EPIGENOMIC AND METABOLIC DRIVERS OF INTESTINAL DEVELOPMENT

By

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A Dissertation submitted to the
Graduate School-New Brunswick

and

Graduate School of Biomedical Sciences
Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in

Cell and Developmental Biology

and

Computational Biology and Molecular Biophysics

written under the direction of

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and approved by

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New Brunswick, New Jersey

January 2017
Embryonic intestinal development is a dynamic process where tissue undergoes drastic transitory changes from morphological and physiological changes, to chromatin restructuring for intestinal specification, to metabolic shifts for supporting growing tissue. My research focuses on two critical themes regulating embryonic intestinal development: Epigenomic and Metabolic.

During early endoderm development, cells exhibit cellular plasticity with the ability to acquire cell fate of multiple endodermal lineages. Lineage-inducing transcription factors (TF) drive tissue-specific transcriptomes, leading to distinct cell types, with the loss of cellular plasticity as tissue matures. However, transcriptional mechanisms governing organ specification and cell fate are poorly understood. We provide evidence that loss of intestinal lineage-inducing TF CDX2 in the developing endoderm at E9.5, using Shh-cre, leads to underdeveloped intestine with cells exhibiting foregut-like cellular identity. This phenomenon is conserved across species with the induction of esophageal and stomach cell fates upon CDX2 loss in human intestinal organoid cultures (HIO). By temporal mapping of Cdx2 knockout in the developing intestine, we highlight the loss of intestine’s ability to transform in esophageal lineages by E9.5; between E9.5-
E13.5 CDX2 loss leads to gastric lineage, while intestine’s window of CDX2-restricted plasticity is entirely lost by E15.5.

Enhancers bound by CDX2 in the developing endoderm, identified using CDX2 ChIP-seq at E13 and E17, regulate genes involved in gut tube development and are enriched for patterning transcription factor motifs. In contrast, enhancers bound by CDX2 in adult epithelium regulate genes involved in metabolic processes and are enriched for mature intestinal transcription factors. This dynamic binding of CDX2 along development is conserved across species, as we identify different regions regulated by CDX2 in early specified hindgut when compared to adult human intestinal cultures, with similar relationships suggesting patterning roles for CDX2 early in development, and mature intestine-specific physiological functions in the adult tissue.

In a temporal survey of developing intestinal chromatin assayed using ATAC-seq, we find that CDX2’s differential binding and intestine’s cellular plasticity coincide with dynamic chromatin restructuring during villus formation. Villogenesis is a transition point in intestinal development, when early embryonic enhancer chromatin condenses while mature intestinal enhancers gain accessibility. We highlight temporal CDX2 bound enhancers highly overlap with dynamic chromatin accessibility. Additionally, in human intestinal cultures, in presence of Wnt/FGF signaling CDX2 binds at enhancers specifying intestinal cell fate, however in absence of Wnt/FGF CDX2 fails to impart intestinal cell fate and loses its ability to bind at intestine-specifying enhancers. These results indicate that CDX2 requires additional factors to drive the intestinal transcriptome, and chromatin accessibility strongly correlates with dynamic CDX2 binding. Enhancer regions with transcriptionally active histone H3k27ac modifications, that become inactive after human hindgut specification, are enriched for foregut transcription factor motifs.
This indicates that genomic regions where foregut transcription factors bind become inactive once intestinal cell fate is established. Furthermore, we analyze forestomach enhancer regions, identified using ATAC-seq from forestomach tissue at post-natal Day 1, for chromatin accessibility in temporally developing intestinal epithelium, assayed using ATAC-seq. We highlight that the foregut enhancer regions are accessible in the developing intestine at E11, which then progressively lose permissive chromatin and are mostly inaccessible by E16. However, in the absence of CDX2, foregut enhancers remain accessible in intestine at E16, permitting ectopic foregut cellular identity to establish in intestine. Our study highlights an unexplored mechanism of chromatin regulation of organ specification and cellular plasticity.

To further decipher mechanisms regulating intestinal development, we investigate the role of transcription factor Yin-Yang1 (Yy1) and metabolism during intestinal development. While, CDX2 is a transcription factor that is appreciated to be involved in intestinal development, the role of YY1 in intestinal development has not been elucidated before. Although, we explore the mechanisms governed by CDX2 and YY1 independently, we highlight that during villogenesis both YY1 and CDX2 regulated processes are critical for intestinal development.

During late gestation, structures called villi extend into the intestinal lumen, significantly increasing the surface area of the intestinal epithelium to prepare the gut for the neonatal diet. Incomplete development of the intestine is a most common gastrointestinal complication in neonates, but the causes remain unclear. We provide evidence that YY1 is critical for intestinal villus development. YY1 loss in the developing endoderm has no apparent consequences until late gestation, after which the intestine differentiates poorly and exhibits severely stunted villi. Transcriptome analysis revealed that YY1 is required for mitochondrial gene expression, and
ultrastructural analysis confirmed compromised mitochondrial integrity in the mutant intestine. We found increased oxidative phosphorylation gene expression at the onset of villus elongation, suggesting that aerobic respiration may function as a regulator of villus growth. Mitochondrial inhibitors blocked villus growth in a fashion similar to Yy1 loss, thus further linking oxidative phosphorylation with late-gestation intestinal development. Interestingly, we find necrotizing enterocolitis patients also exhibit decreased expression of oxidative phosphorylation genes. Our study highlights the greatly unappreciated role of metabolic regulation during organogenesis, and suggests its possible contribution to the pathogenesis of neonatal gastrointestinal disorders.
Acknowledgement

In no words can I express my gratitude for my family, mentors, friends, lab members and collaborators, who have helped me through my PhD.

I would like to thank Mike Verzi, my thesis advisor, for his extraordinary energy, drive and enthusiasm for research. He helped me prioritize and focus on higher impact research leads ensuring that I don’t lose track chasing dead ends. He was always available to discuss experimental plans and results; be it a weekend or Christmas break, which helped move research at a fast pace. Mike has always been a supportive mentor and a great guide. He has been instrumental in me developing critical thinking skills during my PhD. Mike provided freedom for me to pursue my ideas and research directions. My experience in Mike’s lab has not only been intellectually satisfying but also extremely enjoyable. I am greatly thankful to him for his patience and support throughout my PhD.

My wife, Tavleen, has been my pillar of strength. I can not thank her enough for accompanying me from India to US, when I decided to pursue my PhD in US. She provided me with unconditional support when I was trying to finish experiments. She kept me motivated when failed experiments brought the morale down. Tavleen’s unwavering support and love have irrefutably been essential to me finishing my PhD.

Special thanks to Manasa Srivillibhuthur, undergraduate student working in Verzi lab. Manasa worked with me on multiple projects and is also the second lead author of our Development publication. Throughout Manasa was extremely responsible, pro-active and
managed multiple projects well. I thoroughly enjoyed mentoring Manasa because of her intelligence and curiosity for science.

I thank Anbo Zhou, graduate student at Dr. Jinchuan Xing lab at Rutgers. Me and Anbo collaborated on multiple projects and his efforts were instrumental in setting up several bioinformatics pipelines in Verzi lab. He was easily reachable and replied promptly which helped move research at a quick pace. We had interesting discussions about new ways of conducting informatics analysis.

I would like to thank Saurabh Laddha, graduate student at Chan lab at Rutgers, who directed me towards several bioinformatics analysis tools early during my PhD, helping me better channelize my efforts.

Thanks Lei Chan, postdoc in Verzi lab for collaborating on CDX2-epigenomics project; it was fun working with you. Thanks Ansu Perekatt, postdoc in Verzi lab, for collaborating on YY1-metabolism project and answering my countless histology questions. I would also like to thank other members of Verzi lab: Kevin, Oscar, Ritu and all the talented undergrads, who are not only great researchers but also created a lively work atmosphere, making overall lab experience enjoyable.

I would like to thank collaborators of YY1-metabolism project for their critical contributions: Shilpy Joshi, postdoc in Dr. Eileen White’s lab at Rutgers; Dr. Nan Gao and Dr. Edward Bonder at Rutgers Newark; Katherine Walton from Deborah Gumucio’s lab at University of Michigan.
Special thanks to Dr. Jinchuan Xing for his advice on informatics analysis and quantitative methods. I thank members of RUCDR sequencing facility. I would also like to thank Josh Thackray from Dr. Tischfield Lab, for our thought-provoking discussions on ways to analyze epigenomics data.

I express my gratitude to all my thesis committee members: Dr. Sunita Kramer, Dr. Michael Matise, Dr. Ron Hart and Dr. Jay Tischfield, for taking out time for my annual thesis committee meetings and their valuable suggestions and advice throughout my PhD.

I was fortunate to have productive collaborations where we shared research expertise and I also learned a lot from our discussions. I would like to thank Dr. Jason Spence at University of Michigan, for providing his advice and suggestions on CDX2-epigenomics project. I would also like to thank Yu-Huai Tsai from Dr. Jason Spence’s Lab for providing human organoid culture cells. Special thanks to Dr. Ramesh Shivdasani at Dana-Farber Cancer Institute, Boston; for his advice and intellectually rewarding discussions during our joint meetings. I would also like to thank Alessia Cavazza and Kushal Banerjee from Dr. Shivdasani’s lab for sharing sequencing files and results.

Thanks to my younger sister, Neha, for always keeping the mood high-spirited. Lastly, I would like to thank my parents for providing an atmosphere where I could continue doing what I enjoy the most, research.
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CHAPTER 1

Interplay of chromatin and CDX2 regulates intestinal development

Author contributions:

Namit Kumar, Kushal Banerjee, Yu-Hwai Tsai, Lei Chen, Manasa Srivillibhuthur, Anbo Zhou, Alessia cavazza and performed the research; Namit kumar, Kushal Banerjee, Jason Spence, Ramesh Shivdasani, Michael P. Verzi interpreted data; Namit Kumar and Michael P. Verzi conceived the study.
**Introduction**

Early during embryonic development, the three germ layers are formed at gastrulation stage. Endoderm, one of the three germ layer, exists as a layer of cells where regional determining factors pattern the anterior-posterior polarities (Noah et al., 2011). Anterior endoderm forms the epithelium of the stomach, lung, liver, pancreas; while posterior endoderm forms epithelium of small and large intestine. Around E9.5, murine primitive gut tube has a central lumen surrounded by endoderm derived epithelial cells. Epithelium at this stage is multilayer (pseudostratified) and maintains pseudostratified characters till E13.5 (Grosse et al., 2011; Noah et al., 2011) (Fig. 1). E9.5 to E13.5 is also the stage of rapid growth as the gut tube increases in length and circumference (Noah et al., 2011).

Around E14.5 epithelium restructures from pseudostratified to columnar epithelium and starts to form invaginations (Noah et al., 2011; Sancho et al., 2004) (Fig. 1). This leads to formation of villi, which are finger-like projections arising from the previously flat luminal surface, and by E16.5 villi protrusions are formed along the entire small intestine (Grosse et al., 2011; Walton et al., 2012). Cell division becomes restricted to inter-villus regions by E17 (Noah et al., 2011) and around postnatal day 7 (P7), the proliferative pockets of small intestine reshape into crypt structures (Sancho et al., 2004). Murine intestinal development completes around 2 weeks after birth (~P14), and by then Crypts of Lieberkuhn are established that contain all stem and proliferating cells (Noah et al., 2011; Wells and Spence, 2014) (Fig. 1). Intestinal stem cells self-renew throughout life and give rise to transit amplifying progenitor cells which then undergo additional cell divisions and eventually differentiates into mature intestinal cells. Cells continuously
migrate towards intestinal lumen and are extruded, with the entire intestinal epithelium turns over every 5 days in mice (Creamer et al., 1961).

Transcriptional networks regulate the critical events leading to intestinal specification and maturation. Wnt signaling is critical for establishing anterior-posterior polarities in the developing embryo (Huelsken et al., 2000) and is recognized to promote hindgut identity and suppress foregut identity (McLin et al., 2007). Similarly, BMP signaling is required for hindgut development, and BMP antagonist noggin promotes foregut development (Sasai et al., 1996; Tiso et al., 2002; Zorn et al., 1999). Sox17 is reported to be critical for initial endoderm specification in multiple species (Alexander and Stainier, 1999; Clements and Woodland, 2000; Hudson et al., 1997; Kanai-Azuma et al., 2002; Sinner et al., 2004). Sox17 is further shown to cooperate with Wnt signaling, and to activate Foxa2. FoxA2 is regarded as master regulator of anterior primitive gut, as Foxa2 null mutants fail to form foregut endoderm (Dufort et al., 1998; Weinstein et al., 1994). Further, primitive gut tube is then regionalized by expression of transcription factors, Sox2 and Hhex in anterior endoderm and Cdx2 in posterior endoderm (Noah et al., 2011). FGF signaling has been shown to play a crucial role in establishing CDX2 boundary at rostral intestine (Benahmed et al., 2008; Dessimoz et al., 2006). Sox2 and Cdx2 establish the stomach-intestine boundary, as expression of these transcription factors define distinct organ domains. At the time of villus formation (~E14.5) there is epithelium-mesenchymal crosstalk, where PDGF and Hedgehog signals from endoderm are received by adjacent mesenchymal cells, and this cross-talk is critical for positioning
and outgrowth of nascent villi (Karlsson et al., 2000; van den Brink, 2007; Walton et al., 2012) (Fig. 1).

