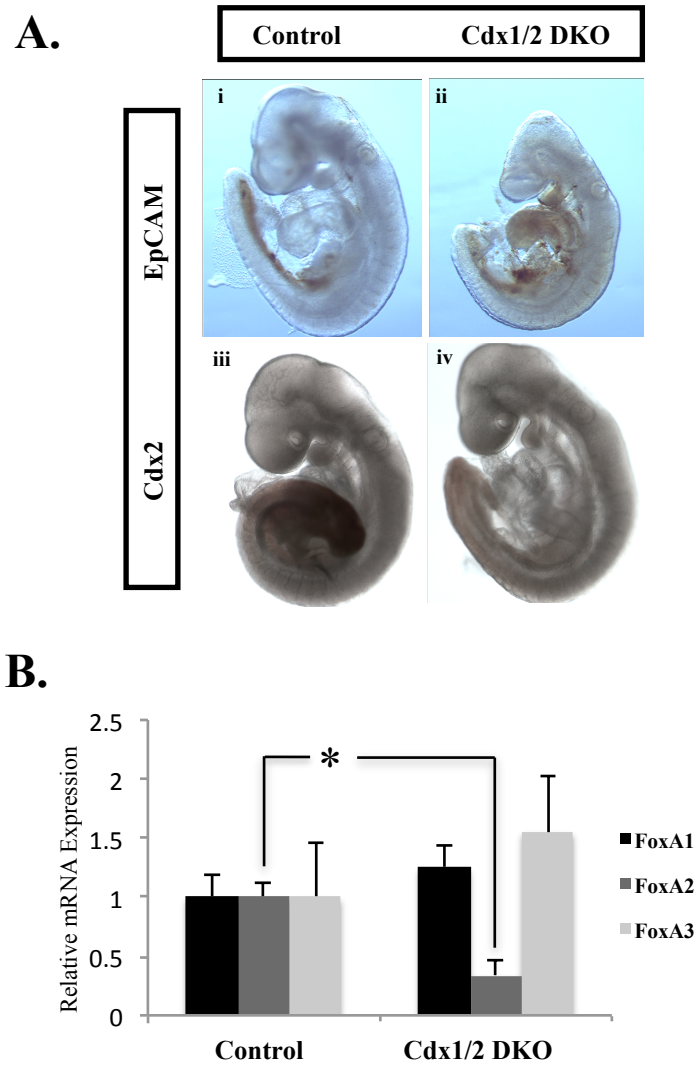

Figure 40: *Foxa* Genes Are Mis-regulated in Cdx-Null Endoderm A. Whole mount immunohistochemistry of control and mutant embryos using EpCAM (i, ii) and Cdx2 (iii, iv) antibodies. Cdx2 expression was severely compromised in E9.5 conditional null mutant as compared to control. Treatment of Cdx1/2 DKO mutants with tamoxifen for 36h results in truncation at caudal end of the embryo. EpCAM expression was reduced as a result of loss of tissue. B. RT-PCR of Foxa2 genes in caudal end of E9.5 $EpCAM^{+}$ control and Cdx1/2 DKO mutants showing that Foxa2 mRNA expression in decrease in mutants. *$p<0.05$ by student's t-test.

further revealed that Cdx2 could associate directly with all of these putative CDREs (Figure 41C). Supershift in the presence of a FLAG-M2 antibody, the loss of binding in the presence of excess unlabeled wild type probe and failure of association to mutant probes indicated that the binding was specific.

To determine if Cdx2 could regulate the *Foxa2* promoter in a tissue culture model, luciferase-based reporter constructs carrying the CDREs from the *Foxa2* promoter sequences were transfected into P19 cells in the absence or presence of a Cdx2 expression vector. The presence of Cdx2 led to an induction of the *Foxa2* promoter in reporters bearing the CDREs from regions A or C, while the reporter construct harboring sequences from region B did not respond to Cdx2 (Figure 42A). Furthermore, mutation of these responsive CDREs led to a loss of Cdx2-dependent expression in the context of the full length reporter when compared to the wild type sequences (Figure 42B). These findings are consistent with direct regulation of *Foxa2* by Cdx2.

Foxa2 and Cdx2 Co-Regulate Target Genes

Foxa proteins have been shown to physically interact with each other in co-immunoprecipitation experiments (Motallebipour et al., 2009) and also cross-regulate at the transcription level, while Foxa2 has also been shown to function in an auto-regulatory loop (Sasaki and Hogan, 1994;Sinner et al., 2004). Examination of the -1kb proximal *Foxa2* promoter indicated that the functional CDREs described above are in close proximity to two Foxa2 response elements, one of which has been previously described (Pani et al., 1992) (Figure 43A). In particular, the more distal Fox response element is approximately 100bp from a CDRE. This observation raised the possibility of a physical

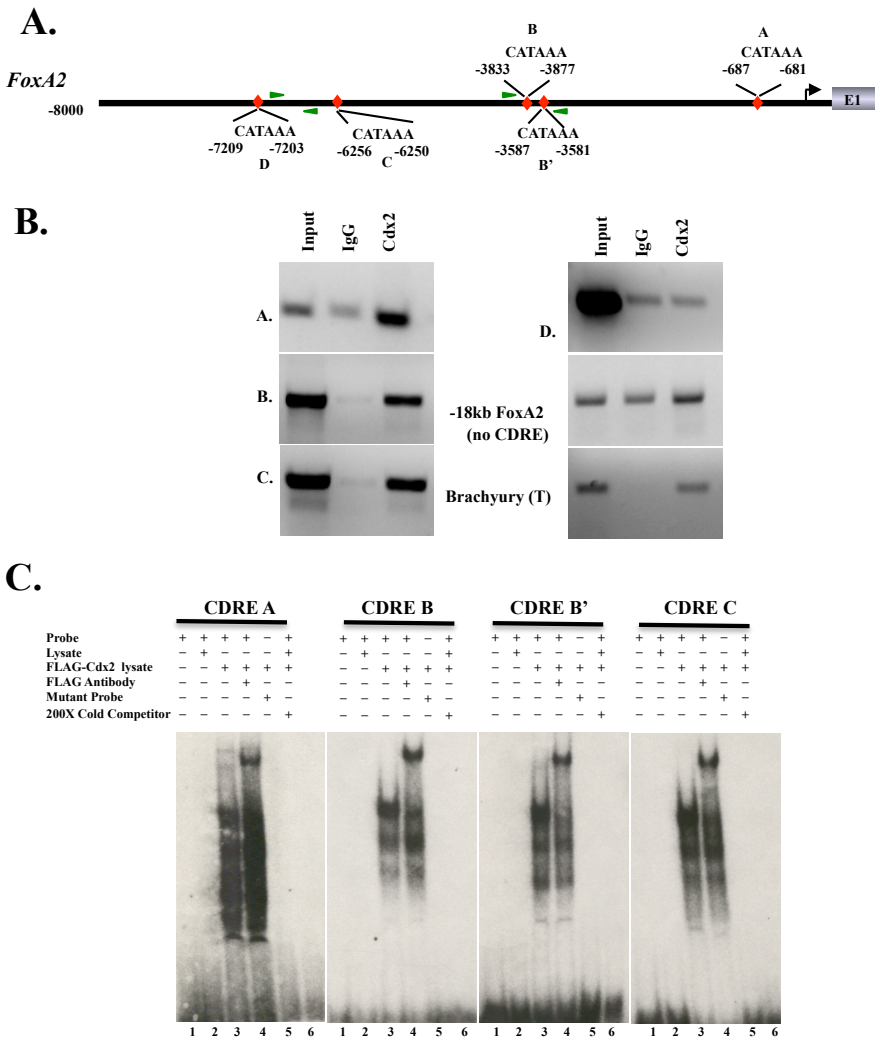

Figure 41: Cdx2 Binds the Foxa2 Promoter. A. Schematic representation of the mouse *Foxa2* promoter showing the position of the putative Cdx response elements (CDREs; Red diamond) as identified by TRANSFAC. B. ChIP of the *Foxa2* promoter using chromatin from E9.5 embryos shows binding of Cdx2 at CDREs A, B, and C but not at D and not at a region 18kb upstream of the transcriptional start site (arrow). C. EMSA analysis shows the ability of Cdx2 to bind primers corresponding to the putative CDREs hi-lighted in A (lanes 3). Binding is out-competed by a mutant primer and by 200X excess of the wild type primer (lanes 5 and 6).

and/or functional interaction between Foxa2 and Cdx2 on the *Foxa2* promoter. To further investigate this, we performed co-immunoprecipitation experiments using Flag-Cdx2 and Foxa2 expressed in Cos7 cells. Flag-Cdx2 was enriched when immunoprecipitated with Foxa2 and in the reciprocal Co-IP, Foxa2 was enriched by immunoprecipiation of Cdx2 compared with rabbit IgG as a control (Figure 43B).

The ability of both Foxa2 and Cdx2 to bind the *Foxa2* promoter and the physical interaction between the two suggests that both proteins interact with each other to form a complex that binds the *Foxa2* promoter. To test the functional significance of this interaction, we investigated the ability of Foxa2 and/or Cdx2 to effect transcriptional activation of the *Foxa2* promoter using varying amounts of *Foxa2* and/or *Cdx2* with a *Foxa2* reporter. Individually, Cdx2 and Foxa2 both activated the Foxa2 promoter albeit with different potencies (Figure 43C). However, co-transfection of Foxa2 together with Cdx2 resulted in an activation of about 30-fold, suggesting that Foxa2 and Cdx2 function in a synergistic manner on the *Foxa2* promoter. Mutation of the CDRE within the -1kb *Foxa2* promoter lead to a loss of this effect (Figure 43C) suggesting a requirement for Cdx2 occupancy of the *Foxa2* promoter for transcriptional synergy.

Cdx2 and Foxa2 have both been shown to be critical for the formation and patterning of the mammalian endoderm (Burtscher and Lickert, 2009;Dufort et al., 1998;Gao et al., 2009;Grainger et al., 2010). The synergistic interaction between Cdx2 and Foxa2 at the *Foxa2* promoter led us to investigate whether they co-regulate other genes. We found that the expression of a number of other known *Foxa2* targets were attenuated in the Cdx1/2 DKO endoderm (Figure 44A). These targets included genes such as *Hhex* (Kubo et al., 2004;Rankin et al., 2011), *Gata 4*

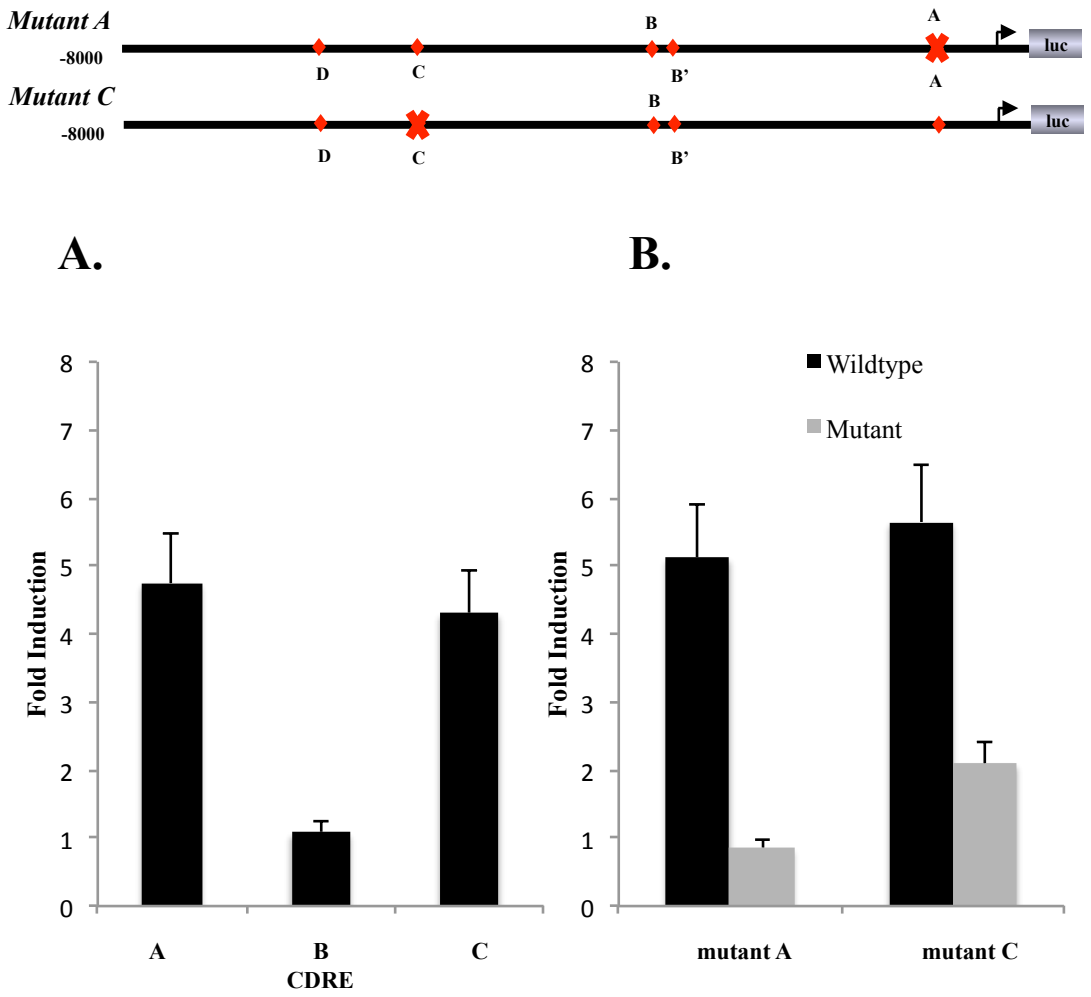

Figure 42: Cdx2 Activates *Foxa2* promoter. Luciferase assays were used to determine the ability of Cdx2 to activate the *Foxa2* promoter. Foxa2 luciferase vector was transiently transfected into P19 cells in the presence or absence of Cdx2 expressing vector. Luciferase reporter activity was measured and normalized to β-galactosidase. A. CDREs in the *Foxa2* promoter regions that show induction in the presence of Cdx2 B. These sites were mutated by site directed mutagenesis (CATAAA➔GAGCTC). Luciferase assays show loss of induction in the mutants when compared to the wild type. Assays were done in triplicate and errors bars indicate s.d. from the mean.