Figure. 1 Landmark stages of intestinal development
Intestinal epithelium at E13.5 exists as pseudostratified epithelium. By E14.5 the epithelium starts to form villus invaginations and by E16.5 villi are formed in small intestine. Mature intestinal morphology is established by P14 with complete villi-crypt structures. (Figure reproduced/adapted with permission (Wells and Spence, 2014)).

Naïve endoderm cells, formed after gastrulation, exhibit cellular plasticity and are yet not committed to any specific cell fate (Zorn and Wells, 2009). By E9.5 patterning/regionalization of the endodermal gut tube occurs and transcription factor domains are established, Sox2 in foregut and CDX2 in mid/hindgut (Sherwood et al., 2009). Transcription factors have been shown to regulate cellular identity, for instance, ectopic expression of CDX2 in stomach or esophageal cells leads to intestinal cellular identity (Liu et al., 2007; Silberg et al., 2002). Mechanisms through which transcription factors partition the endoderm into distinct organs are poorly understood, primarily due to the limited availability of tissue from early embryonic stages.

CDX2 is a homeodomain protein expressed in the posterior endoderm and mesoderm of primitive streak staged embryos (Wells and Melton, 1999), expressed in the posterior gut from E8.5 (Beck et al., 1995) and its expression is confined to endoderm by
E12.5, where it persists throughout the lifetime of the organism. Cdx2 mutants die in-utero at E3.5 before the onset of endoderm development (Chawengsaksophak et al., 1997; Grainger et al., 2010; Tamai et al., 1999), but conditional ablation of CDX2 using Foxa3-Cre (active at E8.5 in the endoderm) leads to ectopic esophageal lineage in intestine and agenesis of colon, with intestine terminating in a blind-ended sac (Lee et al., 2005). CDX2 loss at E8.5 with Foxa3-Cre, leads to expression of otherwise silent genes, Keratin 13 (marker for suprabasal) and p63 (basal squamous epithelial cells); both markers for the esophageal lineage. Thus cellular plasticity exists at E8.5 in intestine, as revealed by lack of CDX2 leading to fate transformation with ectopic esophageal cells formed in intestine (Gao et al., 2009). However, the mechanism of CDX2-mediated intestinal identity is unclear, as CDX2’s genomic binding targets across a developmental continuum have not been explored. Additionally, it is unclear whether CDX2’s induction immediately imparts a mature intestinal imprint on the developing intestinal genome, or if CDX2’s genomic interactions are dynamic over developmental time.

Conditional ablation of CDX2 using Villin-Cre ERT mice induces Cdx2 KO at E13.5, and causes fate transformation of intestinal cells into stomach like identity. Fate change to stomach like identity was confirmed by gastric ATPase (ATP4A), Periodic Acid-Schiff (PAS) and Alcian blue staining; markers that can discern between gastric and intestinal identity. PAS stain for mucins, which are secreted by goblet cells (Mucin2) in the intestine and appear as large singular granules on the apical surface of intestinal goblet cells, scattered throughout the epithelium. By contrast, gastric mucins (such as Mucin5AC) are secreted by a type of stomach cell, the foveolar cells, which are continuous along the
epithelial lining and contain numerous smaller mucin granules. Upon CDX2 loss at E13.5, the Lohnes group reported dispersed PAS stain, and also confirmed increased expression of gastric markers like Sox2, Pdx1, Muc1, Ghrelin (Grainger et al., 2010). While the loss of CDX2 at E13.5 led to ectopic foregut stomach identity, it failed to acquire esophageal identity, as seen after CDX2 inactivation at earlier developmental stages. These findings raise the question of what temporal changes occur between E8.5- E13.5 that restrict the ectopic esophageal transformation of intestine when CDX2 is inactivated at E8.5, and stomach fate in the absence of CDX2 at E13.5. Interestingly, Cdx2 KO in adult epithelium leads to severe intestinal defects, due to lack of intestinal function, causing fatality within weeks, however it fails to induce ectopic foregut fate transformation (Verzi et al., 2010). These observations confirm that there exists a cellular plasticity window during embryonic development which is dynamic and can extend till esophageal foregut fate, depending upon temporal CDX2 ablation. In the current studies, we map temporal requirements of CDX2 in developing intestine, characterizing Cdx2 KO using Shh-Cre beginning at E9.5; which enabled us to explore mechanisms controlling intestinal specification and cellular plasticity.

CDX2 expression in embryonic stem cell leads to trophectoderm formation, expression in endoderm leads to intestinal specification and expression in motor neuron progenitor leads to caudal motor neuron cell fate (Mazzoni et al., 2013; Niwa et al., 2005; Sherwood et al., 2011; Strumpf et al., 2005). Since these lineages and cell types are developmentally very distinct, we speculate that CDX2 might be performing different roles in these varied cell types. Previous studies examining CDX2’s role within the
intestinal lineage, comparing differentiated vs progenitor cell compartments found CDX2 to regulate distinct set of genes by interacting differentially with HNF4A and GATA6 (Verzi et al., 2010). A recent study evaluating CDX2 binding in different cell types used Doxycycline CDX2 expression system, identified CDX2 ChIP-seq sites in ES, endoderm and progenitor motor neuron, and reported CDX2 binding sites to be largely at different regions of the genome in different cell types (Mahony et al., 2014). These reports support the argument of transcription factor CDX2 performing context specific functions.

In the adult intestine, active chromatin histone modifications are lost upon ablation of CDX2, thereby indicating CDX2 is required for maintaining chromatin marks at some of the intestinal genes (Verzi et al., 2010). In embryonic stem cell (ESC) to motor neuron differentiation culture system, it has been reported that Wnt and FGF patterning signal is transmitted in part by CDX2, where CDX2 then removes repressive chromatin mark at Hox clusters, leading to expression of motor neuron specific Hox genes, thereby establishing motor neuron cellular identity. Interestingly CDX2 fails to induce Hox gene expression in the presence of FGF receptor inhibitor PD173074; indicating that some component of FGF signaling is indispensable for CDX2’s function in regulating patterning (Mazzoni et al., 2013). These reports indicate interplay of CDX2 and dynamic chromatin, however CDX2’s relationship to the chromatin landscape is unexplored, particularly across developmental time.

Recent evidence has generated a better appreciation of the requirement for permissive chromatin structures for transcription factors to access DNA, in the developing endoderm (Martino et al., 2009; Shogren-Knaak et al., 2006). Wang et. al reported in
their culture system, upon organ-inductive signals the endodermal gut tube readily differentiates into endodermal organ fates of pancreas, lung, and liver. These results highlight an existence of a cellular plasticity during early endoderm developmental stages, where multiple endodermal cell fates can be acquired. Furthermore, when the same organ-inductive signals were activated at the definitive endoderm stage, they failed to commit to liver, pancreas or lung cell fates upon their respective organ-inductive signals. Enhancers for pancreas, liver and lung became poised in definitive endoderm to gut tube transition, thereby indicating that chromatin landscape needs to be primed for lineage determining transcription factors to be effective. These findings would argue that critical regulation of cellular fate is regulated at the chromatin level, as without permissive chromatin, organ-inducing transcription factors are unable to cause an effect. Another recent study, analyzing different CDX2 binding in ES, endoderm, and motor neuron progenitors reported CDX2 binding sites in ES to be chromatin accessible in ES, which were inaccessible in other cell types and CDX2 failed to bind in these other cell types. These observations provide evidence in support of permissive chromatin landscape regulating where transcription factors can bind (Mahony et al., 2014). However, chromatin regulation of cell fate and transcription factor binding is still an unappreciated field of study, warranting an extensive delineation of pathways involved.

In the current study, we provide a temporal map of intestine’s window of cellular plasticity, beyond which intestine resists foregut fate transformation. We provide evidence in human and mouse, of developmental stage dependent transformation of targets of CDX2 which show strong correlation with chromatin accessibility, that shifts at
the same developmental time when cellular plasticity is lost. CDX2 binds near genes involved in regulating developmental patterning early, and then transitions to genes that drive intestinal functions at a later developmental stage. In addition, we highlight foregut enhancer regions are initially accessible in the developing gut, but become inaccessible in the intestine once intestinal cell fate is locked. However, absence of CDX2 in the developing endoderm keeps foregut enhancers accessible. Our research provides mechanistic insights into how dynamic chromatin architecture regulates lineage specifying transcription factor binding, developmental plasticity and temporal gene expression patterns that are required for tissue development.

Materials and methods

Mice: Cdx2f/f mice (Verzi et al., 2010) and Shh-Cre mice (Harfe et al., 2004; Harris-Johnson et al., 2009) were purchased from Jackson labs. Male Shh-Cre; Cdx2f/+ mice were bred to Cdx2f/f mice for inducing Cdx2 knockout (KO) in the developing intestine beginning E9.5. Villin-CreERT2 transgenic mice (el Marjou et al., 2004) were bred with Cdx2f/f mice and temporal Cdx2 KO in intestinal epithelium was achieved upon tamoxifen treatment (1mg).

Fast genotyping: Pregnant dams were sacrificed and dissected embryos were kept in ice cold PBS. Embryo tail tissue was used for genotyping using KAPA Mouse Genotyping Kits (KK7352).
**Tissue Preparation:** For paraffin processing, duodenum, ileum, or entire small intestine was fixed overnight in 4% paraformaldehyde at 4°C, washed with PBS, and passed through increasing concentrations of an ethanol series and paraffin before embedding, as previously described (Kumar et al., 2016).

**Immunohistochemistry:** Five-µm intestinal sections, cut from paraffin blocks, were processed for immunostaining with the indicated primary antibodies, developed using the Vectastain ABC Kit (Vector Laboratories, PK6101) and counterstained with hematoxylin. A one-hour antigen retrieval step in 10mM sodium citrate solution under 15 psi pressure was used for all stains. Slides were incubated in primary antibody overnight at 4 °C.

The primary antibodies and dilutions used for staining are as follows, CDX2 1:200, PDX1 1:200, H,K,-ATPase 1:200. For a Periodic Acid-Schiff (PAS) stain, slides were incubated in 0.5% periodic acid and stained with Schiff’s Reagent (J612171, Alfa Aesar). For alkaline phosphatase staining, 1-Step NBT/BCIP (34042, Thermo Scientific) was used along with neutral red counterstain. Before developing the alkaline phosphatase, the slides were incubated in a 0.1M Tris-HCL buffer of pH 8. Images were taken using a Retiga 1300CCD (Q-Imaging) camera and a Nikon Eclipse E800 microscope with the QC-Capture imaging software. Oil was used for 60x/1.4 N.A. magnification and air for 10x/0.45 N.A., 20x/0.75 N.A., and 40x/0.75 N.A. Adjustments in contrast and sharpness, when made, were applied to complete figure panels in Adobe Photoshop, as previously described (Kumar et al., 2016).
**Epithelium Cell Isolation:** For embryos E17.5 and younger, tail was used for fast genotyping. Small intestines (proximal stomach to distal caecum) were dissected and cut into small pieces. Tissue pieces were then trypsinized with Trypsin-EDTA 0.05% (ThermoFisher Scientific 25300-054) for 5 minutes and then neutralized with 10% FBS, to isolate single cells. Single cells were incubated with PE-conjugated anti-CD326 (EpCam clone G8.8, eBiosciences, 12-5791-81) for 15 minutes on ice. PE-stained cells were then incubated for 15 minutes with magnetic conjugated anti-PE antibody (Miltenyi Biotec Anti-PE MicroBeads, 130-048-801). Enriched epithelium cells were obtained by passing cells through a column (Miltenyi Biotec, MS Columns, 130-042-201) in a magnetic field to obtain magnetic antibody conjugated, EpCam positive cells, as previously described (Kumar et al., 2016). Purity of magnetic cell isolation was compared to FACS sorted EpCam positive cells and found to be comparable. Cells were then either dissolved in Trizol for RNA processing, used immediately for ATAC-seq, fixed for ChIP experiment or cell pellets were flash frozen for Mnase ChIP experiments.

For mice older than E17.5, we added an additional EDTA enrichment step for better yield, as in older intestinal tissue there is increased muscle and mesenchymal tissue. Small intestines (proximal stomach to distal caecum) were dissected and opened longitudinally to wash the inter lumen space and expose epithelial cells. Tissue was cut in 2-3 large pieces and rinsed once with 1XPBS. Tissue was EDTA treated (2mM) for ~25 mins and mechanical dissociation was applied to release the epithelial cell layer from underlying mesenchyme. Epithelial cells from the solution were collected and used for single cell isolation and EpCam positive enrichment as described above.
**RNA-seq library preparation and informatics processing:** For E12.5 embryonic samples, RNA was prepared using Qiagen RNeasy Micro Kit (74004). RNA quantification and quality assessment was done using Agilent RNA 6000 Pico kit. RNA-seq libraries were prepared using Clontech V4 kit SMART-Seq® v4 Ultra® Low Input RNA Kit for Sequencing (634888) and sequenced on Illumina HiSeq. For E16.5 and older embryos, RNA was prepared by ethanol precipitation using 10µg LPA. RNA-seq libraries were prepared by RUCDR sequencing facilities using Illumina TruSeq RNA Sample prep v2 and sequenced on illumina Hiseq. Sequencing reads (fastq) were aligned using Tophat2 to generate BAM files (tophat2 --output-dir ${out_path} /Bowtie2Index/genome $input_path). Bigwigs were generated using bamCoverage (duplicate reads ignored) from the DeepTools package (Ramirez et al., 2014) and visualized in Integrated Genomics Viewer (IGV, Robinson et al., 2011). BAM files were further processed with Cuffquant to generate cxb files (cuffquant --frag-bias-correct Sequence/WholeGenomeFasta/genome.fa --multi-read-correct -o ${out_path} mm9_genes_archive_2014.gtf ${input_file_prefix}.bam). Cuffnorm was used to generate a normalized RNA-seq FPKM expression value table (cuffnorm --library-norm-method quartile mm9_genes_archive_2014.gtf -L l1,l2 cxb1.cxb, cxb2.cxb). Cuffdiff was used to compute differential gene expression (cuffdiff --multi-read-correct --dispersion-method per-condition --frag-bias-correct Bowtie2Index/genome.fa mm9_genes_archive_2014.gtf -L labels cxb1 cxb2).

**ChIP-seq library preparation and informatics processing:** Epithelial cells were harvested as described above and fixed in a final solution of 1%Formaldehyde, 1XPBS on a rotator
at room temperature for 20 minutes. Cells were washed twice with 1XPBS and centrifuged (300Xg for 3mins at 4°C) to obtain a cell pellet. Cells were lysed in 3X volume of lysis buffer (1%SDS; 10mM EDTA; 50mMTris-HCl pH8.1; Protease Inhibitor 1X) (Gbiosciences 160104). Cells were sonicated using a Diagenode Bioruptor to shear chromatin for 20-25 mins, to obtain DNA fragments in 200-500bp length. Protein A+G beads (15ul each) were washed with 1XPBS,1%BSA solution. Protein A+G beads were pre-loaded with desired antibody (CDX2, H3k4me2, H3k27ac) (Life technology Dynabeads, 6ug/ChIP) on rotator at 4°C for 4 hours. Antibody conjugated beads were washed twice with 1XPBS, 1%BSA solution and sheared chromatin was added to beads. Beads and chromatin were diluted to desired SDS % in dilution buffer (1%Triton X-100; 2mM EDTA; 150mM NaCl; 20mM Tris-HCL pH 8.1). Chromatin and beads were incubated overnight on rotator at 4°C. Antibody conjugated beads bound with chromatin were collected using magnetic separation and unbound chromatin was discarded. Chromatin bound beads were washed five times with RIPA wash buffer (50mM HEPES pH7.6; 1mM EDTA; 0.7% Sodium Deoxycholate; 1% NP-40; 0.5M LiCl), rinsed once with TE buffer (10mM Tris + 0.1mM EDTA). Chromatin bound beads were re-suspended in reverse crosslinking buffer (1% SDS + 0.1M NaHCO3) and incubated at 65°C for 6 hours to release ChIP DNA. DNA was column purified using a Qiagen kit and quantified using Picogreen (Life Technologies). ChIP DNA was used to prepare ChIP-seq libraries using Rubicon Genomics ThruPLEX DNA-seq Kit (R400427/ R400428/R40048) and sequenced on Illumina HiSeq (50-bp reads; single end; ~25M reads). Sequenced reads (fastq) from ChIP-seq were aligned to mm9 or hg19 using Bowtie2 to generate BAM files (bowtie2 -t --very-sensitive -x $bowtieindex -U $fastqpath
Bigwigs were generated using bamCoverage (duplicate reads ignored) from the DeepTools package (Ramirez et al., 2014) (bamCoverage --bam input.bam --outFileFormat bigwig --outFileName output.bw --binSize 10 --ignoreDuplicates --normalizeUsingRPKM). Bigwig traces were visualized in Integrated Genomics Viewer (IGV, Robinson et al., 2011). ChIP-seq peaks were called using MACS (version 1.4) with a P value cutoff at 10^{-5} (macs -t input.bam -n SampleName -c ControlBam -g $macsref -B -S --diag) (Zhang et al., 2008a). Genes associated with ChIP peaks were identified using BETA minus. Motif calling was performed using HOMER (findMotifsGenome.pl ${fastqprefix}_peaks.bed $ref ${bedprefix}-HOMER -preparse -preparsedDir ./preparse) (Heinz et al., 2010).

**Mnase ChIP-seq:** Cell pellets from mouse intestinal epithelium and Human intestinal organoid (HIO) cultures were dissolved in 3X Mnase digestion buffer (50 mM Tris-HCl, pH7.6, 1 mM CaCl2, 0.2% Triton X-100), Na butyrate (5 mM), proteinase inhibitor cocktail (1x), and fresh PMSF (0.5 mM)). Mnase digestion condition was empirically determined and for 100µl cell pellet 0.2 Units of Mnase digestion was performed for 5 mins at 37°C. Digestion reaction was stopped using equal volume of stop buffer (10 mM Tris [pH 7.6], 5 mM EDTA). Micrococcal nuclease digested chromatin is dialysed in 1000X Dialysis buffer (10 mM Tris, pH7.6, 1 mM EDTA, 0.1% SDS, 0.1% Na-Deoxycholate, 1% Triton X-100) for 2 hours at 4°C. Chromatin was added to prewashed antibody-conjugated beads (as described for ChIP experiment) and SDS was added to desired SDS%. Chromatin and beads were incubated overnight on rotator at 4°C. Antibody conjugated beads bound with
chromatin were collected using magnetic separation and unbound chromatin was discarded. Chromatin bound beads were washed once with Dialysis buffer, five times with RIPA wash buffer, and rinsed once with TE. Chromatin bound beads were re-suspended in reverse crosslinking buffer (1% SDS + 0.1M NaHCO3) and incubated at 65°C for 6 hours to release ChIP DNA. ChIP DNA was used to prepare ChIP-seq libraries using Rubicon Genomics ThruPLEX DNA-seq Kit (R400427/ R400428/R40048) and sequenced on Illumina HiSeq (50-bp reads; single end; ~25M reads). Sequencing reads (fastq) were aligned using Bowtie2 (as described for ChIP-seq) to generate BAM files. Bigwigs were generated for visualization as described above. Aligned sequence reads were filtered and reads with mapping quality of <20 were ignored. Nucleosomes containing H3K27ac/H3k4me2 histone modifications were identified using Nucleosome Positioning from Sequencing script (NPS) (Zhang et al., 2008b). The read length distribution is obtained by randomly sampling 1 million reads in each BAM file, and the shift distance for NPS is calculated based on the read length distribution. Nucleosome stability-destability (NSD) scores for each sample pair was calculated by using the BINOCh scripts (Meyer et al., 2011). Most dynamic chromatin regions identified by NSD scores (removing regions with 2kb of TSS) were analyzed for their role as dynamic Enhancer regions regulating developmental transition. Genes associated with dynamic enhancer regions were identified using BETA minus. Motif calling was performed at dynamic enhancer regions using HOMER, as described above.
**K-mean clustering and heatmap:** Bigwigs were regenerated from BAM files using parameters: remove duplicate, no normalization and extended reads. Extended reads were set at 146 bp for Mnase ChIP and for ChIP-seq calculated as: Bioanalyzer determined peak -120bp for adapter -50bp for sequenced read length) . Bigwig files were Quantile normalized using haystack algorithm (haystack_hotspots --bin_size 50 --transformation log2 --input_is_bigwig SampleFileInfo.txt mm9). ComputeMatrix was used to generate a matrix file at the defined ChIP regions (computeMatrix reference-point --referencePoint center -b 2000 -a 2000 --binSize 50 --sortRegions no --sortUsing mean --averageTypeBins mean --missingDataAsZero --skipZeros -R InputBedFile -S normalizeBigwig1.bw normalizeBigwig2.bw --outFileName outputMatrix.gz --outFileNameMatrix output). PlotHeatmap was used to generate heatmap of normalized bigwig files at defined genomic regions input as bed file (plotHeatmap -m $Input_matrix --outFileSortedRegions $Input.bed --kmeans $kclust --sortRegions descend --sortUsing mean --averageTypeSummaryPlot mean --colorMap Purples --dpi 300 --refPointLabel ChIPpeak -out $heatmap.jpeg). Genomic regions associated with desired k-mean clusters were extracted from bedfiles generated by PlotHeatmap, to obtain a selected bed files (genomic regions) which used for further is. Motif is using HOMER2 and Gene ontology on bed files were performed using Stanford GREAT (identifying associate genomic regions with Basal+extension: constitutive 5.0 kb upstream and 1.0 kb downstream, up to 200 kb max extension). For each heatmap, input bigwigs were quantile normalized to ensure intensities on heatmap are comparable and reflect ChIP bindings.
Results

Intestinal identity depends upon Cdx2, but only prior to fetal maturation

Cdx2 ablation in the developing endoderm at E8.5 using Foxa3-Cre leads to ectopic foregut fate transformation of the intestinal epithelial cells, with the epithelium acquiring a keratinized, esophageal-type cellular identity (Gao et al., 2009; Lee et al., 2005). Cdx2 loss around E13.5 using Villin-CreERT2 leads to ectopic foregut identity as well, but with a gastric lineage instead of esophageal histopathology (Grainger et al., 2010). Interestingly, Cdx2 loss in adult mice, although fatal due to loss of intestinal epithelial function, fails to induce foregut fate transformation. Some minor foregut features are still be observed in adult epithelium upon Cdx2 loss as isolated intestinal stem cells and a select subset of foregut genes can be reactivated (Simmini et al., 2014; Stringer et al., 2012; Verzi et al., 2010; Verzi et al., 2011). The timeframe in which ectopic foregut fate transformation must therefore lie in somewhere between E13.5 and adulthood, but the specific temporal requirements of CDX2 in the intestinal fate are imprecisely known.