(Bossard and Zaret, 1998;Bosse et al., 2006;Cirillo et al., 2002;Jacobsen et al., 2002;Zaret, 1999), *Eomesodermin* (Arnold et al., 2008;Kartikasari et al., 2013;Teo et al., 2011) and *Cyp26a1* (Kinkel et al., 2009). To determine if Cdx2 and Foxa2 regulate the promoters of any of the above Foxa2 target genes, we determined which promoters had Cdx and Fox response elements in close proximity to one another (Figure 44B). Assays using *Cyp26a1* (Savory et al., 2009) and *Eomes* reporter vectors revealed that Cdx2 and Foxa2 activate each of these genes individually and can also synergize on these promoters (Figure 44C; Figure 53). We examined the effect of mutating the *Cyp26a1* CDREs on this synergistic response. In this regard, CDRE B has previously been shown to not affect promoter function and therefore was not explored (Savory et al., 2009). Reporter activity in which either CDRE C (mutant 1) or CDREs B and C were mutated (mutant 2) lead to a loss of the synergistic activation in the presence of Cdx2 and Foxa2 (Figure 44C). This suggests that the ability of Cdx2 and Foxa2 to synergize on the *Cyp26a1* promoter was dependent on the ability of Cdx to bind the *Cyp26a1* CDREs, as seen for the *Foxa2* promoter.

Cdx and Foxa2 Interact Genetically

Both Cdx2 and Foxa2 mutants exhibit gastrulation and AP patterning defects (Savory et al, 2009; Ang and Rossant, 19934). In order to determine whether Cdx2 and Foxa2 function in the same genetic pathway, a complementation assay was carried out. A Foxa2$^{f/f}$ (Sund et al, 2000) line was crossed into the *Cdx1*$^{-/-}$*Cdx2*$^{f/f}$ background and floxed alleles deleted to generate *Cdx1*$^{-/-}$*Cdx2*$^{+/-}$ Foxa2$^{+/-}$ compound mutants as well as littermate controls (*Cdx1*$^{-/-}$*Cdx2*$^{+/-}$Foxa2$^{+/+}$ and *Cdx1*$^{-/-}$*Cdx2*$^{+/+}$Foxa2$^{+/-}$). Relative to controls,

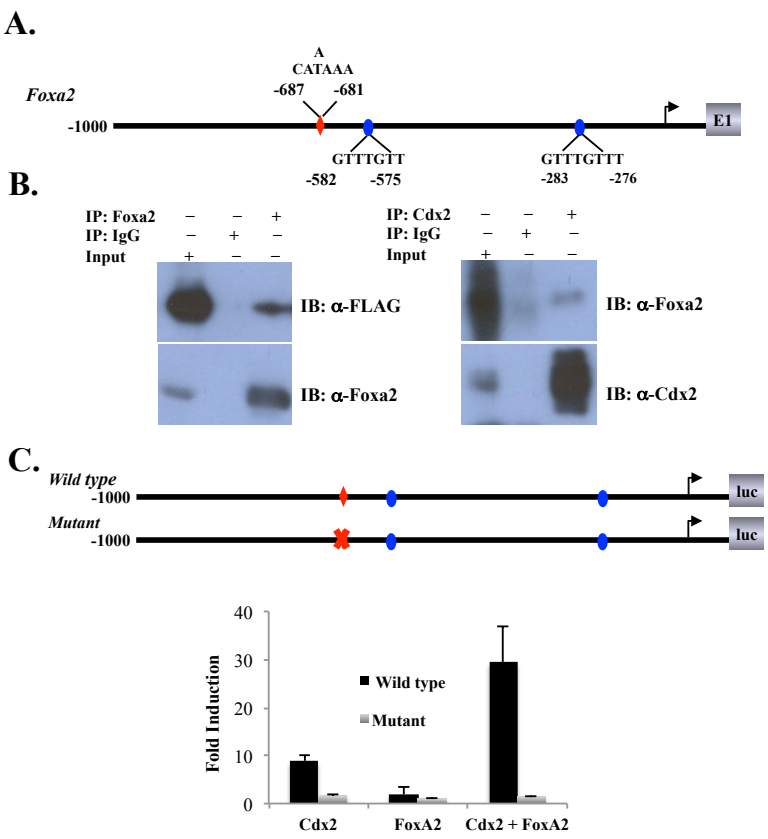

Figure 43: Cdx2 and Foxa2 Co-Regulate the *Foxa2* Promoter. A. Schematic showing the proximal -1kb region of the Foxa2 promoter showing the CDREs (red diamond), the Fox response elements (blue ovals), E1 exon 1 and the arrow at the TSS. B. Cos-7 cells were transfected with Flag-tagged Cdx2 and Foxa2 (left panels). Immunoprecipitation was done with anti-Foxa2 antibody and mouse IgG as a control and immunoblots were carried out with a FLAG and Foxa2 antibody. Gel shows enrichment of Flag-Cdx2 when immunopecipitated with Foxa2 as compared to mouse IgG controls. Cos-7 cells were also transfected with Cdx2 and Foxa2, and protein lysates immunoprecipitated with Cdx2 and rabbit IgG as a control (right panels). Immunoblots were carried out using a Foxa2 and Cdx2 antibody. Gel shows enrichment of Foxa2 when immunoprecipitated with Cdx2 compared to IgG controls. C. Luciferase reporter assays were used to determine whether Cdx2 and Foxa2 act synergistically to activate the Foxa2 promoter. Foxa2 luciferase vectors were transiently transfected into P19 cells in the presence of either Cdx2 alone, Foxa2 alone or both. Luciferase reporter activity was measured and normalized to β-galactosidase. Assays were done in triplicate and errors bars indicate s.d. from the mean. Note that synergy was lost when the CDRE was mutated.

E9.5 *Cdx1*$^{-/-}$*Cdx2*$^{+/-}$Foxa2$^{+/-}$ compound mutant embryos revealed a slight caudal truncation similar to that reported in Cdx1/2 DKO mutants, albeit considerably milder (Figure 45A). Examination of the Cdx2-Foxa2 bipartite target gene *Cyp26a1* further revealed a modest reduction in its expression in the tail bud of *Cdx1*$^{-/-}$*Cdx2*$^{+/-}$Foxa2$^{+/-}$ E9.5 mutants compared to controls (Figure 45A). Consistent with this, RT-PCR analysis showed that the transcript levels of *Cyp26a1,* as well as additional Cdx/Foxa2 target genes, were also attenuated in the compound mutants relative to littermate controls, including *Gata4* and *Wnt6* (Figure 45B). This finding is consistent with Cdx2 and Foxa2 co-regulating the expression of a number of common target genes.

DISCUSSION

In this study, we sought to further our understanding of the means by which Cdx2 impacts endoderm development through the identification of novel target genes. We show that *Foxa2* is a direct transcriptional target of Cdx2 and that there are functional and genetic interactions between Foxa2 and Cdx2 that are important for the regulation of a number of genes critical for endoderm differentiation.

Identification of a Novel Cdx Target Gene in the Mammalian Endoderm

Although essential roles for *Cdx2* in the development and patterning of the mammalian endoderm have been well established (Gao et al., 2009;Grainger et al., 2010;Stringer et al., 2012) little is known about the mechanisms by which Cdx2 exerts these effects. A number of useful markers have been identified that differentiate between

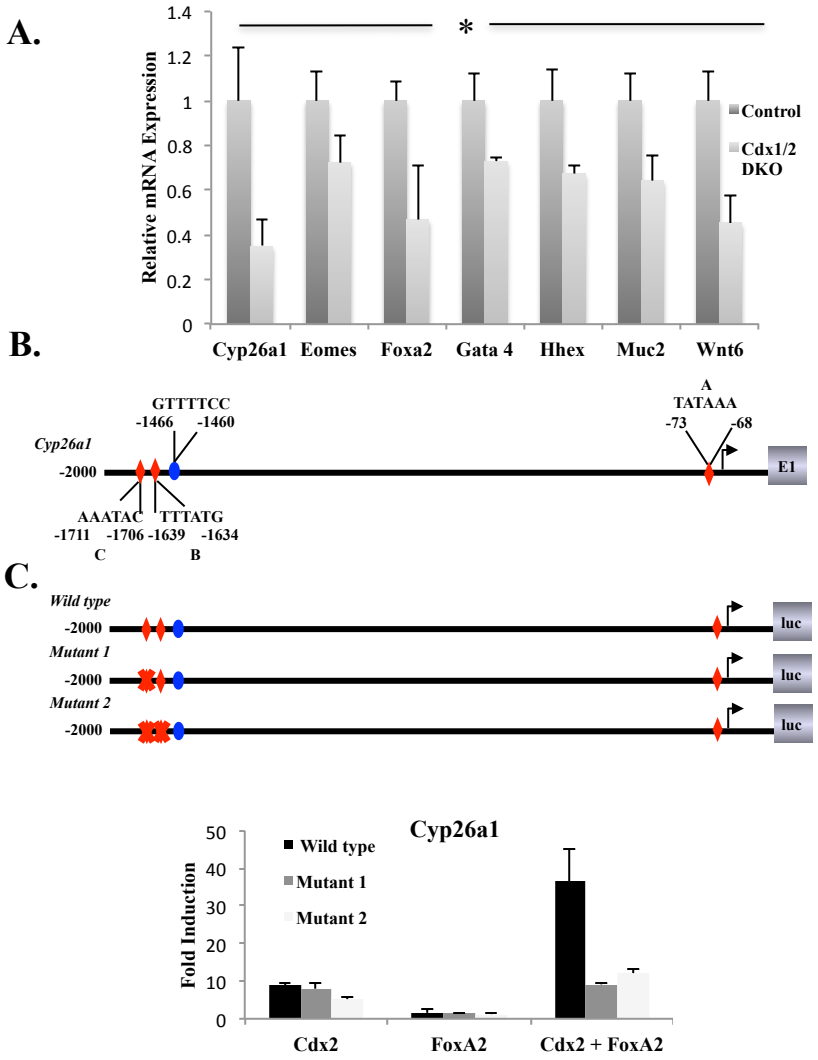

Figure 44: Cdx2 and Foxa2 Converge on Common Targets. A. RT-PCR of RNA from the caudal ends of E9.5 control and Cdx1/2 DKO mutants shows down-regulation of Foxa2 target genes when compared to littermate controls; *p< 0.05 by student's t-test. B. Schematic of -2kb Cyp26a1 promoter showing the CDREs (red diamonds), the Fox response elements (blue ovals); E1 denotes exon 1 and the arrow head indicates the TSS . C. Luciferase assays were used to assess the ability of Cdx2 and/or Foxa2 to synergistically activate the promoters of Cyp26a1. Wild type as well as mutant luciferase vectors were transiently transfected into P19 cells in the presence or absence of Cdx2 and/or Foxa2. Luciferase reporter activity was measured and normalized to b-galactosidase. Assays were repeated in triplicate and errors bars indicate s.d. from the mean. Cdx2 and Foxa2 were shown to synergize on the Cyp26a1 promoter. Again, synergy was lost when CDREs on the cup26a1 promoter were mutated.

the definitive endoderm, the visceral endoderm and other lineages at specific time points (McKnight et al., 2007;Sherwood et al., 2007;Sherwood et al., 2009;Wang et al., 2012). In the present study, we used an anti-EpCAM antibody to isolate endoderm and identified *Foxa2* as a Cdx target gene in this population. These results corroborate zebrafish studies in which attenuation of the Cdx2 ortholog *cdx1b* led to a significant reduction in the number of Foxa2-expressing endodermal precursors (Cheng et al., 2008). Conversely ectopic expression of *cdx1b* resulted in substantial increases in *foxa2* expressing endodermal precursors and an altered distribution pattern.

In the post-implantation embryo, Cdx was initially believed to function primarily through direct regulation of *Hox* genes (Chawengsaksophak et al., 2004;Epstein et al., 1997;Houle et al., 2003;Isaacs et al., 1998;Pilon et al., 2007;Shashikant et al., 1995;Subramanian et al., 1995;Tabaries et al., 2005). However, a number of non-Hox targets have since been identified, including members of the Notch, Wnt and planar cell polarity pathways (Grainger et al., 2010;Savory et al., 2009;Savory et al., 2011). With respect to endoderm, many of the previously identified Cdx2 targets are relevant to later terminal differentiation processes in the intestine and include genes such as *Muc2* (Mesquita et al., 2003), *Muc4* (Jonckheere et al., 2004), *sucrose isomaltase*, *intestinal phospholipase A* (*IPAL*) (Boudreau et al., 2002) and *intestinal alkaline phosphatase (IAP)* (Alkhoury et al., 2005). More recent analysis, however, illustrates critical roles for Cdx in events related to specification, patterning and homeostasis of the intestine (Gao et al., 2009;Grainger et al., 2010;Hryniuk et al., 2012;Verzi et al., 2013).

Little is known about how *Foxa2* itself is regulated. Foxa2 has been shown to participate in an auto- and cross-regulatory loop with other members of the Foxa

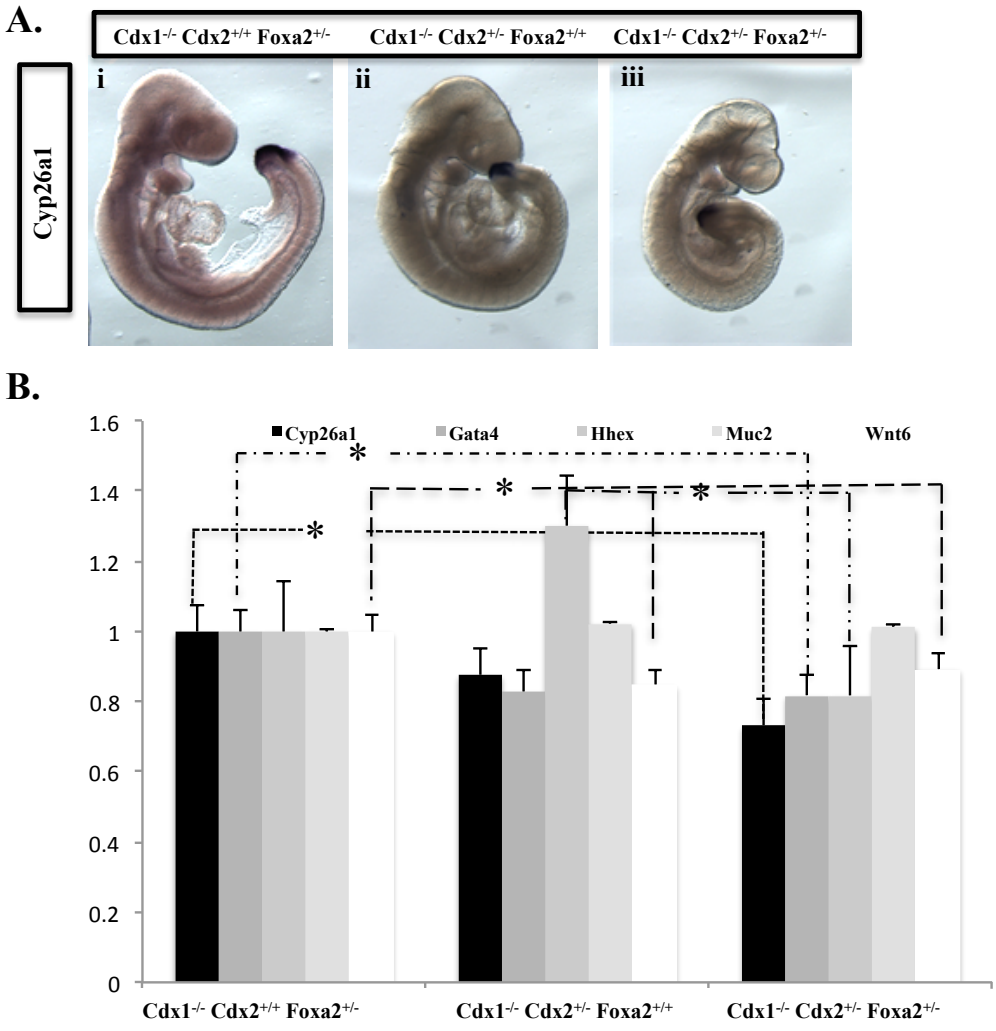

Figure 45: *Cdx2* and *Foxa2* Interact Genetically. A. E9.5 $Cdx1^{+/-}$ $Cdx2^{+/-}$ $Foxa2^{+/-}$ embryos (iii) showing modest axial truncation when compared to litter mate controls (i-ii). Whole mount in situ hybridization showing moderate of loss of *Cyp26a1* mRNA expression in tail bud of $Cdx1^{-/-}$ $Cdx2^{+/-}$ $Foxa2^{+/-}$ E9.5 embryo (iii) when compared to controls (i-ii). B. RT-PCR analysis shows reduced expression of Foxa2 targets in $Cdx1^{+/-}$ $Cdx2^{+/-}$ $Foxa2^{+/-}$ embryos when compared to controls. Error bars indicate s.d. from mean. *$p<0.05$ by student's t-test.