To better understand the temporal requirements of CDX2 in patterning the intestinal epithelium, we investigated CDX2’s loss at two additional developmental stages using the Shh-Cre (inducing Cdx2 loss at E9.5) and using the Villin-CreERT2 (inducing CDX2 loss at E15.5). Temporal ablation of Cdx2 using Shh-Cre (expressed in intestinal epithelium beginning E9.5 (Harris-Johnson et al., 2009), produced gross morphological defects with translucent and distended intestine, compared with littermate controls, when analyzed at E18.5 in Cdx2f/f; Shh-Cre embryos (Fig. 2A). Uniform KO of CDX2 was confirmed using immunostaining (Fig. 2B). Cdx2 mutants exhibited reduced Alkaline
phosphatase activity (Fig. 2C), suggesting an enterocyte differentiation defect. Interestingly, when Cdx2 mutants were assessed for mucins profile using PAS staining, we observed mucins dispersed along the apical surface of the epithelial cells (Fig. 2E), a pattern more typical of gastric foveolar cells rather than mucin-producing goblet cells of the intestine (Fig. 2G). We further assayed the presence of stomach markers in intestinal tissue upon CDX2 loss at E9.5, and found increased –H,-K,ATPase staining (Fig. 2D), a hallmark stomach marker (Fig. 2F). Thus, CDX2 loss at E9.5 leads to an intestinal to gastric fate transformation, with ectopic stomach-like characteristics observed in intestinal cells. Since, CDX2 loss in the adult epithelium fails to induce fate transformation, we induced Cdx2 KO at additional developmental stages (p14, p2, E15.5), going back in developmental time to identify when the window of cellular plasticity is closed during development. Even as early as E15.5, Cdx2-deletion via Villin-CreERT2 resulted in no apparent identity conversion. Cdx2 KO was confirmed via immunohistochemistry (Fig. 2B) and CDX2-loss led to reduced alkaline phosphatase activity indicating an enterocyte differentiation defect, as seen with E9.5 induced Cdx2 KO (Fig. 2C). However, PAS stains evaluating CDX2 loss at E15.5 failed to exhibit dispersed stomach like mucins as seen upon CDX2 loss at earlier time points (Fig. 2E,F) (Gao et al., 2009; Grainger et al., 2010). We further confirmed lack of stomach marker ATPase upon E15.5 CDX2 loss (Fig. 2D,F), thereby indicating failure of fate transformation with CDX2 loss at E15.5.
**Figure 2**

A

Wild type  Cdx2 knock out  Wild type  Cdx2 knock out

s-stomach; d-duodenum; l-ileum; ce-caecum; c-colon

B

<table>
<thead>
<tr>
<th></th>
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<td>E15.5 KO Vill-Cre</td>
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G

**Onset of Cre-driven Cdx2 knockout**

E8.5  E9.5  E13.5  E15.5  Adult  

**Resulting cell fate**

Esophagus  
Gastric  
Retains intestinal identity
**Figure. 2** Cdx2 loss in the developing endoderm leads to abnormal intestinal morphology and foregut fate transformation

(A) Loss of CDX2 at E9.5 in the developing endoderm using Shh-Cre causes a distended and translucent intestine. (B) CDX2 immunostaining in duodenal sections at E18.5 highlights uniform loss of CDX2 in Shh-Cre (activate at E9.5) and Villin-CreERT2 (activate at E15.5) to induce CDX2 loss (C) Reduced Alkaline phosphatase staining upon CDX2 loss indicates enterocyte differentiation defect in Cdx2 mutants, observed in both Shh-Cre (E9.5) and Villin-Cre (E15.5) mediated KO. (D) H,K,-ATPase (stomach marker) immunostaining indicates foregut like cellular identity in Shh-mediated CDX2 loss (E9.5) compared to control intestine. In contrast Villin-CreERT2 induced CDX2 loss beginning at E15.5 does not lead to stomach H,K,-ATPase marker expression by E18.5, highlighting intestinal window of plasticity is closed by E15.5. (E) Periodic acid–Schiff (PAS) assay staining for mucins highlight Shh-Cre mediated Cdx2 KO at E9.5 leads to dispersed mucin as seen in stomach epithelium (F), compared to intestinal goblet cell produced mucin as seen in control. In contrast, E15.5 Cdx2 KO using Villin-Cre produces intestine like mucin and fails to exhibit stomach like dispersed mucin. (F) control stomach PAS stain. (G) Schematic showing relation of onset of Cre driven temporal Cdx2 KO and their corresponding cellular fates. Window of cellular plasticity for intestinal cells to acquire esophageal fate is lost by E9.5, and to acquire gastric cell fate is lost by E15.5.

These results identify a window of cellular plasticity that exists during early embryonic intestinal development. We show the ability of forming ectopic foregut in intestinal tissue is temporally dependent upon CDX2 loss. Earlier CDX2 loss at E8.5 leads to a more foregut esophageal cell fate, and this window to transform into esophageal lineage lost by E9.5. Between E9.5-E13.5, CDX2 loss leads to fate transformation into a gastric cell lineage, and beyond E15.5 intestinal cell fate is locked in and there are no observed lineage restructurings (Fig. 2G).

*Genomic targets of a lineage specifying transcription factor transition over time*

Since loss of CDX2 at different stages of development exhibits differential phenotypes, we wondered if the transcription factor CDX2 regulates different sets of genes at specific stages of intestinal development. We hypothesized that CDX2 binds at different
enhancers, regulating different sets of genes in a stage-specific manner. To test our hypothesis, we performed CDX2 ChIP-seq on isolated intestinal epithelial cells at several developmental stages: E13.5, E17.5, and Adult.

To test stage-specific CDX2 binding at enhancer regions, we concatenated MACS called peaks (P value cutoff $10^{-5}$) from E13.5 (when the epithelium is still permissive to gastric fate conversion), E17.5 (when villogenesis concludes and plasticity is restricted) and Adult (mature intestine) ChIP-seq experiments to obtain a bed file of 33,518 genomic regions. We define these 33,518 genomic sites as “**Cdx2 Enhancer regions**” and applied k-means clustering (K=8) to identify dynamic binding pattern across developmental time (Fig. 3A).

This is revealed sets of regions where CDX2 binds at all developmental stages which we defined as “**Static cdx2 enhancers**” (2252 enhancer regions in cluster1 and cluster2). “**Active until Villogenesis**” enhancers (1305 sites) are sites where CDX2 binds until mid-fetal stages, but binding does not persist in the adult epithelium. Genomic regions where CDX2 binds robustly at E13.5 but fails to bind in the adult epithelium, we have defined as “**Cdx2’s embryonic enhancers**” (2653 enhancer regions in cluster 5). Genomic regions with strong CDX2 binding at E17.5 compared to E13.5 and Adult are defined as E17 enriched (3553 enhancer regions in cluster 6), whereas genomic regions where CDX2 binds at E17.5 but fails to bind at E13.5 and Adult are defined as E17.5 unique (9881 enhancer regions in cluster8). Combined genomic regions with robust CDX2 binding at E17 are defined as “**Cdx2’s villogenesis enhancers**” (combined enhancer regions in cluster 8 and cluster 6). Genomic regions where CDX2 robustly binds in the adult
epithelium but failed to bind during embryonic stages are defined as “\textbf{Cdx2’s adult enhancers}” (8934 enhancer regions in cluster7) (Fig. 3A). Genes within 50kb of CDX2 bound enhancers are considered to be “\textbf{Cdx2 bound genes}”. Genes regulated by CDX2’s embryonic, villogenesis and adult enhancers were evaluated to identify common and unique genes by Venn overlap is, and 434, 3834, 2599 unique genes corresponding to stage dependent CDX2 embryonic, villogenesis and adult enhancers were identified respectively (Fig. 3B).

To evaluate if CDX2 binding at enhancer regions correlates with gene expression, we performed RNA-seq of intestinal epithelium at matching time points, to correlate CDX2’s embryonic enhancers with E12.5 expression; villogenesis enhancers with E16.5 expression and Adult enhancers with Adult expression. (Fig. 3C). Wilcoxon statistical test indicate CDX2’s embryonic enhancer bound genes have higher expression compared to all genes at E12.5 (P value 4.0 X10^{-4}), CDX2 villogenesis bound genes have higher expression compared to all genes at E16.5 (P value 3.2 X 10^{-6}), and CDX2 adult bound genes have higher expression compared to all genes in adult tissue (P value < 2.2 X10^{-16}) (Fig. 3C). These analyses highlights that CDX2’s differential binding at stage dependent enhancers correlate strongly with increased gene expression, thereby indicating that CDX2’s differentially bound enhancers are transcriptionally active.

To gain an understanding of the function of the genes regulated by CDX2 bound stage specific enhancers, we focused our analysis on CDX2’s embryonic–specific enhancers (cluster5) and adult-specific enhancers (cluster7). GO term analysis using Stanford GREAT (identifying associate genomic regions with Basal+extension: constitutive
5.0 kb upstream and 1.0 kb downstream, up to 200 kb max extension) indicated that CDX2 embryonic enhancers regulate genes enriched for epithelial differentiation (\(-\log\) Binomial P value 24) and tube morphogenesis (\(-\log\) Binomial P value 20) ontologies (Fig. 3D). Similar analysis on genes nearby CDX2 adult-specific enhancers found enriched roles for metabolic process (\(-\log\) Binomial P value 220) and lipid metabolic process (\(-\log\) Binomial P value 140) (Fig. 3E). These analyses indicate that CDX2’s differentially bound enhancers lead to stage specific function with gut tube development during embryonic stages and metabolic processes in the adult intestine.

To identify potential CDX2 partner transcription factors, we investigated whether CDX2 bound enhancer regions exhibited differences in their relative enrichment for nearby transcription factor motifs. CDX2’s embryonic enhancers are enriched for patterning transcription factors like Hox-family members (1E-810), and specification transcription factors like FoxA1 (1E-81) and Pdx1 (1E-47) (Fig. 3F). By contrast, Cdx2 adult enhancers are enriched for hallmark mature intestinal transcription factors like HNF4 (1E-1459) and Gata4 (1E-577) (Fig. 3G).

These results highlight differential CDX2 binding at stage-specific enhancers, regulating different sets of genes, which are more transcriptionally active in a stage-specific manner. Stage-specific gene sets perform distinct functions of gut tube development in embryo and metabolic processes in adult. CDX2 potentially partners with patterning/regionalization transcription factors in the embryo and mature intestinal regulatory transcription factors in the adult.
A

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Static cdx2 enhancers (2252)
Active till villogenesis (1305)
Active after villogenesis (3975)

Cdx2's embryonic enhancers (2653)

E17 enriched (3553)

Cdx2's adult enhancers (8934)

E17 uniq (9881)

B

Figure 3

E13.5 (1044) E17.5 (5597)

434 184
221 3834
205 1358
2599 4383

C

p-value = 4.0e-04

p-value = 3.2e-06

p-value < 2.2e-16
Gene ontology: genes associated with CDX2 embryonic enhancers

-\log_{10}(\text{Binomial p value})

0 2 4 6 8 10 12 14 16 18 20 22
Epithelial cell differentiation
Tube development
Tube morphogenesis
Tissue morphogenesis
Negative regulation of cell proliferation

Gene ontology: genes associated with CDX2 adult enhancers

-\log_{10}(\text{Binomial p value})

0 20 40 60 80 100 120 140 160 180 200
Single-organism metabolic process
Small molecule metabolic process
Lipid metabolic process
Organic substance transport
Cellular lipid metabolic process

Motifs at CDX2 embryonic enhancers

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Motifs at CDX2 adult enhancers

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Figure. 3 CDX2 target enhancers transition over developmental time, and suggest stage-specific functions for CDX2.

(A) k-means clustering of (k=8) CDX2 bound regions from E13.5, E17.5 and adult ChIP-seq experiments on isolated intestinal epithelial cells identify dynamic CDX2 target enhancers during murine intestinal development. (B) Stage-specific enhancers, E13.5 (cluster 5), E17.5 (cluster 8 and cluster 6), and Adult (cluster 7), localize nearby different sets of potential target genes (associated genes within 30kb of enhancer). (C) Wilcoxon test of RNA-seq FPKM levels of all genes vs CDX2 bound genes indicate that CDX2 binding significantly correlates with increased gene expression at the indicated time point. E12.5 RNA-seq and E13.5 CDX2 ChIP-seq bound genes; E16.5 RNA-seq and E17.5 CDX2 ChIP-seq bound genes; Adult RNA-seq and Adult CDX2 ChIP-seq bound genes. (D and E) GO term analysis using Stanford GREAT (identifying associated genomic regions with Basal+extension: constitutive 5.0 kb upstream and 1.0 kb downstream, up to 200 kb max extension) on genes associated with CDX2’s embryonic enhancers highlight CDX2’s early binding targets play a role in gut tube development and morphogenesis (cluster 5 regions) whereas genes nearby CDX2’s adult enhancers are associated with terminally differentiated functions of the intestinal epithelium (cluster 7 regions). (F) Selected transcription factor motifs enriched at CDX2’s embryonic enhancers highlight CDX2 partners with regionalization transcription factors, whereas (G) motifs enriched at CDX2’s adult enhancers highlight CDX2 partners with hallmark intestinal transcription factors in the mature stage.

Stage-specific Regulatory roles of Cdx2 are conserved across species

To investigate whether CDX2’s differential binding during intestinal specification and maturation is conserved, we utilized the Human Intestinal Organoid (HIOs) model system in which iPSCs/Embryonic stem cells undergo directed differentiation through a set of intermediate developmental stages and into a mature epithelium (Fig. 4A) (Spence et al., 2011). 3 Day activinA-treated iPSCs/Embryonic stem cells differentiate to endoderm, and a subsequent 4 days of Wnt/FGF treatment upon endoderm imparts intestinal cell fate, leading to hindgut differentiation. Hindgut spheres are then grown in matrigel where they acquire fetal intestine like characters, and upon transplant in kidney capsule organoid cultures differentiate to resemble mature intestine. Enteroid cultures from human adult fresh duodenal biopsy closely resemble adult intestinal tissue. To understand where CDX2
first interacts with the genome of embryonic endoderm cells, CDX2 ChIPs were performed at the equivalent stage of specified hindgut in this system (4 days after Wnt+FGF treatment of defined endoderm). Hindgut specification is the first instance when CDX2 is expressed along this developmental continuum, possibly driving the initiation of intestinal cell fate. To define CDX2 binding in adult human intestinal epithelium, we also performed ChIP-seq for CDX2 in human Enteroid cultures (derived from normal human duodenal epithelium). We called CDX2 ChIP-seq peaks using MACS with a P value cut off of $10^{-5}$ and identified 12,853 CDX2 bound sites in hindgut cultures and 25,358 sites for adult cultures. Only 2607 common sites were observed between these initial and final stages of intestinal development, while 10,128 regions were identified as hindgut-enriched sites and 21,512 as adult-enriched sites (Fig 4B).

We identified unique CDX2 bound enhancers using MaNorm (MACS peak at $10^{-3}$; MaNorm extensity cutoff LFC 1, P value $10^{-3}$) (Shao et al., 2012) and concatenated unique hindgut (3320 regions) and adult enhancers (10183 regions) to obtain 13503 regions, and visualized CDX2’s developmental stage specific dynamic binding patterns using k-mean clustering (k=8) (Fig. 4C). This analysis revealed sets of enhancer regions with robust CDX2 binding in hindgut (specification) and lack of CDX2 binding in adult, which we defined as “CDX2’s hindgut enhancers” (3117 enhancer regions in cluster 8 and cluster4). Similarly, enhancers with CDX2 binding only in adult cultures, were defined as “CDX2’s adult enhancers” (9483 regions in cluster 5, cluster 6 and cluster 7).

To gain insights into the function of genes regulated by CDX2’s specification and adult enhancers, we performed GO term analysis using Stanford GREAT (identifying
Figure. 4 CDX2’s stage-specific regulatory role is conserved across species
(A) Schematic of human intestinal organoid (HIO) culture. 3Days of ActivinA treated Embryonic stem cells differentiate to endoderm, 4 days of subsequent Wnt + FGF treatment upon endoderm imparts intestinal cell fate leading to hindgut differentiation. Hindgut spheres then bud into the supernatant and are then grown in matrigel where they acquire fetal intestine-like cell fate, and upon transplant in kidney capsule, these organoids mature further to adult-like intestine. Enteroid cultures from human adult fresh duodenal biopsy retains adult intestinal properties in matrigel “organoid” culture. (B) Overlap analysis of CDX2’s target genomic regions identified by ChIP-seq in specified human hindgut (HG) cells and in intestinal enteroid cultures derived from adult duodenal biopsies. (C) CDX2’s differentially bound genomic regions were identified using MaNorm and shown via k-means clustering (K=8) to reveal dynamic CDX2’s target genomic regions between HG and Adult cultures. (D) GO term analysis using Stanford GREAT (identifying associate genomic regions with Basal+extension: constitutive 5.0 kb upstream and 1.0 kb downstream, up to 200 kb max extension) on genes bound by CDX2’s hindgut-specific targets indicate that when CDX2 is first expressed in endoderm, CDX2 likely regulates genes involved in morphogenesis and gut tube development (cluster 4 and 8), whereas (E) genes nearby CDX2’s adult-specific enhancers are involved in metabolic processes (cluster 5, 6 and 7). (F) Transcription factor motifs enriched at CDX2’s hindgut enhancer highlight CDX2 partners with regionalization transcription factors, whereas (G) motifs enriched at CDX2’s adult enhancers highlight CDX2 partners with hallmark mature intestinal transcription factors.
for patterning transcription factors like HOXB13 (P value 1E-465) and HOXA9 (1E-246) (Fig. 4E), whereas CDX2’s adult enhancers are enriched for hallmark intestinal transcription factors, which are associated with physiological functions of the mature intestine, like HNF4 (1E-304), GATA4 (1E-142) (Fig. 4F).

These results indicate that, like in mouse, CDX2 binds differentially at stage specific enhancers. Genes regulated by CDX2’s specification and adult enhancers perform distinct functions, with digestive track morphogenesis and developmental processes during specification, and metabolic processes in adult tissue.

*Chromatin accessibility regulates stage specific Cdx2 binding*

CDX2 is expressed at similar levels in the intestinal epithelium throughout development, from early embryo to adult mice; what might regulate CDX2’s stage specific binding at different genomic regions? We hypothesized that maybe CDX2 is unable to access genomic regions similarly at all developmental stages. To test our hypothesis, we performed ATAC-seq to monitor chromatin accessibility across developmental time in isolated murine epithelial cells (E11, E14, E16, P1, Adult). We tested whether CDX2’s embryonic enhancers and CDX2’s adult enhancers (from Fig. 3) correlate with regions of the genome which exhibit differential chromatin accessibility during development. The rationale being that genomic regions performing adult intestinal functions, which are not required at early embryonic stages, may be inaccessible as a potential regulatory mechanism ensuring a stage-specific transcriptome (and vice versa for embryonic regions).
k-means clustering (k=5) of ATAC-seq experiments across the developmental time course was performed at the union of CDX2’s embryonic enhancers and CDX2’s adult enhancers (11,588 genomic regions) (from Fig. 3). We identified chromatin accessible regions are most dynamic before and after E16.5, suggesting the emergence of villi serves as an inflection point at which the intestinal epigenome is restructured towards the mature state. CDX2-bound genomic regions which have accessible chromatin only until villogenesis are defined as “Enhancers accessible till villogenesis” (1908 regions in cluster 3). Whereas CDX2 bound genomic regions which have accessible chromatin only after villogenesis, are defined as “Enhancers accessible after villogenesis” (2720 genomic regions in cluster 4) (Fig. 5A). Average ChIP-seq signal profiles highlight that chromatin accessibility drastically transitions at E16.5, where Enhancers accessible till villogenesis lose accessibility and Enhancers accessible after villogenesis becoming accessible (Fig. 5A).

We then determined whether chromatin accessibility at Enhancers accessible till villogenesis and Enhancers accessible after villogenesis, selectively correlated with CDX2 binding specific embryonic enhancers vs adult enhancers (from Fig. 3). Indeed, ATAC-seq sites (yellow) from Enhancers accessible till Villogenesis are more likely to be occupied by CDX2 embryonic enhancers (blue; 73% of sites from Enhancers accessible till Villogenesis have CDX2 binding during embryonic stage; Fig. 5B). In contrast, ATAC-seq sites (yellow) from Enhancers accessible after villogenesis are more likely to be occupied by CDX2’s adult enhancers (blue; 96% of the ATAC sites that are chromatin accessible after villogenesis have CDX2 binding in the adult epithelium; Fig. 5C)
Figure 5

A

B

Chromatin accessible only before Villogenesis C3 and Cdx2 embryonic enhancers

Accessible Chromatin: 972
Cdx2 regions: 73%

C

Chromatin accessible only after Villogenesis C4 and Cdx2 adult enhancers

Accessible Chromatin: 2279
Cdx2 regions: 96%

D

Cdx2 embryonic enhancers and Chromatin accessible only after Villogenesis (C4)

Accessible Chromatin: 70
Cdx2 regions: 4%

E

Cdx2 adult enhancers and Chromatin accessible only before Villogenesis (C3)

Accessible Chromatin: 308
Cdx2 regions: 4%
Figure. 5 Dynamic chromatin accessibility corresponds with developmental stage specific CDX2 binding

(A) Around E16.5, when Villus formation occurs, an inflection point is observed, where embryonic and adult chromatin form distinct chromatin accessible regions at CDX2’s binding sites, as revealed by k-means clustering (k=5) of ATAC-seq chromatin accessibility signal at genomic regions from CDX2’s embryonic and adult enhancers, identified in Fig 3. (B) Venn overlap of Enhancers accessible till villogenesis (cluster3) and CDX2 embryonic enhancers highlight chromatin at CDX2’s embryonic targets are accessible only before villogenesis. (C) Venn overlap of Enhancers accessible after villogenesis (cluster 4) and CDX2 adult enhancers highlight chromatin at CDX2’s adult targets are accessible only after villogenesis. (D) Venn overlap of CDX2 embryonic enhancers with Enhancers accessible after villogenesis, highlight CDX2’s embryonic enhancers do not have chromatin accessible after villogenesis (E) Venn overlap of CDX2 adult enhancers with Enhancers accessible till villogenesis, highlight that CDX2 adult targets are not chromatin accessible before villogenesis.

Conversely, only 4% of CDX2’s embryonic bound enhancers (blue) occupy ATAC-seq identified Enhancers accessible after villogenesis (yellow; Fig. 5D). Similarly, only 4% of CDX2’s adult enhancers (blue) occupy ATAC-seq identified Enhancers accessible till villogenesis (yellow; Fig. 5E). These results revealed that a dynamic chromatin landscape accompanies a lineage-specific transcription factor’s differential binding pattern, and this major shift in transcription factor occupancy occurs at a developmental time in which cellular fate is restricted to the mature lineage. These findings also raise the question of whether the transcription factor instructs chromatin accessibility, or is restricted by chromatin accessibility, or the two processes are co-dependent.

**CDX2 requires permissive chromatin to impart intestinal fate, and loss of CDX2 causes foregut fate transformation**

To begin investigating the relationship between CDX2 binding and chromatin accessibility, we used the human culture system where 4 days of Wnt/FGF signaling leads to intestinal
fate specification (hindgut), increased CDX2 expression, reduced PDX1 expression and silencing of SOX2. We first investigated whether loss of CDX2 during human intestinal specification can lead to foregut fate transformation, like observed in mouse models. We induced CDX2-loss using the Crisper-Cas9 system (SFig. 1A) and revealed that CDX2 ablation in HIOs led to foregut cellular identity with increased PDX1 and SOX2 expression (Fig. 6A). This analysis highlights that the cellular plasticity is a conserved phenomenon and CDX2 loss leads to foregut fate transformation.

To ascertain the role of CDX2 at this critical juncture (hindgut specification) where the balance between foregut vs intestinal cell fate is established, we identified genomic regions bound by CDX2 in specified intestine (hindgut) by ChIP-seq (12853 genomic regions, MACS P value cutoff at $10^{-5}$). We performed DAVID gene ontology analysis on CDX2 regulated genes (genes in 5kb range of CDX2 bound enhancer) and identify that genes associated with CDX2 at early specification events are enriched for GO associated with regionalization and patterning functions (Fig. 6B). We further evaluate whether genes regulated by CDX2 bound enhancers are functionally active, by GSEA correlation with RNA-seq transcriptome analysis of genes differentially expressed during Endoderm to Hindgut specification event. This analysis highlights a strong correlation of CDX2 binding with increased gene expression (NES=1.85, FDR=0) upon specification (Fig. 6C,E). These results show CDX2 regulates patterning genes during Hindgut specification, which correlates with increased gene expression, revealing that CDX2-regulated genes are functionally active at hindgut specification event, similar to CDX2’s functionality during murine intestinal specification stages.
Figure. 6 CDX2 loss leads to foregut fate in humans as well; both CDX2 and Wnt + FGF signaling are required to impart intestinal identity

(A) Crisper-mediated CDX2 loss of function prevents iPS/ES cell development into intestine, and instead leads to increased expression of P63 (esophageal marker) Pdx1, and Sox2 (stomach), highlighting foregut fate transformation upon directed human intestinal development (4 days Wnt/FGF) in the absence of CDX2. (B) GO term analysis of genes bound by CDX2 ChIP-seq in human hindgut (5kb range at enhancer binding) are regulating regionalization and patterning, when CDX2 is first expressed in endoderm. (C) GSEA shows that CDX2 bound genes in hindgut are more likely to be differentially expressed during endoderm to hindgut transformation, indicating CDX2 is binding at intestine specifying enhancers in presence of Wnt/FGF morphogens. (D) Venn overlap analysis of genomic targets identified by CDX2 ChIP-seq in presence of Wnt/FGF with CDX2 induced via Doxycycline in absence of Wnt/FGF, highlights that without morphogens CDX2 fails to bind at targets associated with intestinal cell fate.

To then evaluate if stage-specific binding can be accomplished by CDX2 alone, without a permissive chromatin architecture, we removed the intestinalizing Wnt and FGF morphogens from our cultures and overexpressed CDX2 using a Doxycycline inducible promoter. High expression of CDX2 was confirmed using qPCR and immunostaining (Fig. S1 B,C). ChIP-seq identified CDX2-bound genomic targets in the absence of Wnt and FGF at a large number of genomic sites (144,228 ChIP-seq peaks called at P value $10^{-5}$). Interestingly, when we compared CDX2 bound regions in absence of Wnt/FGF, in spite of occupying large numbers of genomic regions, only 3% of CDX2 enhancers in absence of Wnt/FGF occupy genomic regions where CDX2 binds in the presence of intestine-inducing Wnt and FGF morphogens (Fig. 6D). GSEA analysis revealed that CDX2 bound genes in the hindgut-directed differentiation condition were transcriptionally activated as the cells transition from endoderm to hindgut identity, consistent with intestinal specification. In contrast, when CDX2 expression is induced in endoderm cells not instructed to become hindgut (absence of Wnt/FGF morphogens), no correlation is observed, indicating that
other factors are required for priming intestinal chromatin for CDX2 to impart intestinal cell fate.