family (Odom et al., 2006;Pani et al., 1992;Sasaki and Hogan, 1994) and is also thought to be downstream of Sox17 in the mouse (Kanai-Azuma et al., 2002;Sinner et al., 2004). However *Foxa2* is unaffected in *Sox 17* null embryos (Howard et al., 2007) suggesting that its expression may be regulated by other factors. Foxa2 has also been shown to be regulated by miRNAs, such as miR-335 (Yang et al, 2014). We show here that *Foxa2* can also be regulated by Cdx2.

Foxa2 is a Transcriptional Co-Factor for Cdx2

Our results suggest that Cdx2 interacts with Foxa2 *in vitro*. Although it is yet to be determined whether this interaction is direct and dependent on the ability of either protein to bind DNA, we propose that Cdx2 and Foxa2 form a complex at the *Foxa2* promoter. The co-regulation of gene expression by physically interacting transcription factors is not an uncommon theme, including in the endoderm. One such example is the interaction between HNF6 and Foxa2, leading to transcriptional activation in a p300/CBP dependent manner (Rausa et al., 2003). Similarly, Foxa2 can also interact and synergize with Neurogenin3 to enhance its auto-activation loop during pancreatic differentiation (Blanpain and Fuchs, 2009;Kanai-Azuma et al., 2002).

On a more global level, ChIP-seq studies have shown Foxa2 enriched motifs associated with HNF4a, HNF6 and GATA binding motifs. Furthermore, interactions amongst these proteins were revealed by co-immunoprecipitation (Wallerman et al., 2009). Cdx2 has also been shown to cooperate with other transcriptional co-factors such as p300 (Hussain and Habener, 1999), LEF/TCF members (Beland et al., 2004;Grainger et al., 2013) and Oct3/4 in the trophectoderm (Niwa et al., 2005). With respect to the

endoderm, Cdx2 has been shown to form complexes with Gata4 and HNF1-A (Benoit et al., 2010;Mitchelmore et al., 2000) as well as with LEF/TCF members (Grainger et al., 2013;Verzi et al., 2010). Consistent with a function for such complexes in transcription, regions of Cdx2 occupation in the intestine are highly enriched for GATA, HNF and LEF/TCF binding motifs (Verzi et al., 2010).

Both Cdx2 and Foxa2 target different genes in a number of tissues where they affect gene expression. Although Foxa proteins bind a specific consensus sequence, they also show significant nonspecific binding (Sekiya et al., 2009), suggesting additional mechanisms to ensure functional specificity. It is possible that additional co-factors are key determinants of such specificity and that Cdx2 may contribute such a role within the endoderm.

As mentioned previously, there are a number of endodermally expressed Foxa2 target genes such as *Foxa1, Foxa2, Muc2, Muc4, Gata4, Isl1, Cyp26a1* and *Eomes* (Burgeson, 1993;Burgeson and Christiano, 1997;Fuchs and Raghavan, 2002;Niehrs, 2006;Nishiyama et al., 2000). A number of these have been shown to be down-regulated in the absence of Cdx2 and/or have been postulated to be Cdx2 target genes (Gao et al., 2009;Savory et al., 2009) consistent with a critical role for Cdx2 upstream of *Foxa2*. Notably, a number of these target genes have also been shown to be directly regulated by Cdx2 (Malissen et al., 2014;Marinkovich et al., 1993;Schultheiss et al., 1995;Tamoutounour et al., 2013). Our present findings suggest that there are subsets of genes, such as *Cyp26a1* and *Eomes* that are co-regulated by Cdx2 and Foxa2. These findings are also consistent with a role for both Foxa2 and Cdx2 in the development of the definitive endoderm (Ang et al., 1993;Hallonet et al., 2002) as supported by

complementation analysis showing loss of expression of *Cyp26a1* and *Gata4* in Cdx-Foxa2 compound mutants relative to controls. Similar to what we propose here, genome-profiling studies have shown that the Foxa motif is overrepresented at Cdx2 sites suggesting that they may co-regulate a number of genes (Verzi et al., 2010).

With respect to onset of expression within the developing embryo, Foxa2 is expressed much earlier than Cdx2 (Kaestner et al., 1994;Lohnes, 2003). In fact, recent time-lapse imaging studies have shown that Foxa2 is expressed in the presumptive definitive endoderm cells before migration through the primitive streak and is maintained in these cells as they go on to populate the developing gut tube (Viotti et al., 2014). This suggests that Foxa2 function in the endoderm is before that of Cdx2. These observations can be aligned with our data when the "pioneer factor" activity of Foxa2 is considered. As a pioneer factor, Foxa2 is one of the first transcription factors to bind chromatin to either act as a place-holder for other factors to engage the promoter or actively recruit these factors for transcription initiation (Sekiya et al., 2009;Zaret and Carroll, 2011). Foxa2 is therefore important for the specification of cell lineages during development. In the endoderm, it has been suggested that Foxa proteins cause the tissue to become 'competent' to respond to inductive signals from the mesoderm that are necessary for tissue-specific differentiation into organs (Cirillo et al., 2002). Genome profiling studies suggest that Foxa2 does this by occupying the regulatory regions of thousands of silent genes in a tissue and time dependent manner to convey competence for subsequent tissue-specific transcriptional programs (Alder et al., 2014). In the absence of Foxa, gene regulatory regions cannot be assessed by other transcription factors. Foxa2 can also regulate gene expression by acting as a 'classical' transcription factor and a large number

of Foxa2 targets have been identified (Friedman and Kaestner, 2006). This function is carried out subsequently to its role as a pioneer factor. Although a few studies suggest that Cdx2 may have some pioneer factor attributes, as it has been shown to control enhancer chromatin structure to direct access of other TFs (Lewis et al., 2014;Verzi et al., 2013), it is not clear whether Cdx2 can initiate chromatin access. It is therefore more likely that Cdx2 and Foxa2 operate as co-factors in the more traditional ways of genes regulation.

Cdx2 and Foxa2 in Axial Elongation

Both Cdx2 and Foxa2 mutants show gastrulation defects and disruption in AP patterning (Cookson, 2004;Hudson et al., 1997;Xavier et al., 2008). Consistent with both transcription factors impacting a common pathway reflected in these phenotypes, we also found increased axial truncation in the $Cdx1^{-/-}Cdx2^{+/-}Foxa2^{+/-}$ embryos, relative to controls. This truncation occurred concurrently with reduced expression of *Cyp26a1,* which is expressed in the tail bud during axial elongation. *Cyp26a1* is a direct Cdx target gene (Savory et al., 2009;Young et al., 2009) and inactivation of *Cyp26a1* leads to tail truncations as seen in Cdx mutants, as well as abnormalities of the kidneys, urogenital tract and posterior gut (Abu-Abed et al., 2001). Similarly, *Cyp26a1* is down-regulated in Foxa2 null mice (Tamplin et al., 2008) and has been suggested to be a Foxa2 target (Xu et al., 2012). The overlap in axial phenotypes seen in the Cdx2 and Foxa2 mutants suggests that they not only cooperate in the endoderm alone but also in other lineages. Finally, studies in zebrafish show that in the absence of the Cdx2 ortholog *cdx1b*, Foxa2

expression was reduced in the prechordal plate as well as in the endoderm, further suggesting that Cdx2 may regulate *Foxa2* in multiple lineages (Cheng et al., 2008).

In conclusion, our present findings show that *Foxa2* is regulated by *Cdx2*. In addition, the cooperation between Foxa2 and Cdx2 on the promoters of target genes provides a mechanism by which Cdx2 regulates *Foxa2* function in anterior-posterior patterning of the embryo.

Acknowledgements

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CHAPTER 7-GENERAL DISCUSSION II

The *Cdx* gene family of transcription factors has been shown to play a critical role in the development and patterning of the endoderm and the mature intestinal epithelium. In this study, we sought to better understand how *Cdx* impacts endoderm. We show that *Cdx2* regulates the *Foxa2* promoter and that both proteins physically and genetically interact to activate genes that are involved in endoderm formation.

Novel Cdx Targets in the Endoderm

Until recently, *Cdx* was believed to operate in developmental processes primarily through the regulation of Hox genes (Charite et al., 1998;Davidson and Zon, 2006;Deschamps et al., 1999;Gaunt et al., 2004;Isaacs et al., 1998;Tabaries et al., 2005;Young et al., 2009). A number of non-Hox targets have now been identified including genes involved in planar cell polarity and posteriorization of the embryo (Savory et al., 2009;Savory et al., 2011). In addition, several Cdx targets involved in the development of the intestine have also been identified (Hryniuk et al., 2014). However, very few *Cdx* targets involved in the early events of endoderm patterning and formation have been identified. The development of the definitive endoderm is poorly understood largely due to a relative paucity of markers available. Over the last few years, a number of gene profiling studies have sought to identify candidate genes that could be used to label the endoderm at specific time points (Hou et al., 2007;Sherwood et al., 2007;Wang et al., 2012). One of the genes identified in these studies, *EpCAM*, was used to mark the mammalian endoderm in our experiments. Using *EpCAM* to mark the Cdx1/2 DKO endoderm, we identified by microarray analysis a number of targets that were down-regulated in the Cdx1/2 DKO endoderm compared to the control. Included in this list are

known *Cdx2* targets genes such as *fabp2/ifabp* and *Dll1* and novel *Cdx2* target *Foxa2*. In addition, our microarray studies also showed that a number of genes enriched in the definitive endoderm were also down-regulated in the Cdx1/2 DKO endoderm (Table 8). These include genes coding for proteins such as Embigin, a member of the immunoglobulin super family important for integrin mediated cell substratum adhesion (Huang et al., 1993), Spink3, a serine protease inhibitor expressed in the pancreas, kidney and lung (Neuschwander-Tetri et al., 2004), Tmem30b, a little known transmembrane protein associated with malignant peritoneal mesothelioma (Perez-Magan et al., 2012), Desmoglein 2 (Dsg2) a membrane associated desmosome protein (Arnemann et al., 1992) linked to apoptosis in the intestinal epithelium (Nava et al., 2007), cardiomyopathy (Awad et al., 2008) and a number of skin conditions (Amagai, 2010) as well as peptide-tyrosine tyrosine (Pyy), a small peptide released by cells in the gastrointestinal tract and stomach, important for digestion and eating behavior (Manning and Batterham, 2014;Price and Bloom, 2014;Tatemoto, 1982) (Table 8). Although the roles of these genes in the endoderm are mostly unknown, it would be interesting to investigate if they are direct Cdx targets.

Given its critical role in the development of endoderm-derived organs, we were most interested in *Foxa2* as a potential *Cdx2* target. Although the endoderm is specified in Foxa2 mutant embryos, the foregut and midgut are severely disrupted while the hindgut is less affected (Ang and Rossant, 1994;Dufort et al., 1998;Weinstein et al., 1994). This suggests that other factors may function with Foxa2 to regulate posterior endoderm patterning. We postulated that Cdx2 is a very strong candidate for such a cooperative factor. Loss of Cdx2 at E9.5 in the early endoderm results in abnormal

patterning of the A-P axis where the more posterior endoderm takes on properties of the more anterior endoderm (Gao et al., 2009). If Foxa2 and Cdx2 function in the same processes, then the compound mutant should phenocopy the single mutants. Conversely, the presence of a different phenotype would suggest that the combination of Cdx2 and Foxa2 mediate programs distinct from those regulated by either factor on its own. The Cdx-Foxa2 compound mutants examined in this study show that neither *Foxa2* nor *Cdx2* is able to compensate for one another with respect to endoderm gene expression suggesting that these genes require both transcription factors for optimal expression. Such non-allelic non-complementation implies that both genes function in a common pathway. It is important to note however, that examination of the A-P patterning of the endoderm beyond E9.5 was precluded by the nature of the Cre used, as loss of *Foxa2* and *Cdx2* in other tissues confounded the interpretation of any observable phenotype.

Cdx2 Co-Regulators

Combinatorial control of gene expression is often necessary for the regulation of complex biological processes. In addition, such transcription factors can also physically interact. There have been over 10,000 Foxa2 and Cdx2 binding sites identified throughout the genome (Nishiyama et al., 2009;Wederell et al., 2008) and some of these sites are occupied in association with other TFs. Indeed, Cdx2 and Foxa2 occupied regions are generally enriched for motifs that bind other endodermal transcription factor families (Alder et al., 2014;Verzi et al., 2010). Although it is not entirely clear how protein-interaction decisions are made, the ability to bind other co-factors is often a key determinant of specificity of action for a TF factor with thousands of binding sites. In

addition, tissue-specific gene expression can be governed by TFs that may either be restricted to certain cell types or may be more broadly expressed. In the latter case, specificity can be effected by collaboration with other more restricted TFs or co-factors. A number of studies have shown that Cdx2 participates in obligate pairings with more widely expressed factors to modulate distinct functions in the intestine (San Roman et al., 2014;Verzi et al., 2010;Verzi et al., 2010). With respect to Cdx2 and Foxa2, Cdx2 expression becomes increasingly restricted to the posterior gut as the intestine develops while Foxa2 remains expressed more broadly within the endoderm. Given these expression patterns, Cdx2 may help to direct Foxa2 activity within the endoderm. We show here that Cdx2 interacts with Foxa2 *in vitro* and propose that Cdx2 may contribute to the specificity of action of Foxa2 within the endoderm.

Although our studies suggest that *Cdx2* is upstream of *Foxa2* in the transcriptional hierarchy, another study has shown that Foxa2 regulates *Cdx2* expression by binding at a shadow intronic enhancer 7kb from the transcriptional start site (Watts et al., 2011). This complicates our interpretation of the relative position of both TFs within the transcriptional hierarchy. In considering this, we must recognize the ability of Foxa2 to function either as a typical transcription factor or as a pioneer factor. As a transcription factor it binds and regulates the promoter regions of target genes in the classical manner. However as a pioneer factor, Foxa2 binds chromatin to facilitate binding of other factors or actively recruit these factors for transcriptional initiation (Cirillo et al., 1998;Sekiya et al., 2009;Zaret, 1999;Zaret and Carroll, 2011). Foxa2 therefore occupies the regulatory regions of thousands of genes that are not yet being transcribed, in a tissue and time dependent manner (Alder et al., 2014). Once these genes are activated, Foxa2 can then

change its distribution across the genome. In this fashion, Foxa2 may function upstream of Cdx2. In addition, a reciprocal activation between Foxa2 and Cdx2 could serve to strengthen the regulatory circuit responsible for the development of the endoderm. Our studies support such a feed-forward model in which *Cdx2* directly activates the expression of its transcriptional binding partner, Foxa2.

Cdx2 and Foxa2 in Other Lineages

Foxa2 and Cdx2 have both been shown to function in tissues other than the endoderm. Cdx mutants have varying severities of axial patterning defects and posterior truncation representative of dysfunction in the paraxial mesoderm (Table 5). For example, the Cdx1/2 DKO mutants used in this study exhibit malformed, fused and improperly segmented somites, and the neural tube defect craniorachischisis in which the neural tube fails to close (Savory et al., 2011). Foxa2 nulls do not form a node or a notochord and show defects in the organization of somites and neural tube (Ang and Rossant, 1994;Weinstein et al., 1994). Specifically, the Foxa2 nulls have defects in dorsal-ventral patterning of the neural tube and in the development of the axial mesoderm (Ang and Rossant, 1994;Filosa et al., 1997;Weinstein et al., 1994). Finally, loss of Foxa2 and Foxa3 abolishes formation of all axial derivatives in zebrafish (Dal-Pra et al., 2011). The $Cdx1^{-/-}Cdx2^{+/-}Foxa2^{+/-}$ compound mutants examined in this study also exhibited a modest caudal truncation further evidenced by reduced expression of *Cyp26a1* and reduction in number of somites (data not shown). Although these observations necessitate further investigation, it suggests that Foxa2 and Cdx2 function together in the axial mesoderm as well as in the endoderm.

Future Directions

The development of the mammalian endoderm is a very intricate process that is still not well understood. Progress in the study of these processes is further complicated by the fact that many of the relevant genes are expressed in both the definitive and visceral endoderm and/or mesoderm. The lack of suitable molecular probes for fundamental aspects of endoderm development has also been a significant hindrance. In addition, reciprocal interactions between the endoderm and adjacent mesoderm, which are crucial for endoderm differentiation, must be taken into consideration (Lewis and Tam, 2006). It will therefore become very important to develop an informative panel of markers that can be used to label and define the definitive endoderm at specific time points.

In this study, we performed microarray on RNA isolated from endoderm tissue by FACS analysis. One major shortcoming of flow cytometry is the low cell throughput rate, as even high-speed sorters only sort a few thousand cells per second (Givan, 2011;Ibrahim and van den Engh, 2007). To obtain enough cells and RNA for microarray analysis, carried out over long periods of time. This may affect the quality of the RNA as it can deteriorate over time. Although the RNA was amplified to obtain more material, and we checked that the relative transcript levels of genes after amplification was similar to levels before amplification, the quality control biometrics suggest that the fluorescent signals obtained from the Affymetrix chips were low. To circumvent these shortcomings, an alternative source of RNA could be obtained, such as from the Cdx1/2 null ESCs that have been created in the lab (J.A.G.S, D.L., unpublished observations). These cells can be differentiated along the endodermal lineage using well-defined protocols that yield a

population of cells, 80% of which are double positive for definitive endoderm markers Sox17 and Foxa2 (Borowiak et al., 2009;Mfopou et al., 2014;Naujok et al., 2014). This not only provides a uniform and relatively pure source of cells but also provides sufficient material that does not need prior amplification. Alternatively, transcriptome-profiling studies can be done using the more sensitive RNA-seq. Advantages of RNA-seq over microarray are that it not only provides a more dynamic range of sensitivity, but it also requires smaller amounts of starting material and allows detection of novel transcripts even at the single cell level (Chepelev et al., 2009;Ozsolak and Milos, 2011;Treutlein et al., 2014).

There are still many outstanding questions regarding Cdx biology within the endoderm. To determine Cdx mode of action, its other protein interacting partners will have to be determined. This can be done by proximity-dependent biotin identification (BioID) (Roux et al., 2012;Roux, 2013) or affinity purification followed by mass spectrometry (AP-MS) optimized for chromatin-associated proteins (Armean et al., 2013;Gingras et al., 2007;Lambert et al., 2014) using appropriate cell types. It is also important to identify other Cdx targets within the endoderm. In order to do so, ChIP-seq experiments can be paired with the gene profiling studies described in this thesis or using the alternative source of RNA as described above. This will allow us to determine Cdx occupancy across all genes within the endoderm. Direct targets are genes that are both down-regulated and occupied at promoter/enhancer regions by Cdx protein. Such targets can then be verified by RT-PCR, directed ChIP, reporter assays and transgenesis.

The role of Foxa2 in specifying liver progenitor cells and maintaining liver function in the adult is well documented. However, there still remain some unanswered

questions about its role in the mammalian endoderm. Such studies have been complicated by the lack of an effective endoderm-specific Cre. The *Foxa3*-Cre for example, leads to incomplete excision of *Foxa2* because *Foxa3* is not expressed uniformly throughout the entire endoderm (Lee et al., 2005). In order to more thoroughly address the roles of Foxa2 in the early endoderm, a Foxa2 knockout mouse could be created using the more efficient *villin*-Cre (el Marjou et al., 2004;Grainger et al., 2010). Foxa2 targets within the endoderm can then be identified by carrying out gene expression experiments similar to those outlined above. In addition, the complementation studies performed here could be carried out using the *villin*-Cre. This would allow us to more closely examine the effects of *Cdx2* and *Foxa2* on the A-P patterning of the endoderm.

In this thesis, we have identified some genes dependent on both *Cdx2* and *Foxa2*. To determine the entire subset of genes co-regulated by Cdx2 and Foxa2, independent Cdx2 and Foxa2 ChIP-seq experiments can be performed. Binding peaks obtained from both experiments can then be overlaid to determine genes that are occupied by both proteins. Again, this genome occupancy data can then be paired with mRNA expression data to determine genes that are targets of both proteins. It will be interesting to see if Cdx2 and Foxa2 co-regulation is a more general mechanism by which endoderm development is regulated. Other transcription factors such as GATAs, Sox17 and HNF4 are known to be involved in endoderm formation. In order to obtain a more complete picture of how these TFs interact, motif enrichment can be carried out on the Cdx2 and Foxa2 ChIP-seq data sets. Enriched TF motifs close to the Cdx2 or Foxa2 peaks can be identified to provide clues as to which other TFs could potentially cooperate with Cdx2 or Foxa2 in the regulation of the mammalian endoderm. In many instances, a number of

these sequence motifs occur at active or poised promoters and enhancers as well as at transcribed regions. These regions often exhibit informative histone marks or differential DNA methylation to indicate whether they are activated or modifications that reveal mode of chromatin repression such as those mediated by Polycomb or heterochromatin proteins. In many cases, these genome-wide chromatin states are associated with developmental cues (Chen and Dent, 2014;Zhu et al., 2013). It might therefore be informative to perform ChIP-seq on endoderm tissue using antibodies for some of these activating or repressive markers. The peaks obtained from this experiment can be compared to those from a Cdx2 or Foxa2 ChIP-seq to determine which of the regions co-bound by both TFs are transcriptionally active or silent.

Finally, to fully understand how the Cdx and Fox gene families impact on endoderm formation, the roles of the other family members will have to be considered. Foxa2 is undoubtedly the main Foxa player in endoderm function, however Foxa1 and Foxa3 are also expressed in the endoderm and studies have shown functional redundancy amongst the Fox family (Dal-Pra et al., 2011;Gao et al., 2008;Lee et al., 2005;Maier et al., 2013). Indeed, ChIP studies show that Cdx2 occupies the Foxa1 promoter as well (Figure 54). Therefore it might be necessary to investigate Cdx2 and Foxa2 interactions in the endoderm within the context of Foxa1. With respect to functional redundancy within the *Cdx* family, Cdx1 and Cdx2 can target and regulate the same genes (Kakizaki et al., 2010;Verzi et al., 2011). There are also some examples in which a gene is differently responsive to Cdx members (Alkhoury et al., 2005;Koslowski et al., 2009;Ma et al., 2012). Although most of the mouse models described above suggest that Cdx1 and Cdx2 have distinct and independent functions, a number of studies using Cdx compound

mutants support functional overlap among Cdx members. Generally, these mutants show increased severity of vertebral and intestinal phenotypes when compared to their corresponding single mutant counterparts (Hryniuk et al., 2012;Savory et al., 2011;van den Akker et al., 2002;van Nes et al., 2006). In addition, gene substitution studies in which the *Cdx2* gene was knocked into the *Cdx1* locus show that *Cdx2* can rescue the axial patterning defects observed in the Cdx1 null mice, suggesting that *Cdx2* is functionally equivalent to *Cdx1* function in vertebral patterning (Savory et al., 2009). This is however not the case in the gut where *Cdx2* cannot be expressed from the *Cdx1* locus at levels that support gastrointestinal function (Grainger et al., 2013). It is still unclear whether *Cdx1* can functionally compensate for *Cdx2*. To this end, a knock-in allele in which *Cdx1* is placed under the control of the *Cdx2* promoter has been constructed for creation of a *Cdx1* knock-in *Cdx2* knock-out mouse; ES clones for creation of the mouse line are currently being analyzed (Figure 55). It might therefore be informative to determine whether Foxa genes are also Cdx1 targets and explore other possible interaction between Cdx1 and Fox family members.

In conclusion, cooperative control among two or more transcription factors allows for the various specific transcriptional outcomes that are necessary for complex processes such as endodermal development. In this study, we show that the combinatorial activity of *Cdx2* and *Foxa2* is important for optimal development of the mammalian endoderm.

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APPENDICES

APPENDIX I

Table 1. Selective Ion Permeability of Claudins. Table shows effects of Claudins on ion permeability.

Cldn	Ion Selectivity	References
Decreased Permeability; Barrier-Forming (Tight)		
1	Less permeable to cations	Inai et al, 1999; McCarthy et al, 2000
3	Less permeable to cations	Milatz et al, 2010
4	Less permeable to cations	Van Itallie et al, 2001; Hou et al., 2009; Hou et al., 2010
5	Less permeable to cations	Wen et al., 2004
6	Less permeable to cations	Sas et al., 2008
8	Less permeable to cations	Angelow, 2005; A. S. L. Yu, 2003
9	Less permeable to cations	Sas et al., 2008
11	Less permeable to cations	Van Itallie et al, 2003
14	Less permeable to cations	Ben Yosef, 2003
18	Less permeable to cations	Jovov et al., 2007
7	Less permeable to cations	Alexandre et al., 2005; Alexandre et al., 2007
19	Less permeable to anions*	Hou et al., 2008; Angelow and Yu, 2007
Increased Permeability; Pore Forming (Leaky)		
2	More permeable to cations	Amasheh et al., 2009; Colegio et al., 2002; Furuse et al., 2001; Yu et al., 2009
10	More permeable to cations	Van Itallie and Anderson, 2006
15	More permeable to cations	Colegio et al., 2002; Colegio et al., 2003; Van Itallie et al., 2003; Tamura et al., 2011; Inai et al., 2011
16	More permeable to cations *	Hou, 2005; Ikari et al., 2007; Hou et al., 2008; Kausalya et al., 2006
17	More permeable to anions	Krug et al., 2012

* indicates conflicting data

Table 2. Summary of Claudin Mouse Models and Associated Phenotypes.