*Foregut enhancers lose permissive chromatin to lock intestinal cell fate, but remain accessible in the absence of Cdx2*

To better appreciate the chromatin progression across developmental time in humans, we evaluated dynamic active chromatin in organoid cultures by k-mean clustering (k=5) of H3K27ac ChIP-seq experiments (Endoderm, Hindgut, Fetal, Mature, Adult biopsy). Genomic regions focused for analysis were identified using MACS called peaks from corresponding H3K27ac ChIP-seq (253,697 regions identified with active histone modification across developmental stages of HIOs, promoters ignored). We identified enhancers that lose active chromatin after hindgut specification, and defined them as “*Human enhancers active till specification*” (40869 regions in cluster 5) (Fig. 7A). We evaluated enrichment of motifs at identified human enhancers active till specification, to ascertain which lineage-inducing transcription factors have lost access to their enhancers (figA-cluster5) once intestinal cell fate is established (hindgut). Human enhancers active till specification are highly enriched for foregut enhancers like Sox10 (1E-142), Sox2 (1E-127), FoxP1 (1E-145), Eomes (1E-10) (Fig. 7B). This analysis highlights that in human intestinal developmental, enhancers that become inactive after establishment of intestinal cell fate are regions where foregut specific transcription factors bind, presumably to drive a foregut transcriptome. These results, provide further support that foregut chromatin is no longer permissive after the intestine matures.
To directly evaluate whether chromatin accessibility depends upon CDX2, we investigated chromatin accessibility by ATAC-seq in mouse intestine across developmental time (E11, E14, E16, P1, Adult), at P1 forestomach ATAC-seq identified enhancers (9781 regions). By P1, forestomach’s cellular identity is established, which implies that regions identified by ATAC-seq would contain regions specific to foregut tissue. We identify E11 intestine is highly permissive to genomic regions of foregut enhancers, consistent with broad plasticity at this developmental time point, but these regions are sequentially lost and mostly become inaccessible by the time of intestinal villus formation at E16. This observation matches temporal closing of the window of plasticity, indicating that intestinal cell fate is locked and foregut fate transformation is restricted once foregut specific enhancers lose accessibility. We then compared ATAC-seq signal in intestinal epithelium of Shh-Cre; Cdx2 mutant intestines (permits gastric fate) and littermate controls (retains intestinal fate), at P1 foregut enhancers. By E16 most of foregut enhancers are chromatin inaccessible in control tissue, however in the absence of CDX2, foregut enhancers remain accessible in the intestine. We then evaluated foregut enhancers that retain chromatin accessibility in Cdx2 mutants (cluster2, 9781 regions) for motif enrichment. We highlight that regions which retained accessibility, when gastric fate transformation is permitted upon CDX2 loss, are enriched for foregut transcription factors like p63 (1E-430), Pdx1 (1E-79), Sox2 (1E-55). These results identify that dynamic chromatin permits broad endodermal specification, and CDX2 loss allows foregut chromatin to remain accessible even beyond the development stage when intestinal cell
Figure. 7 Foregut Enhancers become inactive after intestinal cell fate is locked, but remain active in the absence of CDX2

(A) k-means clustering (k=5) of active chromatin regions marked by H3K27ac ChIP-seq signal across human developmental cultures (Endoderm, hindgut, fetal, mature, adult biopsy). Enhancers in cluster 5 identified as enhancers that become inactive after hindgut specification, when the tissue still exhibits cellular plasticity. (B) Motif enriched at Human enhancers active till specification in intestinal tissue (FigA-cluster5) are enriched for foregut transcription factors, indicating these genomic sites possibly regulate foregut transcriptome in human and are inactivated as intestinal development proceeds. (C) k-means clustering (k=2) of mouse ATAC-seq chromatin accessibility signals across the developmental time (E11, E14, E16, p1 and Adult), with E16 control and Cdx2 mutant ATAC-seq profiles plotted adjacent, at forestomach Enhancers identified using ATAC-seq on forestomach tissue at p1 when forestomach fate is fully established. Cluster 2 highlights forestomach enhancers are accessible in intestinal tissue at E11 and become inaccessible beyond E16, however, in absence of CDX2, forestomach enhancers are readily accessible in intestinal tissue, allowing foregut cell fate to be imparted on intestinal tissue. (D) Motifs at forestomach enhancers, that become inaccessible by E16, but gain accessibility upon CDX2 loss at E16 in mouse intestine, are enriched for foregut transcription factors indicating that foregut enhancer regions become accessible, in the absence of CDX2, and may facilitate foregut cell fate upon intestinal cells when CDX2 is inactivated early in intestinal development.

fate should be specified. In the absence of CDX2, there is a failure of inactivating foregut enhancers and foregut transcriptome is established, permitting gastric fate transformation. Thus CDX2 appears to operate in a Wnt/FGF defined chromatin landscape to specify intestine and maintain an intestinal chromatin landscape while restricting a program of foregut-driven chromatin accessibility.

Discussion

An unanswered question of embryonic organ development is how are cell fates established and maintained? In 1987, single cell transplantation experiments in Xenopus confirmed germ layer commitment is progressive, as blastula staged individual vegetal cells contributed to all three germ layers, but gastrula staged cells were restricted to one
germ lineage only (Wylie et al., 1987). A similar plasticity window exists in the anterior endoderm, where ectopic expression of CDX2 in stomach leads to intestinal identity (Grapin-Botton, 2005; Liu et al., 2007; Silberg et al., 2002). Sherwood et al. reported that Wnt signaling induces CDX2 expression in the developing foregut around E7.5-E8.5, using genetic (β-catenin exon 3 deletion with Sox17-CreER at E6.5) and chemical approaches (GSK3 inhibitor XV); both approaches also reduced SOX2 expression. These observations of increased CDX2 and decreased SOX2 are characteristic of posterior gut. However, activating Wnt signaling in E9.5 stomach fails to induce CDX2, indicating that a window of cellular plasticity in the foregut is lost by E9.5 (Sherwood et al., 2011). Studies uncovering cellular plasticity in intestinal tissue are still under-explored.

Previous reports observed CDX2 loss by E8.5 leads to a more anterior foregut esophageal cell fate, but E13.5 CDX2 loss leads to fate transformation into the gastric cell lineage. These attempts to uncover molecular and epigenetic mechanisms regulating cellular plasticity in hindgut had severe limitations as the early-endoderm Foxa3-Cre (used for E8.5 Cdx2 KO) has mosaic expression, whereas Villin-CreERT2 induced uniform Cdx2 KO in rostral intestine but mosaic KO in caudal intestine at E13.5 (Gao et al., 2009; Grainger et al., 2010; Lee et al., 2005). In this study, we use Shh-Cre (Harris-Johnson et al., 2009) to induce uniform KO of Cdx2 at E9.5, which is characterized here for the first time. We highlight that CDX2 loss at E9.5 resists esophageal fate and rather leads to gastric fate conversion. Our mouse model thus enables us to understand the mechanisms governing dynamic cellular plasticity in the intestinal epithelium and the role of Cdx2 in this process. We refine the temporal map of intestinal window of plasticity where E8.5 intestine
exhibits cellular plasticity to acquire esophageal cell fate, which is now lost by E9.5 (using Shh-Cre). Between E9.5-E13.5 intestine still exhibits cellular plasticity, however, it is restricted to only gastric lineage transformation and by E15.5 the entire window of plasticity is closed. In a physiological context, understanding ectopic fate transformation is of pressing need as it’s often diagnosed with stomach metaplasia and Barrett’s esophagus, and while the diagnosis is becoming increasingly common, their etiologies remain largely unknown.

CDX2 is an Intestinal lineage-inducing transcription factor, however it is expressed in several other cells types like trophectoderm, endoderm, and motor neuron progenitors (Beck et al., 1995; Mazzoni et al., 2013; Sherwood et al., 2009). Since these tissues are developmentally distinct; it raises the question: does CDX2 bind at the same genomic targets in these functionally diverse cell types? Our research probes a similar question, where embryonic intestine is distinct from adult intestine, and CDX2 is known to be critical in both developmental stages. We elucidate through our results, that CDX2 in fact binds at different genomic regions in embryo compared to adult intestines, and regulates stage specific functions in adult and embryo. We further, identify that this phenomenon is evolutionarily conserved, as we find similar results in human cultures and mouse models.

Transcription factors only bind at a small fraction of all genomic regions that carry their consensus binding motifs, highlighting the existence of a higher regulatory mechanism dictating where and when transcription factors bind (Carr and Biggin, 1999; Iyer et al., 2001). Wang et. al reported recently, that enhancers are first poised, then
recognized by pioneer transcription factors like FOXA1 and FOXA2, and subsequently lineage-inducing transcription factors are recruited (Martino et al., 2009; Shogren-Knaak et al., 2006). We highlight through our work that CDX2 bound enhancer regions during mouse early embryonic development and human hindgut specification stage, are enriched for FOXA motifs, indicating that CDX2 is binding at regions pre-primed by FOXA transcription factors for imparting intestinal identity. CDX2’s recruitment at intestinal sites, is however ablated when chromatin is not primed using Wnt/FGF morphogens in human hindgut cultures, further enhancing the field by identifying chromatin’s regulation of transcription factor binding. However, it appears that once morphogens gradients and pioneer factors expose chromatin for lineage-specific regulators, the maintenance of lineage-specific chromatin requires lineage-specific transcription factors, as CDX2 is required for maintaining adult intestinal chromatin structures (Verzi et al., 2013).

Villogenesis is a critical transitory phase in intestinal development, where intestine goes through drastic restructuring with development of enterocytes, goblet cells and shift in metabolism from glycolysis to oxidative phosphorylation (Kumar et al., 2016). We here identify that concurrent with villus formation, dynamic changes occur in chromatin of the intestinal epithelium, with a set of enhancers becoming inactive while a separate set becomes active; changes that also coincide with the closure of window of plasticity. We mechanistically highlight that forestomach enhancers are accessible at E11 when endoderm largely exhibits cellular plasticity, however by E16 these forestomach enhancers are largely lost. However, in absence of CDX2, these forestomach enhancer regions remain active in intestine at E16. This identifies that foregut chromatin is closed
for intestinal cell fate to be locked, by a yet unexplored mechanism. Current literature provides support for our proposed mechanism as CDX2 loss during intestinal development leads to increased expression of foregut transcription factors like PDX1 and SOX2. Since foregut chromatin is accessible upon CDX2 loss, the foregut transcriptome can be initiated and thus ectopic cell fate is established (Gao et al., 2009; Grainger et al., 2010; Lee et al., 2005). Additionally, SOX2 expression in the early developing intestine, in the presence of CDX2, also leads to stomach like cells in the intestine, possibly since foregut enhancers are still accessible (Raghoebir et al., 2012). Once the cell fate is locked, CDX2 binding is transformed in human and mouse, where it binds to enhancers associated with intestinal maturation and function. These enhancer regions highly overlap with chromatin regions that become accessible only after villogenesis, thus proposing an unrecognized mechanism where chromatin regulates cell fate by directing lineage-inducing transcription factors to bind at their mature effector sites.

Our work provides several new paths to explore in future. Mechanisms that regulate modulation of chromatin and their cues will be of great interest. One possibility is that Wnt/FGF signaling has a more global impact leading to chromatin modulation. Wnt/FGF signaling has been shown to play a critical role in establishing anterior-posterior polarities and induction of cell fates. Thus, a global role of establishing lineage specific chromatin landscape by Wnt/FGF might explain their expression in several tissue types, at specific developmental stages. Future experiments investigating chromatin accessibility using ATAC-seq can be performed in human enteroid cultures, in the presence of Wnt/FGF, Wnt/FGF with Cdx2 KO and absence of Wnt/FGF with Doxycycline
induced CDX2. Comparing chromatin accessibility in the presence of Wnt/FGF with cultures without Wnt/FGF (with Doxycycline induced CDX2) might provide further evidence of Wnt/FGF’s impact on chromatin structures. Comparing chromatin accessibility in the presence of Wnt/FGF, in control vs Cdx2 KO cultures can provide further evidence for CDX2’s requirement in maintaining intestinal enhancer regions. Cultures in the absence of the Wnt/FGF condition will also shed light on CDX2’s ability to initiate chromatin structures de novo. Together, these suggested experiments might provide further evidence in support of our proposed mechanism whereby chromatin regulates cell fate specification.