Claudin	Mouse Model	Phenotype	Reference
1	Down regulation in skin	Psoriasis and Dermatitis	De Benedetto et al, 2011, Watson et al, 2007
1	Loss of function mutation	Liver dysfunction and neonatal scaly skin (ichthyosis)	Hadj Rabia et al, 2004
1	Knockout	Barrier dysfunction, dehydration and death	Furuse et al, 2002
2	Knockout	Hypocalciuria	Muto et al, 2010
5	Knockout	Death within 10 hours	Morrow et al, 2010
6	Inhibition by clostridium perfringens enterotoxin	Inhibits blastocyst formation via trophectoderm barrier breakdown	Moriwaki et al, 2007
6	Knockout in early endoderm	No observable phenotype; animals viable and fertile	Anderson et al, 2008
6	Overexpression in epidermis	Barrier dysfunction, dehydration and death	Turksen and Troy, 2002
7	Knockout	Urinary ion wasting and dehydration; death within 10 days	Tatum et al, 2009
9	Missense mutation	Deafness associated with high perilymph potassium levels	Nakano et al, 2009
11	Knockout	Sterile, slow nerve conduction, inner ear deafness	Gow et al, 1999
14	Knockout	Deafness due to degeneration of outer hair cells, renal absorption problems	Gong et al, 2012; Ben Yosef et al 2003
15	Knockout	Enlargement of small intestine	Tamura et al, 2008
16	Mutant	Familial hypomagnesemia, hypocalciuria and nephrocalcinosis (FHHNC)	Gunzel et al, 2009
16	Knockout	Renal divalent cation wasting	Will et al, 2010
18	Knockout	Osteoporosis due to increased bone resorption	Linares et al, 2012
19	Knockout	Peripheral neuropathy	Miyamoto et al, 2005
19	Mutant	Severe FHHNC with ocular defects	Konard et al, 2006

Table 3. Summary of Claudin 6 Over-expression Mouse Models

Transgenic Mouse Model	Phenotype	Reference
Cldn 6 (Homozygous)	-Death 24-48 h after birth due to dehydration -Thicker epidermis but thinner skin due to decreased subcutaneous fats. -Disorganized epidermal layers	Turksen and Troy, 2002
Cldn6 (Heterozygous)	-Sufficient barrier function for survival at birth. -Thinner skin at birth -Epidermal hyperkeratosis and decreased dermal collagen. -Altered hair type-distribution and hair cycle -Epidermis repaired by day 4	Troy et al., 2005
CΔ187 Cldn6 truncation (minus last 32 AA)	-Failure of Cldn6 to localize to the membrane -Indistinguishable from wild type at birth -Epidermal thickening and hyper-proliferation with age	Arabzadeh et al., 2006
CΔ196 Cldn6 truncation (minus last 23 AA) HIGH transgene level	-Barrier defect at birth -Death 1month after birth -Nude mice -Hair follicles replaced by cysts. -Epidermal hyperplasia and hyperkeratosis.	Troy and Turksen, 2007
CΔ196 Cldn6 truncation (minus last 23 AA) LOW transgene level	-Normal barrier at birth -2 week lag in epidermal maturation. -Thickened epidermis with aging -Sensitized to injury -Dermatitis at 2 mths	Troy et al., 2009
CΔ206 Cldn6 truncation (minus last 13 AA)	-Sufficient barrier function at birth -3 day lag in epidermal maturation.	Enikanolaiye, et al., 2010

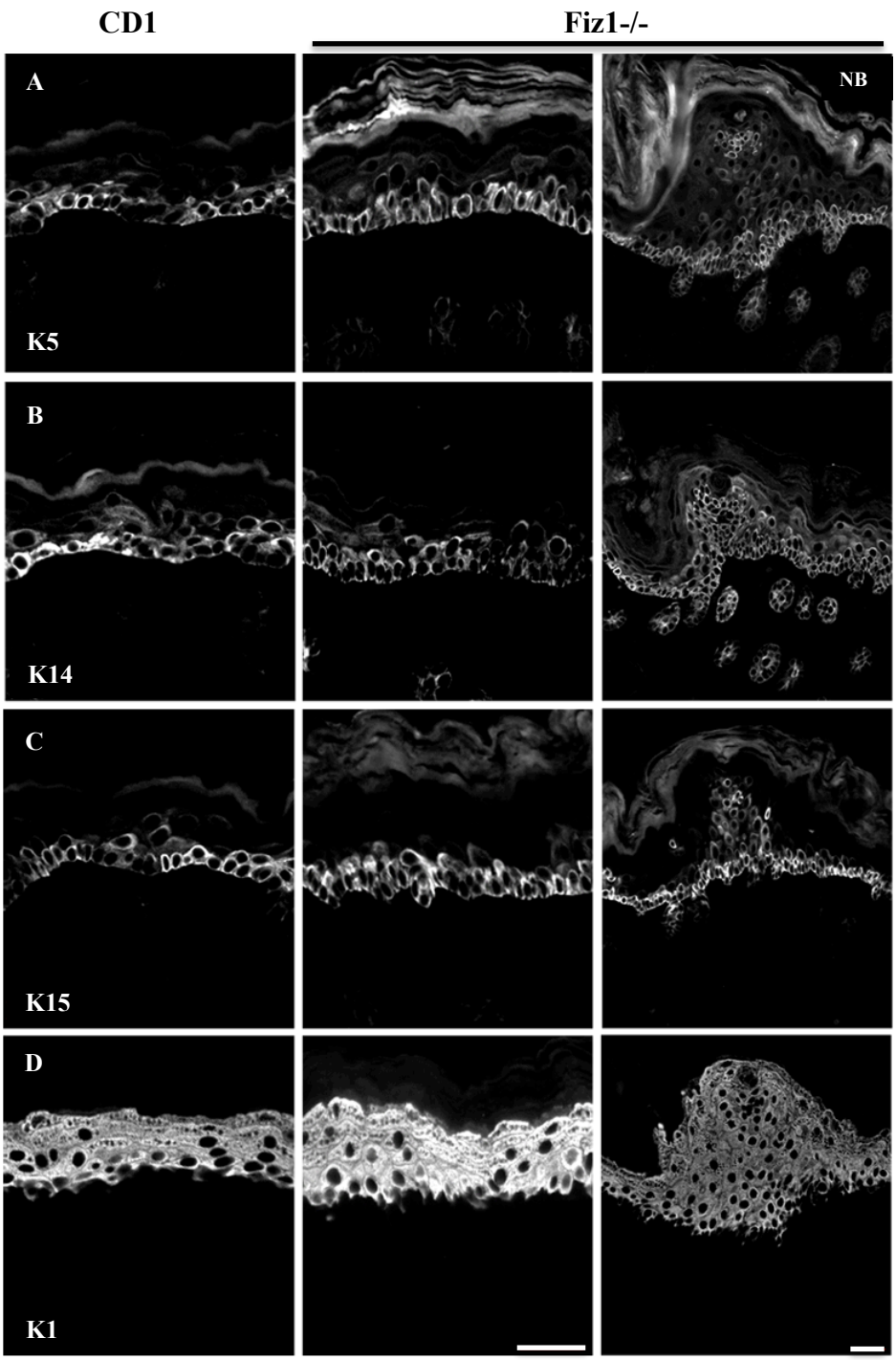

Figure 46: Immunohistochemistry of Newborn Fiz1 Knock-out Mouse Epidermis Using Keratin Antibodies. Keratin expression profile is expanded in the Fiz1 knock-out epidermis indicating an immature epidermis.

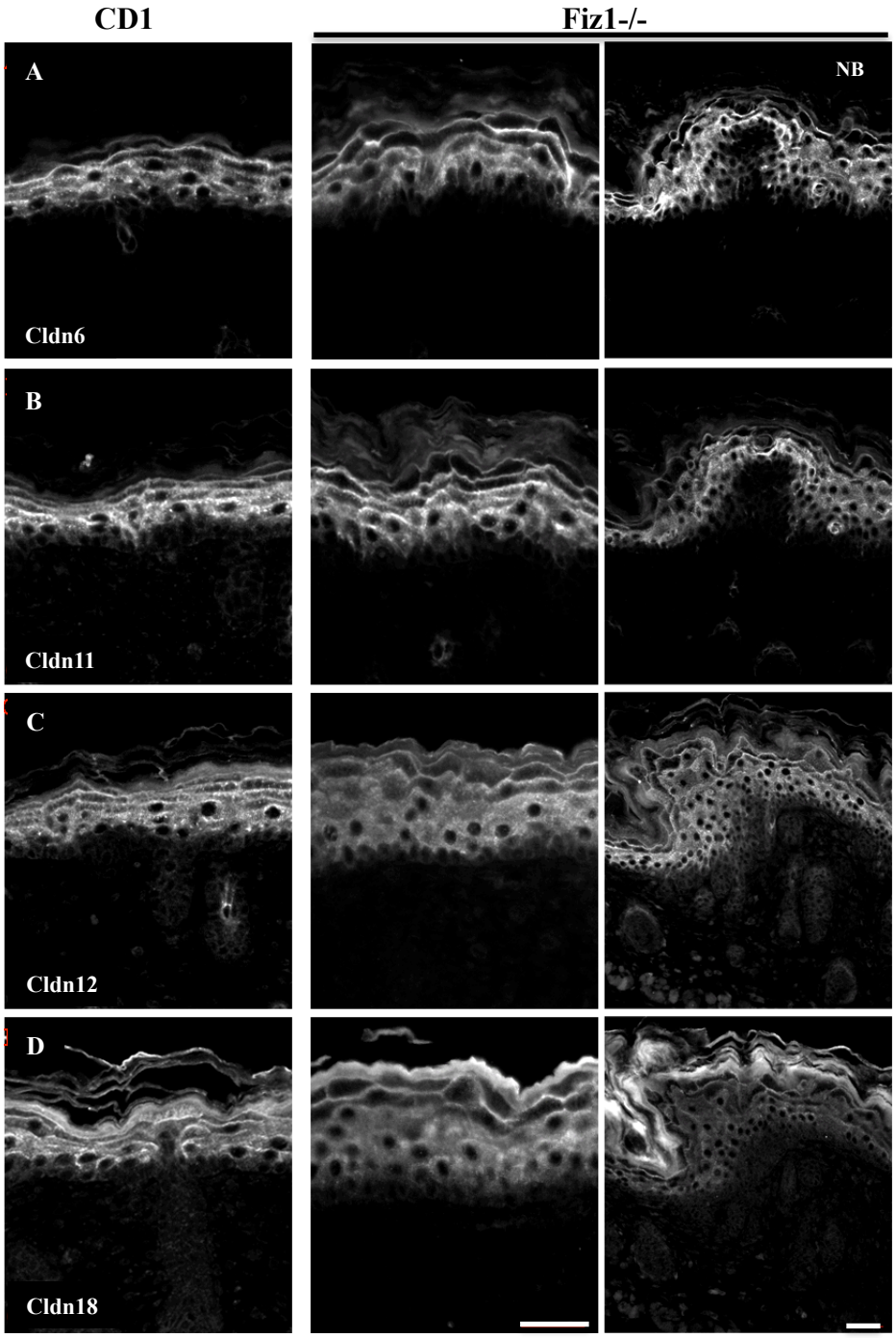

Figure 47: Immunohistochemistry of Newborn Fiz1 Knock-out Mouse Epidermis Using Claudin Antibodies. Expanded expression profile of Claudins as well as loss of association with the membrane in the knock-out epidermis when compared to the wild type, suggests abberant epidermal differentiation.

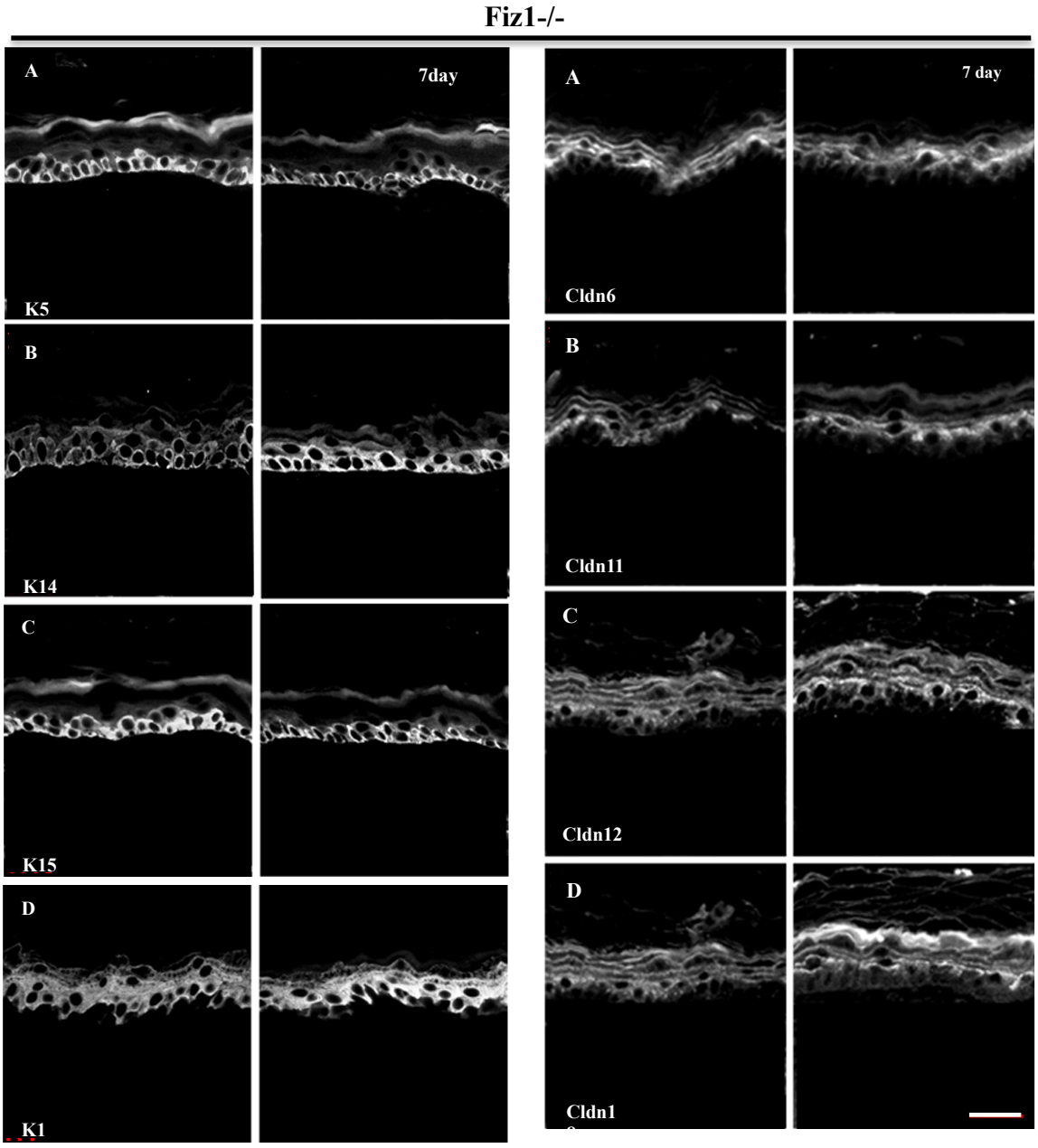

Figure 48: Immunohistochemistry of 7-day old Fiz1 Knock-out mouse Epidermis using Keratin and Claudin Antibodies. By day 7, Fiz1 knock-out epidermis has thinned in a manner comparable to the wild type. In addition, Keratin and Claudin expression is now similar to that of the wild type.

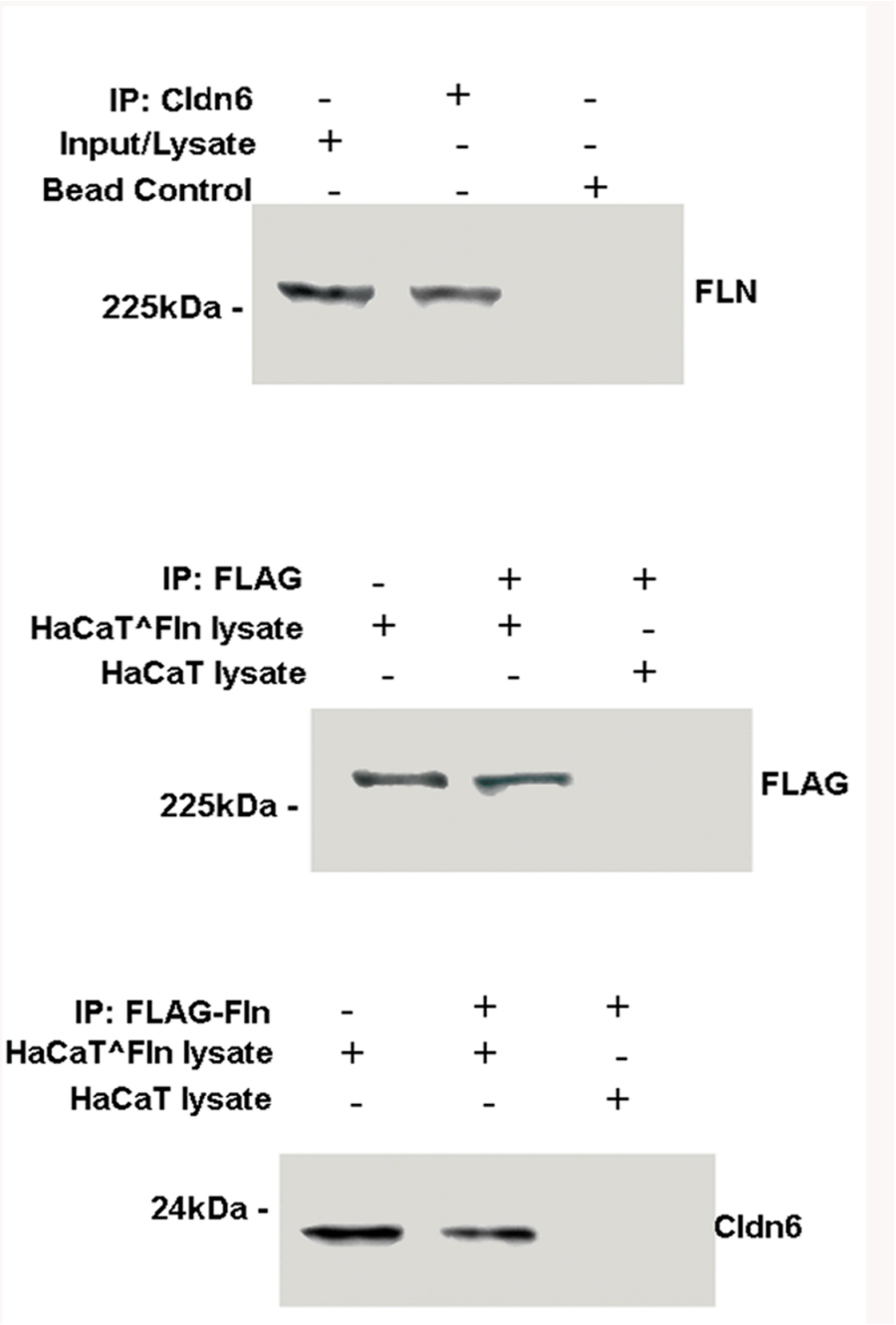

Figure 49: Co-immunoprecipitation of Claudin 6 (Cldn6) and Filamin (Fln) in HaCaT cells. Experiments show interaction between endogenous Cldn6 and Fln in first panel and between Cldn6 and FLAG-tagged Fln in last panel.

APPENDIX II

Table 4: Summary of Foxa2 Conditional Knockout Mouse Models. Adapted from Friedman and Kaestner, 2006.

Mouse Model	Phenotype	References
Foxa2 +/-	Normal lifespan and fertility if on favorable genetic background Increased adiposity on a high-fat diet Decreased adipocyte glucose uptake and glycolysis	Wolfrum *et al*, 2003.
Foxa2-/-	Death at E10-11 Severe defects in node, notochord, neural tube, and gut tube	Weinstein *et al*, 1999.
Foxa2loxP/loxP Ins.Cre (β-cells)	Death at P9-P12 Hypoglycemia Hyperinsulinism	Lantz *et al*, 2004. Sund *et al*. 2001.
Foxa2loxP/loxP Alfp.Cre (hepatocytes)	Diminished induction of gluconeogenic enzymes during fasting	Zhang *et al*, 2005.
Foxa2loxP/loxP Alb.Cre (hepatocytes)	Normal	Sund *et al*, 2000.
Foxa2loxP/loxP Foxa3-Cre (endoderm)	Death at P0-P5 Hypoglycemia Hypoglucagonemia	Lee *et al*, 2005.
Foxa2loxP/loxP SP-C-rtTA; (tetO)7 Cre (respiratory epithelium)	Death at P0-P1 Respiratory distress syndrome	Wan *et al*, 2004
Foxa1–/–; Foxa2loxP/loxP Foxa3-Cre	Death at E9.5-10.5 Loss of liver specification	Lee *et al*, 2005.
Foxa1–/–; Foxa2loxP/loxP SP-C-rtTA; (tetO)7Cre	Abnormal branching morphogenesis and epithelial differentiation of lung	Besnard et al, 2005

Table 5: Summary of Cdx Mouse Models.

Mouse Model	Phenotype	References
Cdx1$^{-/-}$	No intestinal Phenotype Anterior homeotic transformation of vertebral column	Subramanian *et al*, 1995
Cdx2$^{+/-}$	Vertebral homeotic transformation in cervical and thoracic vertebrae Anterior homeotic shift of intestine to stomach	Chawengsaksophak *et al*, 1997
Cdx2 Conditional	Defect in axial elongation Anterior homeotic shift of intestine to stomach (knock out at E13.5)/stomach to esophagus (knock out at E8.5)	Gao *et al*, 2009 Grainger *et al*, 2010
Cdx1$^{-/-}$Cdx2$^{-/-}$	Severely truncated anteroposterior axis	Savory *et al*, 2011
Cdx1$^{2ki/2ki}$	Rescue of Cdx1-/- axial patterning phenotype but not of intestinal phenotype	Savory *et al*, 2009 Grainger *et al*, 2012
Cdx1$^{-/-}$ Cdx2$^{-/-}$ Cdx4 $^{-/-}$	Death at E10.5. Severely truncated A-P axis	Rooijen et al, 2012

Table 6: Genes Down Regulated in Cdx1/2 DKO Endoderm Microarray

RefSeq	Gene Symbol	Fold-Change (log2)
NM_019538	Plac1	-11.8151
NM_001164058	Prl7a1	-9.52287
NM_013697	Ttr	-9.13739
NM_001159487	Rbp4	-7.66461
NM_010330	Emb	-7.22804
NM_009258	Spink3	-5.87782
NM_007607	Car4	-5.05416
NM_016867	Gipc2	-4.76456
NM_025273	Pcbd1	-4.1947
NM_139198	Plac8	-4.16259
AK034234	Fut11	-3.77937
NM_007423	Afp	-3.74859
NM_025622	Lgals2	-3.68993
NM_007469	Apoc1	-3.56377
NM_009984	Ctsl	-3.55012
NM_031170	Krt8	-3.34183
NM_174993	Fmr1nb	-3.32045
NM_001045539	Xlr5a	-3.04027
NM_019432	Tmem37	-2.95983
NM_175105	Aqp11	-2.90914
NM_023530	Pla2g12b	-2.90871
NM_177601	Tmem60	-2.78495
NM_010016	Cd55	-2.77159
NM_018821	Socs6	-2.70478
NM_023585	Ube2v2	-2.63405
NM_023585	Ube2v2	-2.59271
ENSMUST00000048486	Fgg	-2.47605
NM_028749	Npl	-2.44915
NM_010360	Gstm5	-2.43876
NM_017382	Rab11a	-2.43648
ENSMUST00000030651	Sh3bgrl3	-2.37386
NM_025607	Rnf181	-2.36823
NM_178715	Tmem30b	-2.36401
NM_009105	Rsu1	-2.31638
NM_029814	Chmp5	-2.30406
NM_028567	1700094D03Rik	-2.29643
NM_011635	Trap1a	-2.29585
BC099528	0610010B08Rik	-2.27712
BC099528	0610010B08Rik	-2.27712
NM_001099327	100043387	-2.27712

BC099528	0610010B08Rik	-2.27712
BC099528	0610010B08Rik	-2.27712
BC099528	0610010B08Rik	-2.27712
BC099528	0610010B08Rik	-2.27712
NM_028696	Obfc2a	-2.24726
NM_026325	Tmem179b	-2.21272
NM_008260	Foxa3	-2.20324
NM_026521	Zfp706	-2.2012
NM_178218	Hist3h2a	-2.2004
NM_026904	Anapc10	-2.19882
NM_183250	Ccdc72	-2.16077
NM_011254	Rbp1	-2.13956
NM_020585	Golga7	-2.13871
NM_016753	Lxn	-2.13732
NM_019922	Crtap	-2.11452
NM_013896	Timm9	-2.11348
NM_138589	Ubfd1	-2.10433
NM_001085493	2310030N02Rik	-2.09224
BC002022	Casp6	-2.0914
NM_021291	Slc7a9	-2.06907
NM_001164220	Trim13	-2.06167
NM_025969	1700034H14Rik	-2.05715
NM_007793	Cstb	-2.05527
ENSMUST00000150042	Mrpl48	-2.05108
NM_010371	Gzmc	-2.04062
NM_175009	Eny2	-2.0369
NM_001112715	Ifitm1	-2.02146
NM_025447	Dimt1	-2.02032
NM_001199996	4933402E13Rik	-2.01423
ENSMUST00000160640	Gm7664	-1.99672
NM_007709	Cited1	-1.9961
NM_022023	Gmfb	-1.97527
NM_019920	Lamtor3	-1.95901
NM_009681	Ap3s1	-1.94513
NM_175400	Sephs1	-1.94227
NM_026070	Ccdc53	-1.93884
NM_013587	Lrpap1	-1.93796
NM_019586	Ube2j1	-1.93212
NM_026879	Chmp2b	-1.92991
NM_001104531	Cyp2d11	-1.92707
NM_133805	Cops8	-1.92305
NM_025417	Commd4	-1.91997
NM_011149	Ppib	-1.91812

NM_019443	Ndufa1	-1.91416
NM_025668	Spcs2	-1.90212
NM_009155	Sepp1	-1.89899
BC024353	0610009B22Rik	-1.89129
NM_133900	Psph	-1.88942
NM_028176	Cda	-1.88926
NM_007980	Fabp2	-1.88913
NM_027231	Polr2f	-1.87326
NM_011309	S100a1	-1.87171
NM_010562	Ilk	-1.8712
NM_026632	Rpa3	-1.85242
NM_033073	Krt7	-1.85001
NM_007620	Cbr1	-1.84443
NM_009031	Rbbp7	-1.83801
NM_029508	Pcgf5	-1.83729
NM_026732	Mrpl14	-1.83433
NM_027352	Gorasp2	-1.82426
NM_009681	Ap3s1	-1.8184
NM_011712	Wbp5	-1.81797
NM_007801	Ctsh	-1.81255
NM_007413	Adora2b	-1.80596
NM_027052	Slc38a4	-1.80492
NM_174995	Mgst2	-1.80346
NM_027002	Polr2d	-1.80261
NM_031180	Klb	-1.79871
NM_012060	Bcap31	-1.79612
NM_010726	Phyh	-1.79511
NM_178700	Grsf1	-1.79188
NM_016958	Krt14	-1.79098
NM_181796	Gstp2	-1.7831
NM_026845	Ppil1	-1.76744
NM_001098227	Sdcbp	-1.75158
AK006430	Cdc42ep5	-1.75062
NM_029341	Capsl	-1.75045
NM_025683	Rpe	-1.74321
NM_026053	Gemin6	-1.74257
NM_025806	Plbd1	-1.73909
NM_025846	Rras2	-1.73801
BC027506	2010012O05Rik	-1.73568
NM_019920	Lamtor3	-1.73302
NM_028394	Hspb11	-1.72951
NM_144903	Aldob	-1.71349
NM_007883	Dsg2	-1.71019

NM_028355	Tmem48	-1.70745
NM_012002	Cops6	-1.70057
NM_026921	Isca1	-1.69998
NM_028064	Slc39a4	-1.69878
ENSMUST00000047697	Trim17	-1.68704
NM_199008	Cox11	-1.67814
NM_008163	Grb2	-1.67367
NM_145435	Pyy	-1.6712
ENSMUST00000087511	Tmem128	-1.66999
NM_134123	Scaf8	-1.66775
ENSMUST00000063204	1700025K23Rik	-1.66662
NM_001177568	Gm14420	-1.66196
NM_008817	Peg3	-1.66183
NM_001127346	Ndufaf2	-1.66072
ENSMUST00000118209	Atp5f1	-1.65652
NM_010952	Oaz2	-1.64851
NM_007502	Atp1b3	-1.63954
ENSMUST00000110293	Serpinb6b	-1.63724
NM_025617	2210012G02Rik	-1.63611
NM_080556	Tm9sf2	-1.63312
NM_007503	Fxyd2	-1.62567
NM_029541	Arxes1	-1.623
NM_001115132	Ncaph2	-1.62065
NM_025498	Psenen	-1.62062
ENSMUST00000174651	Moap1	-1.61384
NM_001162903	2010109K11Rik	-1.60447
NR_027872	4930515G01Rik	-1.5987
NM_026992	Dnajc24	-1.59726
NM_133220	Sgk3	-1.59376
NM_011293	Polr2j	-1.58526
NM_197992	Pcgf1	-1.5788
ENSMUST00000020699	Gatsl3	-1.57671
NM_138743	Fam165b	-1.57586
NM_001017429	Cox17	-1.57479
NM_145630	Pdk3	-1.56743
NM_019405	Cetn2	-1.56625
NM_009460	Sumo1	-1.55721
NM_019577	Ccl24	-1.55696
NM_026950	Ociad2	-1.55263
NM_019727	Snx1	-1.55257
NM_023697	Rdh14	-1.55235
NM_024219	Hsbp1	-1.54621
NM_173779	Sowahd	-1.54486