Another level of regulation during organ specification could be an expression gradient of Wnt/FGF effector proteins. In adult intestine, gradient of Wnt/BMP signaling exist along crypt-villi axis, with high Wnt at crypt and high BMP signaling at villi structures. Thus, broad signaling could establish tissue fate first and then gradient of effector proteins could further specify cell fates at a finer resolution, similar to how intestinal stem cells are exposed to high Wnt and differentiated cells are in a low Wnt environment. Another likely regulator for transition from embryonic to adult intestinal chromatin landscape could be metabolism. Metabolic shifts from glycolysis to oxidative phosphorylation occurs around villogenesis, which also morphologically restructures the intestine, and could also have chromatin modulatory role, which is still not explored.

CDX2 is widely accepted as a master regulator of intestinal cell fate, and CDX2 bound sites after villogenesis are enriched for the intestinal transcription factor HNF4α’s motifs. Experiments can be conducted to evaluate if CDX2 maintains chromatin structures
exclusively or if other factors like HNF4α/γ are capable, in part or exclusively, in maintaining intestinal enhancers.

We highlight that CDX2 loss at E9.5 leads to greater permissive chromatin at foregut enhancers when investigated at E16.5. Future work can increase the resolution of analysis and investigate stomach and esophageal enhancers individually. This would further test the proposed notion that extension of the foregut plasticity exhibited by the intestine is temporally dependent upon Cdx2 loss. Experimentally, we would compare chromatin accessibility using ATAC-seq in the intestinal epithelium, focusing at esophageal and stomach enhancers, upon Cdx2 loss at E9.5 using Shh-Cre and E14.5 loss using Villin-CreERT2. Presumably CDX2 loss at E9.5 might leads to permissive chromatin at both stomach and esophageal enhancers, while loss at a later time ~E14.5 will permit accessible chromatin only at stomach enhancers. These future studies will further corroborate the relation of temporal loss of chromatin accessibility with cellular plasticity.

Further, repressive factors may work to restrict differentiation in the developing intestine, and may play a role in restricting maturation before the chromatin transition. Factors such as Blimp1 have been shown to function in this role (Mould et al., 2015; Muncan et al., 2011) and it will be interesting to see whether repressive factors localize to CDX2’s late enhancers and facilitate its transition from a patterning to a differentiation factor.

Finally, from a healthcare perspective, ectopic cell fate when established in adult tissue leads to metaplasia as seen in case of Barrett’s esophagus and stomach metaplasia. Although these disorders are often diagnosed in the clinic, their etiology remains poorly
understood and a chromatin basis for metaplasia is still unexplored. Our work provides evidence for chromatin regulation of cellular plasticity and thus chromatin profiling of metaplastic tissue might provide insights into mechanisms, read-outs for early diagnosis and possibly candidate targets.

**Supplemental Figure 1. Uniform crisper mediated CDX2 KO and efficient CDX2 overexpression using Doxycycline inducible promoter, in human hindgut culture.** (A) Hindgut differentiation upon Crisper mediated CDX2 ablation show absence of CDX2 in hindgut using immunostaining, indicating crisper mediated CDX2 KO is efficient. Also confirmed via western blot against anti-CDX2 antibody. (B) qPCR testing relative gene expression of CDX2 at hindgut upon different treatment conditions. ES as baseline reference, continued ActivinA treatment with no Wnt/FGF morphogens, Doxycycline inducible promoter lead CDX2 expression with no Wnt/FGF morphogens, 4 days Wnt/FGF morphogens inducing hindgut, 4days Wnt/FGF and Doxycycline induced CDX2. These
results highlight that Doxycycline inducible promoter system is efficient in overexpressing CDX2 in the absence of Wnt/FGF morphogens.
References


CHAPTER 2

A YY1-dependent increase in aerobic metabolism is indispensable for intestinal organogenesis

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Author contributions: Namit Kumar, Manasa Srivillibhuthur, Shilpy Joshi, Katherine D. Walton, Anbo Zhou, William J. Faller, Ansu O. Perekatt, Edward M. Bonder performed the research; Namit Kumar, Manasa Srivillibhuthur, Shilpy Joshi, Katherine D. Walton, Owen J. Sansom, Deborah L. Gumucio, Jinchuan Xing, Edward M. Bonder, Nan Gao, Eileen White, Michael P. Verzi interpreted data; Namit Kumar, Manasa Srivillibhuthur and Michael P. Verzi drafted the manuscript; Namit Kumar and Michael P. Verzi conceived the study.
**Introduction**

Necrotizing enterocolitis (NEC) is the most common gastrointestinal condition prevalent among newborns, affecting ~7% of pre-term infants with a mortality rate between 20-30% (Neu and Walker, 2011; Zani and Pierro, 2015). The etiologies of NEC remain unclear, but they are expected to be multi-factorial and correspond closely with an underdeveloped intestine that is predisposed to deficiencies in digestion, barrier function, and immune defenses. The immature intestine is susceptible to an excessive inflammatory response upon exposure to diet and colonization of microbiota, and ultimately manifests in necrosis of the tissue (Neu, 2014). Why a subset of pre-term babies may present with an immature intestine is unclear, necessitating a better understanding of intestinal development and maturation.

The intestine arises from a specified field of endodermal tissue that is shaped into a tube lined with a rapidly proliferating, pseudostratified epithelium by embryonic day 9.5 in mouse (E9.5) (Grosse et al., 2011; Spence et al., 2011; Wells and Melton, 1999). Over the next 6 days of development, proliferation in the epithelium accompanies a tremendous expansion in length and circumference of intestine. By E14.5 – E16.5, condensations of mesenchymal cells arise and serve as signaling centers that trigger the overlying epithelium to withdraw from the cell cycle, undergo a transition to a simple columnar morphology, and extend into luminal projections called villi (Burns et al., 2004; Kaestner et al., 1997; Madison et al., 2009; Mathan et al., 1976; Noah et al., 2011; Pabst et al., 1997; Pabst et al., 1999; Shyer et al., 2015; Walton et al., 2012; Walton et al., 2016). Cells between villi remain proliferative and will ultimately undergo a morphological
restructuring to form Crypts of Lieberkhün. Meanwhile, villus cells are post-mitotic and express differentiated genes that accompany their function in secretion or absorption; epithelial cells transit towards the villus tips, where they ultimately delaminate into the lumen. A number of developmental signaling molecules and transcriptional regulators have been implicated in villus development (Choi et al., 2006; Kaestner et al., 1997; Karlsson et al., 2000; Kim et al., 2007; Lepourcelet et al., 2005; Madison et al., 2005; McLin et al., 2009; Ormestad et al., 2006; Shyer et al., 2013; Spence et al., 2011; van den Brink, 2007; Walker et al., 2014; Walton et al., 2012; Walton et al., 2016), however the role of metabolic regulators in villus development has yet to be investigated.

Otto Warburg first recognized that cancer cells ferment much of their glucose supply into lactate regardless of the presence of oxygen, a phenomenon termed the Warburg effect, or aerobic glycolysis (Warburg, 1956). Recently it has become better appreciated that metabolic shifts are also critical in developmental processes. At the earliest stages of life, glycolysis is preferred as seen in the ESC-to-EpiSC transition (Zhou et al., 2012) and in a switch from oxidative phosphorylation to glycolysis that accompanies the conversion of differentiated cells into iPSCs (Folmes et al., 2011; Shyh-Chang and Daley, 2013). Metabolic processes have also been recently coupled to other developmental transitions, including macrophage activation (Tannahill et al., 2013), differentiation of mesenchymal and hematopoietic stem cells, and in the transition of satellite stem cells in muscle (Bracha et al., 2010; Chen et al., 2008; Qian et al., 2016; Takubo et al., 2013; Tormos et al., 2011); and as reviewed by (Shyh-Chang et al., 2013). These studies highlight that metabolic processes are potentially driving regulators of
development. In the adult intestine, a shift from glycolysis to oxidative phosphorylation correlates with the cell position along the crypt to villus axis (Stringari et al., 2012), and the suckling-weaning transition coincides with metabolic changes, probably related to dietary changes (Mould et al., 2015). However, a role for metabolism has not been explored in intestinal organogenesis.

Yy1 is a transcription factor that has been shown to play a significant role during cell differentiation, proliferation, and other major biological processes such as V(D)J rearrangement (Atchison, 2014), viral gene repression (Shi et al., 1991) and X-inactivation (Jeon and Lee, 2011). YY1 also regulates mitochondrial genes, as YY1 has been implicated in the maintenance of mitochondrial structure and function as well as in regulating oxidative phosphorylation and other ATP-yielding processes (Blattler et al., 2012; Perekatt et al., 2014). The molecular pathway through which Yy1 acts to regulate these functions is unclear. Some studies have shown that Yy1 functions downstream of mTOR and PGC-1α to control the transcription of these mitochondrial genes (Cunningham et al., 2007). mTOR plays a critical role in intestinal regeneration after injury in adult mice, whereas mice with genetic ablation of mTOR components during mid-gestation have minimal deficiencies in normal adult homeostasis, particularly in the proximal gut, where villi are most pronounced (Faller et al., 2015; Sampson et al., 2016). However, the consequence of mTOR or YY1 disruption in early intestinal development has not been closely examined. Elucidating YY1 function in intestinal development therefore presents an opportunity to understand how a loss of a metabolic regulator might impact organogenesis in this tissue.
In the current study, we observe a regulatory shift in expression of oxidative phosphorylation genes that coincides with the process of villus formation. Genetic ablation of YY1 in the developing intestinal epithelium results in a failure to elevate oxidative phosphorylation gene expression and in compromised villus elongation. To substantiate the link between aerobic metabolism and intestinal organogenesis, we show that pharmacological inhibition of the electron transport chain (ETC) similarly compromises villus development. Finally, we demonstrate that expression of oxidative phosphorylation genes is decreased in NEC patient intestines, and propose that a deficient metabolic transition could underlie this prevalent neonatal condition.

**Materials and methods**

**Mice:** Yy1<sup>f/f</sup> mice (Affar et al., 2006) and Shh-cre mice (Harfe et al., 2004; Harris-Johnson et al., 2009) were purchased from Jackson labs and male Shh-cre; Yy1<sup>f/+</sup> mice were bred to Yy1<sup>f/+</sup> and Yy1<sup>f/f</sup> mice for experimental litters.

**Tissue Preparation:** For paraffin processing, duodenum, ileum, or full small intestine were fixed overnight in 4% paraformaldehyde at 4 °C, washed with PBS, and passed through increasing concentrations of an ethanol series and paraffin before embedding. For explant cultures, histogel (ThermoFisher Scientific HG-4000-012) was used during processing and embedding. In case of tissue prepared for BrdU immunohistochemistry, the mice were injected with BrdU (1 mg) 1 h, 48hrs or 96hrs before euthanasia. For immunofluorescent stains, E16.5 small intestines were fixed overnight in 4%
paraformaldehyde at 4 °C, washed with PBS then embedded in 7% agarose and vibratome sectioned at 100 µm.

**Rapamycin** (LC laboratories, R-5000) treatment was given for 5 days at 5mg/kg beginning at E13.5, dissolved in Tween-80 and PEG-400. To induce RptorKO, pregnant dams were injected with tamoxifen (1mg) for 4 days beginning E13.5 and harvested at E18.5.

**Fast genotyping:** Pregnant dams were sacrificed and dissected embryos were kept in ice cold PBS. Embryo tail tissue was used for genotyping using KAPA Mouse Genotyping Kits (KK7352).

**Immunohistochemistry:** Five-µm intestinal sections, cut from paraffin blocks, were processed for immunostaining with the indicated primary antibodies, developed using the Vectastain ABC Kit (Vector Laboratories, PK6101) and counterstained with hematoxylin. A one-hour antigen retrieval step in 10mM sodium citrate solution under 15 psi pressure was used for all stains. Slides were incubated in primary antibody overnight at 4 °C. The primary antibodies and dilutions used for staining are as follows: YY1 (E0511, 1:500, Santa Cruz), CC3 (1:200, Cell Signaling, 9661S), BrdU (MCA2060GA, 1:500, AbD Sterotec), Tomm20 (sc-11415, Santa Cruz), and Ki67 (1:300, Abcam ab15580). For Cleaved Caspase 3, the primary antibody was diluted in the Signal Stain® Ab Diluent (Cell Signaling, 8112S). For a Periodic acid-Schiff (PAS) stain, slides were incubated in 0.5% periodic acid and stained with Schiff’s Reagent (J612171, Alfa Aesar). For alkaline phosphatase staining,
1-Step NBT/BCIP (34042, Thermo Scientific) was used along with neutral red counterstain. Before developing the alkaline phosphatase, the slides were incubated in a 0.1M Tris-HCL buffer of pH 8.