NM_011514	Suv39h1	-1.544
NM_001025263	Tpd52	-1.54158
NM_008062	G6pdx	-1.541
NM_021876	Eed	-1.54025
ENSMUST00000059899	Mmgt1	-1.54022
NM_016861	Pdlim1	-1.54004
NM_007865	Dll1	-1.53867
NM_010902	Nfe2l2	-1.53821
NM_001039368	Polr2k	-1.53505
NM_013614	Odc1	-1.53328
NM_018808	Dnajb1	-1.53175
NM_001081668	Nup62cl	-1.53043
NM_025335	Tmem167	-1.52911
NM_030203	Tspyl4	-1.5265
ENSMUST00000043962	Cdc16	-1.52635
NM_133994	Gstt3	-1.52328
NM_199042	Thap1	-1.52273
NM_025419	1110059G10Rik	-1.5224
NM_207000	H2afy2	-1.52139
NM_011252	Rbmx	-1.52038
NM_001005847	Aga	-1.52036
NM_019731	Nme4	-1.51436
NM_178798	Slc7a6	-1.5115
NM_011427	Snai1	-1.51048
NM_001009935	Txnip	-1.50672
NM_026556	Dynll2	-1.506
NM_009874	Cdk7	-1.50392
NM_028761	Parn	-1.50391
NM_022983	Lpar3	-1.50044
NM_001146212	Mrps10	-1.49935
BC028765	0610010O12Rik	-1.49803
NM_031197	Slc2a2	-1.49716
ENSMUST00000064667	Rap1b	-1.49604
NM_001025261	Tpd52	-1.49581
ENSMUST00000054213	Timm8a1	-1.49294
NM_001033988	Ncoa4	-1.492
NM_183140	Zfp691	-1.4907
NM_024190	Chmp1b	-1.48948
NM_181391	Chchd7	-1.48768
NM_173781	Rab6b	-1.48725
NM_022996	Ndfip1	-1.48274
NM_026487	Atad1	-1.48197
NM_009052	Bex1	-1.47662

NM_001127376	Wisp3	-1.47473
NM_026211	Tmed9	-1.47286
NM_001159617	Pigp	-1.47195
NM_029767	Rps9	-1.46911
NM_001159595	Ints8	-1.46839
NM_011920	Abcg2	-1.46481
NM_172762	Rbm34	-1.46352
ENSMUST00000108656	Sat2	-1.4572
NM_023383	Aadac	-1.45668
ENSMUST00000016088	Gatsl2	-1.45619
BC071241	9430016H08Rik	-1.4535
NM_022889	Pes1	-1.45274
NM_019403	Rnf5	-1.45231
ENSMUST00000087321	Ppp1r3c	-1.44988
ENSMUST00000039729	Lyrm5	-1.44759
NM_033521	Laptm4b	-1.44748
NM_198021	Scyl2	-1.44729
NM_029891	Nkrf	-1.44578
NM_001033166	2700094K13Rik	-1.44408
NM_029992	Tchp	-1.44097
NM_007668	Cdk5	-1.44034
NM_001099664	Npw	-1.43826
NM_010446	Foxa2	-1.43422
NM_019579	Mpp5	-1.4328
NM_010168	F2	-1.43215
AK011956	2610301H18Rik	-1.43049
NM_011030	P4ha1	-1.42998
NM_029979	Trim35	-1.42806
NM_019998	Alg2	-1.42692
NM_025667	Tmem222	-1.42636
NM_029985	Lrrc42	-1.42411
NM_025576	Ptpmt1	-1.42324
NM_008720	Npc1	-1.42084
NM_023906	Asb3	-1.41835
NR_027978	4930465K10Rik	-1.41543
NM_010757	Mafk	-1.4128
BC024851	1110038D17Rik	-1.4115
ENSMUST00000085591	Pdx1	-1.41015
NM_024170	Cxx1a	-1.40931
NM_026155	Ssr3	-1.40698
BC004797	0610040J01Rik	-1.40359
NM_025370	1110018J18Rik	-1.40347
NM_172943	Alkbh5	-1.40307

NM_027889	Vps11	-1.40286
NM_010291	Gjb5	-1.40126
NM_023054	Utp3	-1.40126
NM_027652	Ept1	-1.39999
NM_008303	Hspe1	-1.39982
NM_001025250	Vegfa	-1.39782
DQ080431	1700003F12Rik	-1.39749
ENSMUST00000115660	Slc35a2	-1.39268
NM_001042407	Pex10	-1.3909
NM_001168668	Fam114a2	-1.38917
NM_009558	Zfp51	-1.38554
NM_146115	A830007P12Rik	-1.38502
NM_007497	Atf1	-1.38377
ENSMUST00000100314	Cldn10	-1.38305
NM_172474	Tyw3	-1.38299
NM_007467	Aplp1	-1.38226
NM_025823	Pcyox1	-1.37977
NM_025582	2810405K02Rik	-1.37431
NM_001038230	Anapc11	-1.37399
ENSMUST00000014220	Tctex1d2	-1.37247
NM_008303	Hspe1	-1.37046
NM_029357	Pcdh1	-1.36808
ENSMUST00000153148	Wdr54	-1.36586
NM_013814	Galnt1	-1.36558
BC080301	2210418O10Rik	-1.36556
BC080301	2210418O10Rik	-1.36556
NM_013492	Clu	-1.36514
NR_003960	Gm5478	-1.36314
NM_183126	6030498E09Rik	-1.36209
NR_033802	2610002J02Rik	-1.36183
NM_001005223	Znhit3	-1.36044
NM_175423	Orai1	-1.35942
NM_009460	Sumo1	-1.35303
NM_011526	Tagln	-1.35288
NM_001004436	Wapal	-1.35271
NM_172529	Gnptg	-1.35162
NM_030114	Herc4	-1.35137
NM_145934	Stap2	-1.34999
NM_177448	Mogat2	-1.34994
NM_145211	Oas1a	-1.34871
NM_198190	Ntf5	-1.34231
NM_146008	Tcp11l2	-1.34188
ENSMUST00000100314	Cldn10	-1.34166

ENSMUST00000123296	Ndufab1	-1.33979
BC038268	BC038268	-1.33838
NM_008542	Smad6	-1.33711
NM_145587	Sbk1	-1.33625
BC020125	D030056L22Rik	-1.33492
NM_198625	Mtss1l	-1.3343
NM_008216	Has2	-1.33313
NM_001029990	Mettl17	-1.33291
NM_021279	Wnt1	-1.33181
NM_007747	Cox5a	-1.33108
NM_008631	Mt4	-1.32848
NM_027116	Nkpd1	-1.32688
ENSMUST00000117069	Ppil3	-1.32582
NM_001033380	Itpripl2	-1.32391
NM_001172113	Traf7	-1.32264
NM_029271	Mrpl32	-1.32246
NM_177677	Dnajc5g	-1.32227
ENSMUST00000056924	Plagl2	-1.31993
NM_010590	Jub	-1.317
NM_031869	Prkab1	-1.31618
NM_027415	Tmem70	-1.31607
NM_133700	Btbd10	-1.31531
NM_011294	Sub1	-1.31479
NM_029654	Atg2b	-1.3126
NM_175552	Wdr3	-1.31185
NM_029023	Scpep1	-1.30897
NM_010070	Dok1	-1.3064
NM_010581	Cd47	-1.30627
NM_145415	Diexf	-1.30262
NM_172288	Nup133	-1.3026
NM_033560	Vps37a	-1.30199
NM_146285	Olfr801	-1.30063
NM_024200	Mfn1	-1.30012
NM_021710	Ap4s1	-1.30002
NM_025676	Mcm8	-1.29994
NM_001136070	Eif2d	-1.29978
NM_026498	Mrps11	-1.29781
NM_013796	Nagpa	-1.29759
NM_012006	Acot1	-1.29504
NM_007959	Etv2	-1.29234
NM_020493	Srf	-1.29144
NM_019955	Ripk3	-1.29089
NM_172446	Skor1	-1.29023

NM_023644	Mccc1	-1.29023
NM_027828	Fam110c	-1.29008
NM_145401	Prkag2	-1.28905
NM_001033543	Il20rb	-1.28862
NM_011578	Tgfbr3	-1.28789
NM_010489	Hyal2	-1.2865
NM_175247	Zfp28	-1.28612
NM_007508	Atp6v1a	-1.28581
NM_001081213	Ermp1	-1.28394
NM_134257	Rgs3	-1.28314
NM_153196	Rbks	-1.28281
NM_178278	Caps2	-1.27909
NM_011544	Tcf12	-1.27773
NM_001110197	Rnf146	-1.27763
NM_001002011	Lmna	-1.27759
NM_031161	Cck	-1.27711
NM_001163611	Nps	-1.27636
NM_025614	Rwdd1	-1.2755
NM_001252481	Smad2	-1.27498
NM_009200	Slc1a6	-1.27442
NM_001110142	Cul4b	-1.27361
NM_010483	Htr5b	-1.27026
NM_001012704	Wfdc13	-1.26854
NM_001177901	Zfp513	-1.26771
NM_028440	3110003A17Rik	-1.26761
NM_007789	Ncan	-1.26666
NM_144511	Ces3b	-1.26495
ENSMUST00000027467	Serpine2	-1.26193
NM_028809	Arpc5l	-1.2599
BC023359	3632451O06Rik	-1.25386
NM_145449	Ifi27l2b	-1.25298
NM_025852	Rexo1	-1.25293
NM_198654	Nsl1	-1.25214
NM_001174107	Map3k9	-1.25123
ENSMUST00000030884	Mfn2	-1.24981
NM_019564	Htra1	-1.24919
NM_199198	Hdac10	-1.24838
NM_021437	1700123O20Rik	-1.24749
NM_178113	Ncapd3	-1.24358
NM_025956	1700011H14Rik	-1.24201
NM_001205181	Abhd4	-1.24034
NM_029098	Lmbr1l	-1.23969
NM_172717	Chfr	-1.23844

NM_025768	Grtp1	-1.23739
NM_033324	Dgcr8	-1.23694
NM_027149	Wdr20a	-1.23428
NM_010125	Elf5	-1.23307
NM_010552	Il17a	-1.23241
NM_133973	Cog4	-1.23075
NM_199475	Fam63a	-1.23048
NM_026448	Klhl7	-1.22837
NM_023647	Nipa2	-1.2276
NR_002455	Snord34	-1.22742
NM_020330	Adam21	-1.22567
NM_007568	Btc	-1.22558
NM_134102	Pla1a	-1.22533
ENSMUST00000161430	Ttc16	-1.22511
NM_030598	Rcan2	-1.22288
NM_173445	Rccd1	-1.22226
NM_001005421	Amica1	-1.22008
ENSMUST00000026554	1190003J15Rik	-1.21993
NM_028058	Fundc1	-1.2178
NM_027975	Fam83d	-1.21418
NM_177471	Ccdc69	-1.21256
NM_172286	6430548M08Rik	-1.21138
NM_010739	Muc13	-1.20726
NM_026858	Xrcc6bp1	-1.2065
NM_026393	Nmral1	-1.20497
NM_053196	Sfxn2	-1.2043
NM_001205369	Casc4	-1.2026
NM_172488	9030625A04Rik	-1.20077
NM_009710	Art1	-1.20051

Table 7: Primers Used for RT-PCR, EMSA and ChIP Analysis

Gene	RT-PCR	Forward	Reverse
β-Actin		GCGGGAAATCGTGCGTGACATT	GATGGAGTTGAAGGTAGTTTCGTG
Cyp26a1		AAGATCCGCCGGCTTCAGGCTA	TTGCAAACTCTTTGCCTACACAGCTCC
Eomesodermin		ACGGTGTGGAGGACTTGAAT	CTTTGGCGAAGGGGTTATGG
Epcam		GTGAAGGGGGAGTCCCTGTTC	ATCTCACCCATCTCCTTTATC
Foxa1		CAGGGCTGGATGGTTGTATT	GCCTGAGTTCATGTTGCTGA
Foxa2		CCCTACGCCAACATGAACTCG	GTTCTGCCGGTAGAAAGGGA
Foxa3		GAAGATGGAGGCCCATGAC	GGGGGATAGGGAGAGCTTAG
Gata 4		CTGGAAGACACCCCAATCTC	TGATAGAGGCCACAGGCATT
Hhex		CGAGACTCAGAAATACCTCTCCC	CTGTCCAACGCATCCTTTTTG
Ifabp		GATCATGGCATTCGACGGCA	GCTTTTACTTCTTTAGCTTTGAC
Mash 1		GATGGAGAGTGGAGCCGGCC	GCTCTTGTTCCTCTGGGCTAAGAG
Muc2		CTGACCAAGAGCGAACACAA	CATGACTGGAAGCAACTGGA
Pax 6		GTCACAGCGGAGTGAATCAGCTTGG	GGCGTGTGCCCCAGCTTCC
Wnt6		CGGTAGAGCTCTCAGGATGC	CCTGCAGATGCTGGTAGGAT
T (bracyhury)		CGAGATGATTGTGACCAAGAACGGCA	GGGAATACCCCGGCTGCTGG
	CHIP	**Forward**	**Reverse**
Foxa2 CDRE A		CAGTACCTGCCACCTCTATGT	ATCTGTGTTCTCTGCCTGCAG
Foxa2 CDRE B		GCAGAGTCCATAGGCTGATAC	AGGCTGCATGTGTTTGTAAAA
Foxa2 CDRE C		AGGCAAACTTCACTGAAAACT	GGAGGCCCCTCTTCAATTTCT
Foxa2 CDRE D		GGCCCCAAACCCTTCACTCCT	AGGCCCAGAAGTTTAGACTTG
Foxa2 -18kb		TGATGTAATAATCGGACACGA	TGCCTAGGGAATCCAGGTGGG
T (Bracyhury)		GGGCCCGCCCGGCCAGTC	GAGATCGGGATGAGGGGGGAAGGGGG
	EMSA	**Wild type**	**Mutant**
Foxa2 CDRE A		ACTGTCCACATAAAAACGCCG	ACTGTCCAGGGCCCAACGCCG
Foxa2 CDRE B		TTCTTTCCCATAAATCTTGCC	TTCTTTCCGGGCCCTCTTGCC
Foxa2 CDRE B'		GGATGGAACATAAACAATATA	GGATGGAAGGGCCCCAATATA
Foxa2 CDRE C		CCTGTGCACATAAAAGGTTAA	CCTGTGCAGGGCCCAGGTTAA

Table 8: Endoderm Genes Down-Regulated in Cdx1/2 DKO Endoderm Microarray

Gene Symbol	RefSeq	Fold-Change
Ttr	NM_013697	-9.13739
Emb	NM_010330	-7.22804
Spink3	NM_009258	-5.87782
Pcbd1	NM_025273	-4.1947
Afp	NM_007423	-3.74859
Lgals2	NM_025622	-3.68993
Krt8	NM_031170	-3.34183
Pla2g12b	NM_023530	-2.90871
Tmem30b	NM_178715	-2.36401
Trap1a	NM_011635	-2.29585
Foxa3	NM_008260	-2.20324
Krt7	NM_033073	-1.85011
Krt14	NM_016958	-1.79098
Dsg2	NM_007883	-1.71019
Grb2	NM_008163	-1.67367
Pyy	NM_145435	-1.6712
Cebpa	NM_007678	-1.65821
Foxa2	NM_010446	-1.43422
Ncoa4	NM_001033988	-1.492
Mogat2	NM_177448	-1.34994
Has2	NM_008216	-1.33313
1700011H14Rik	NM_025956	-1.24201

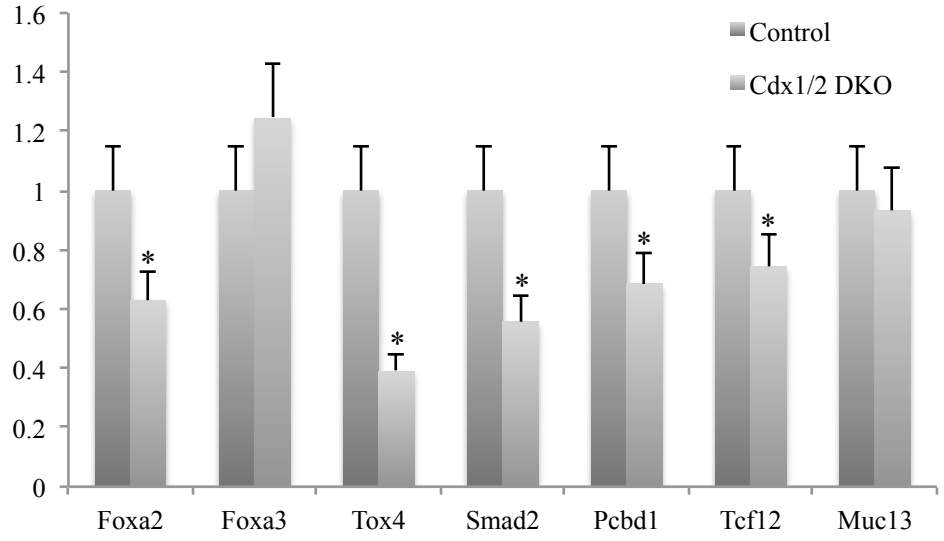

Figure 50: Validation of Targets Down-regulated in Cdx1/2 DKO Endoderm Microarray by RT-PCR. *Foxa2*, *Tox4*, *Smad2*, *Pcdb1* and *Tcf12* were all significantly down-regulated in the Cdx1//2 DKO endoderm when compared to control. *p <0.05 by student's t-test.

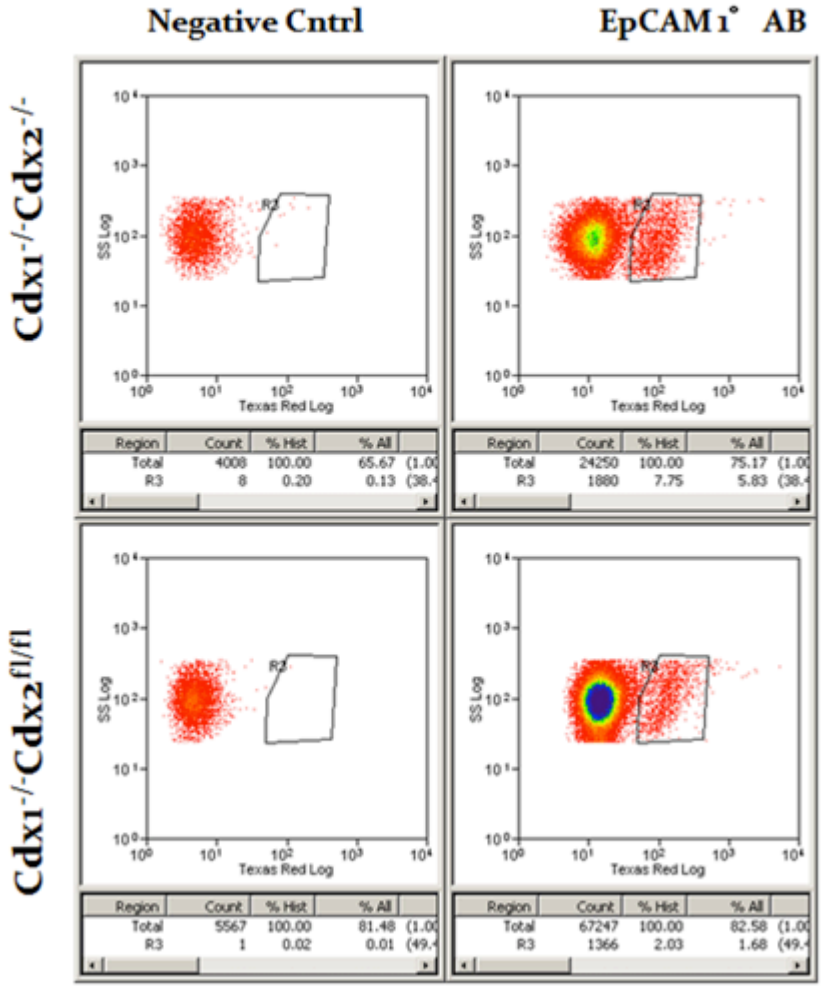

Figure 51: Isolation of Endodermal Cells by Fluorescent Activated Cell sorting (FACS). Representative FACS analysis of cells from the caudal ends of E9.5 Cdx1/2 DKO and control embryos stained with EpCAM primary antibody and Goat anti-mouse Texas Red secondary antibody. EpCAM positive cells are found in the enclosed region denoted by R3 and represents 5.83% and 1.78% of the total cell population in Cdx1/2 DKO and control embryos respectively

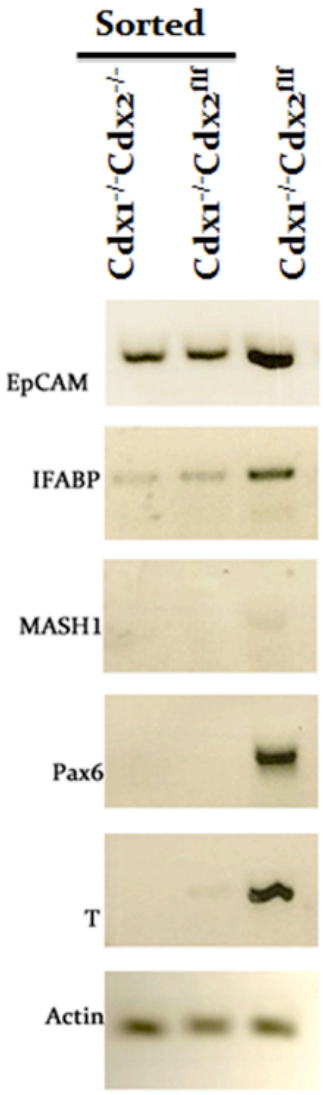

Figure 52: Validation of FACS Analysis. Semi-quantitative RT-PCR of RNA isolated from FACS sorted EpCAM positive Cdx1/2 DKO and control embryos. The first two columns represent cDNA from sorted cells while the last column represents cDNA from non-sorted control cells. Intestinal fatty acid binding protein (IFABP) was used as a marker for endodermal lineage, Ascl1/Mah1 and Paired box 6 (Pax6) were used as markers for neuroectodermal lineage while Bracyhury (T) was used as a marker for mesodermal lineage

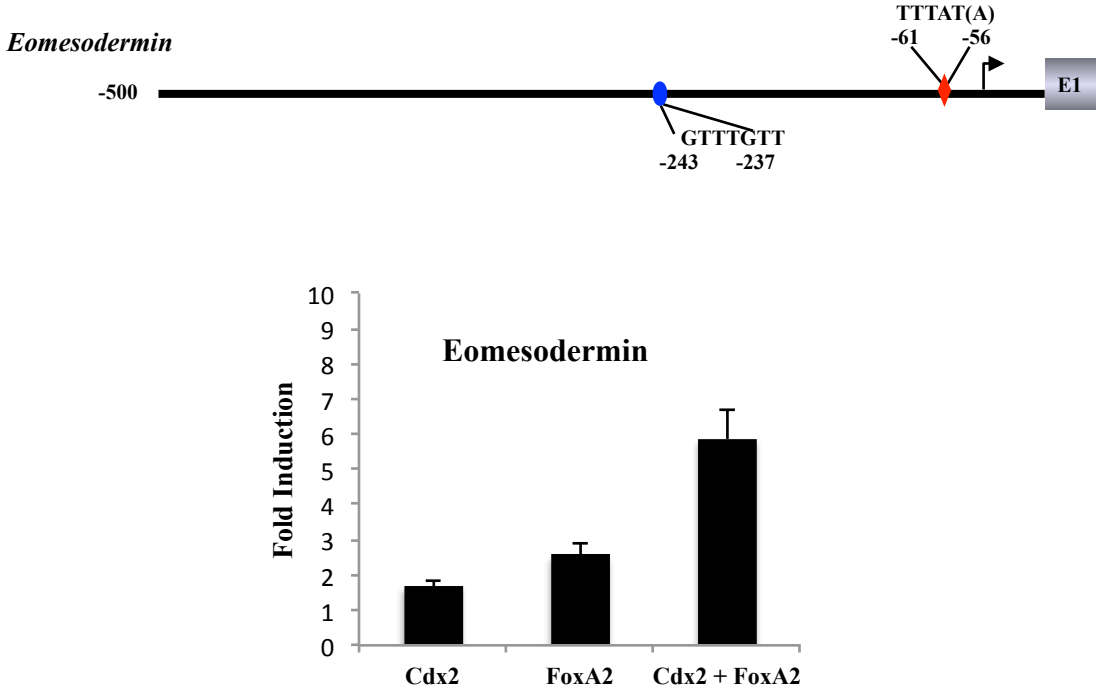

Figure 53: Cdx2 Activates *Eomes* Promoter. Eomes luciferase vector was transiently transfected into P19 cells in the presence or absence of Cdx2 expressing vector. Luciferase reporter activity was measured and normalized to β-galactosidase. CDRE and Foxa2 response element in the -500bp proximal *Eomes* promoter show induction in the presence of Cdx2 and/or Foxa2. Assays were repeated in triplicate and errors bars indicate s.d. from the mean.

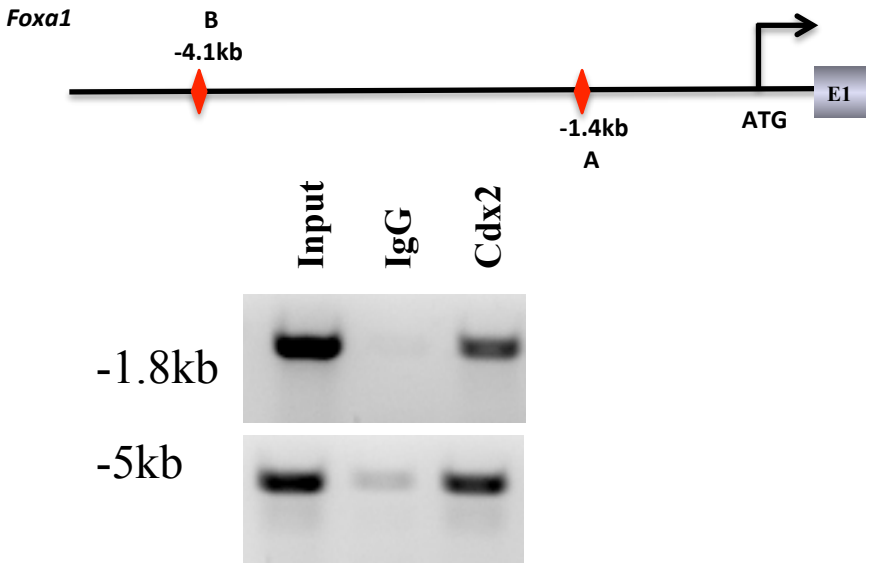

Figure 54: Cdx2 Binds the *Foxa1* Promoter *in vivo*. Schematic representation of the mouse Foxa1 promoter showing the position of the putative Cdx response element (CDRE) as identified by TRANSFAC. Red diamond indicates CDRE, arrow indicates TSS and E1 denotes exon 1. ChIP of the Foxa1 promoter sequences using chromatin from E9.5 embryos showing binding of Cdx2 at -1.8 and -5kb downstream of the TSS.

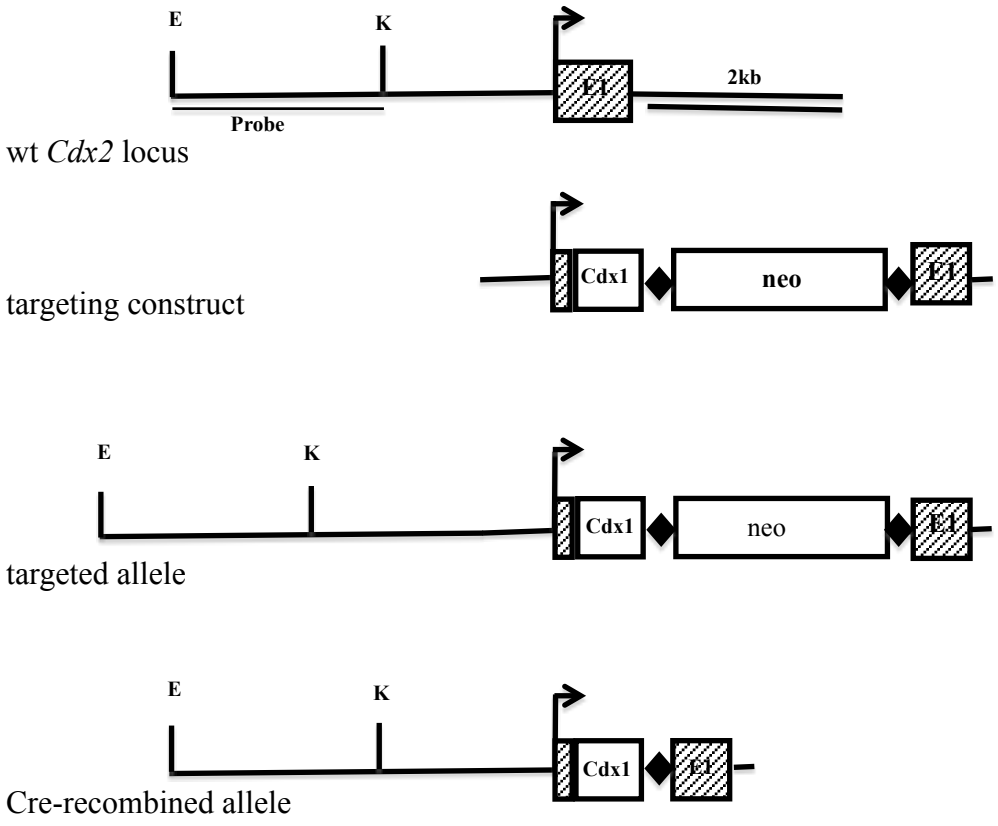

Figure 55: Schematic of $Cdx2^{1ki/1ki}$ Locus. The wildtype locus, the targeted allele and the expected product after homologous recombination and Cre-mediated recombination are indicated above.