Images were taken using a Retiga 1300CCD (Q-Imaging) camera and a Nikon Eclipse E800 microscope with the QC-Capture imaging software. Oil was used for 60x/1.4 N.A. magnification and air for 10x/0.45 N.A., 20x/0.75 N.A., and 40x/0.75 N.A. Adjustments in contrast and sharpness, when made, were applied to complete figure panels in Adobe Photoshop.

Vibratome sections were permeabilized in 0.1% TritonX-100 for 25 minutes, washed three times for five minutes each with PBS, and blocked with 5% normal goat serum before primary antibody was added overnight at 4°C. Primary antibodies were Ecadherin (BD Transduction Labs 610181 at 1:1000), PECAM/CD31 (BD Transduction Labs 557355 at 1:1000), and PDGFRa (Santa Cruz sc338 at 1:200). Sections were then washed three times for 15 minutes each in blocking solution before DAPI and secondary antibodies diluted 1:1000 were added for one hour at room temperature. Secondary antibodies were donkey anti-mouse-555, goat anti-rabbit-488, and goat anti-rat-647 (all Invitrogen). Finally, sections were washed three times for 15 minutes each in PBS and mounted on slides with Prolong Gold for confocal imaging on a Nikon A1 Confocal with Nikon Elements software. Three-dimensional images were reconstructed using Imaris 8.0.2 software.
Quantification: Image-J software was used to calculate villi and microvilli length measurements. Only villi that were complete and protruding from the basement membrane were considered. Only microvilli with complete longitudinal sections were measured. For epithelial cell counts (Fig. S2F), 0.2mm long duodenal regions (E14.5) and 0.663mm duodenal regions (E18.5) were quantified along one side of the intestine and then normalized to 1mm. For explant experiments, only sections of tissue containing intact epithelium, mesenchyme, and the basement membrane were measured. Regions of growth in explant assays were characterized as areas with villi protruding above the flat epithelial layer. Explant experiments were performed 5 times and multiple counts were averaged for each biological replicate to measure region of villus growth (control=3, rotenone=6, oligomycin=2) and biological replicates were analyzed for significance using a two-tailed t-tests based on the results of an F-test for sample variances between biological replicates.

For the BrdU pulse-chase experiment (Fig. 2I), average number of BrdU positive cells per villus were identified using immunohistochemistry and grouped depending upon BrdU positive cell’s position from the intervillus base. (group1- cells up to position 5, group2- cells in position 6-15, group3- cells in position 16-25). Averages were taken from up to 4 biological replicates of each condition.

Western blot: 50 μg of whole cell lysate (Control, Rapamycin-treated, RptorKO; whole small intestine tissue) was heat denatured in 5× SDS sample buffer, separated by 4-12% gradient SDS-PAGE, and transferred onto a PVDF membrane (ISEQ00010; Millipore). The
membrane was blocked with 5% skim milk in PBS containing 0.1% Tween-20 for 1 h, and then pS6 antibodies (1:1000), Tomm20 (1:1000) and B-actin (1:5000) were used to detect proteins.

**Microarray:** Mouse embryos of the same genotypes were pooled (up to 2) to obtain 2 biological replicates of Y\textsuperscript{f/f}Y1 and Y\textsuperscript{f/f}; \textit{Shh-Cre}. Small intestines (proximal stomach to distal caecum) were dissected and cut into small pieces. Tissue pieces were then trypsinized with Trypsin-EDTA 0.05% (ThermoFisher Scientific 25300-054) for 5 minutes and then neutralized with 10% FBS, to isolate single cells. Single cells were incubated with PE-conjugated anti-CD326 (EpCam clone G8.8, eBiosciences, 12-5791-81) for 15 minutes on ice. PE-stained cells were then incubated for 15 minutes with magnetic conjugated anti-PE antibody (Miltenyi Biotec Anti-PE MicroBeads, 130-048-801). Enriched epithelium cells were obtained by passing cells through a column (Miltenyi Biotec, MS Columns, 130-042-201) in a magnetic field to obtain EpCam positive, magnetic antibody conjugated cells. Cells were then dissolved in Trizol and RNA prepared by ethanol precipitation using 10\mu g LPA. RNA was then processed for microarray on Affymetrix Mouse 430 \_2 arrays according to manufacturer’s instructions. Data was analyzed using R Bioconductor (Gentleman et al., 2004) with gcrma normalization (Wu et al., 2004) and limma package (Smyth, 2004). 1495 unique genes were differentially expressed with \log_{2}FC >1 and adjusted Benjamini Hochberg p-value <0.05 (Table S1). 800 genes were identified as downregulated (\log_{2}FC < -1 and adjusted Benjamini Hochberg p-value <0.05) and were analyzed for gene ontology using DAVID (Dennis et al., 2003).
**Transmission EM:** Intestinal tissues were freshly dissected, flushed in cold PBS, minced into 1-3 mm fragments, and immediately fixed overnight at 4 °C in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde and 2.0% (vol/vol) paraformaldehyde. Fixed tissues were washed briefly with 0.1 M sodium cacodylate buffer and postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4 °C. Tissues were washed with excess distilled water and En bloc-stained with 1% aqueous uranyl acetate for 30 min in the dark. Tissues were then washed with distilled water and dehydrated through a series of ethanol and propylene oxide. Tissues were then transferred into EMBed 812 (Electron Microscopy Sciences 14120) and propylene oxide (mixed at 1:1) overnight at room temperature in tightly capped vials on a shaker. Tissues were then transferred into 100% EMBed 812 overnight at room temperature. Tissues were embedded in embedding molds at 65°C for two nights. Ultrathin sections of 70 nm were cut; grids were stained with uranyl acetate and lead citrate and observed under an FEI Tecnai 12 Transmission Electron microscope with Gatan Cooled-CCD camera. Multiple grids were analyzed for each intestinal sample. The brightness was edited in Adobe Photoshop adjusting the Lightness to +7, using the Hue/Saturation adjustment tool, the Brightness and Contrast to +28 and +7 respectively, using the Brightness/Contrast adjustment tool, and finally by using the Levels adjustment tool and selecting ‘Increase Contrast 1’.
Informatics analysis: Genes involved in oxidative phosphorylation (135 genes) were obtained from the GSEA-KEGG oxidative phosphorylation list (Liberzon et al., 2011) (Kanehisa et al., 2016) (Kanehisa and Goto, 2000). Genes involved in lysosomal ontologies (23 genes), not directly implicated in oxidative phosphorylation, were ignored to use a final oxidative phosphorylation gene list (112 genes) (Table S2). A custom R-script was used to prepare heatmaps of oxidative phosphorylation gene expression in mouse colon of CD1 and C57BL6 genetic backgrounds (GSE5204) (Kaiser et al., 2007).

Necrotizing enterocolitis patient’s microarray CEL files (GSE46619) were obtained from previous study where whole genome microarray expression profiling was performed on bowel tissue from neonates diagnosed with necrotizing enterocolitis and surgical controls (Chan et al., 2014). We processed the CEL files using R Bioconductor (Gentleman et al., 2004) with gcrma normalization (Wu et al., 2004) and limma package (Smyth, 2004). Principal component analysis and hierarchical clustering were performed as quality control and one replicate found to be an outlier was ignored from further analysis. Our study analyzed 4 biological NEC replicates and 4 surgical controls. Genes downregulated in NEC patients were analyzed using DAVID (Dennis et al., 2003). Correlation study of NEC patients and YY1 downregulated genes was performed using Gene Set Enrichment Analysis (scoring scheme=weighted, permutations=10000) (Subramanian et al., 2005). NEC patient’s log2FC of oxidative phosphorylation genes and all genes was calculated and Wilcoxon Test was used to evaluate statistical significance, using a custom R-script. Schematic of oxidative phosphorylation genes dependent on YY1 was prepared by modifying pathway workflow obtained from IPA [IPA®, QIAGEN Redwood City,
www.qiagen.com/ingenuity] using genes downregulated upon YY1 loss (log$_2$FC < -0.27 and adjusted Benjamini Hochberg p-value <0.05, 2475 genes).

YY1 ChIP-seq peaks were obtained from previous study that identified YY1 binding sites in adult intestinal epithelium (GSM1295000) (Perekatt et al., 2014). Genes within 30kb regions of YY1 binding sites were overlapped with genes downregulated in intestinal epithelium upon YY1 loss (log$_2$FC < -0.27 and adjusted Benjamini Hochberg p-value <0.05, Table S3).

**Mitochondrial Activity Assays:** Complex I (NADH-dehydrogenase - NADH-coenzyme Q reductase complex) activity was applied to mouse cryosections using previously described methods (Christensen and Diemer, 2003; Hench et al., 2011). In brief, flash frozen tissues were prepared for cryosections (4 microns). Sections were covered with reaction medium (0.2 M Tris buffer, pH 7.4 containing 1 mg/ml tetranitroblue tetrazolium (NBT), 5 mM magnesium chloride, 25 mM cobalt chloride and 2 mg/ml NADH) at room temperature. After development of the blue color (8-10 min), reaction was stopped using 4% formalin (10-15 min), washed twice with distilled water and mounted in water based mounting media. Complex IV (Cytochrome C oxidase) activity was performed as previously described (Seligman et al., 1968). Briefly, cryosections (4 microns) were incubated in reaction buffer (2.5 ml of 0.2 M phosphate buffer, pH 7.6, 20 mg catalase, 10 mg cytochrome c, 5 mg of 3, 39 diaminobenzidine tetrahydrochloride hydrate (DAB) and 750 mg of sucrose in 7.5 mL of deionized H2O) for an hour, rinsed thrice with water, dehydrated and mounted in xylene based mounting media.
**Explants:** Proximal duodena were dissected from E14.5 and E15.5 embryos, connective tissue was separated, and intestines were placed on transwell membrane (Costar 3428) in BGJb media (Invitrogen 12591-038) supplemented with 1% pen/strep (vol/vol) and 0.1 mg/mL ascorbic acid (Sigma A4403). For mitochondrial inhibition base media was supplemented with rotenone (1 µM; Sigma 45656) or Oligomycin (1µM; Sigma O4876). Intestines were cultured for 72 h (starting at E15.5) or 96 hours (starting at E14.5) at 37 °C with 5% CO2 with media changes every 24 h. For qPCR analysis, intestinal explants were cultured for 48 h (starting at E15.5). Tissue was dissolved in Trizol and RNA prepared using RNeasy Micro Kit (Qiagen 74004). The RNA was reverse transcribed using SuperScript III First-Strand Synthesis System (Invitrogen 18080051) to prepare cDNA for quantitative PCR analysis using gene-specific primers (available on request) and SYBR green PCR master mix (Applied Biosystems 4309155).

**Results**

**YY1 expression in the developing endoderm is required for villus development**

To characterize the role of Yy1 during intestinal development, we employed the *Shh-cre* driver, which is expressed throughout the intestinal epithelium starting around E9.5 days (Harris-Johnson et al., 2009). At E18.5, *Yy1<sup>+/−</sup>; Shh-cre* pups (further referred to as YY1<sup>KO</sup>) exhibited gross morphological defects with a translucent and distended intestine, indicating an underdeveloped intestine compared to littermate controls (Fig. 1A). Uniform, epithelial-specific YY1 loss was confirmed using immunostaining (Fig. 1G-K), yet
despite YY1 loss early in intestinal development, no morphological phenotype was observed until late gestation. We first observed reduced villus growth at E16.5 in YY1 mutant embryos, though villus height at this stage was not significantly shorter (Fig. 1 B-E). However, by E18.5, a dramatic failure of villus elongation was evident (Fig. 1F) and villus height in the mutants was significantly reduced compared to littermate controls (Fig. 1L), a pattern consistent from duodenum to ileum (Fig. S1).

To further explore intestinal development upon YY1 loss, we examined proliferation and differentiation markers. Yy1 mutants exhibited an enterocyte differentiation defect as seen by reduced alkaline phosphatase staining (Fig. 2A, S2A); whereas Periodic Acid-Schiff staining suggested similar Goblet cell development in mutants and controls (Fig. 2B, S2B). To evaluate whether the villus growth defect could be attributed to reduced proliferation or increased cell death, we assayed proliferation using Ki67 (Fig. 2C,D,E, S2C) and BrdU (Fig. 2F,G,H), and found proliferation to be unaffected upon YY1 loss (Fig. S2G). We also did not find significant apoptosis upon YY1 loss, as measured by cleaved caspase 3 staining (Fig. S2D). The epithelium also had fewer cells (Fig. S2F), suggesting that the villus elongation deficit was not attributable to pressure accumulating in the lumen. To better understand why the Yy1 mutant epithelium had fewer cells, we traced epithelial cells over time using BrdU pulse-chase labelling at 1, 48, and 96 hours of chase time concluding at E18.5. In both Yy1 mutants and control littermates, BrdU cells migrated towards villi tips. However, BrdU-labeled cells were eliminated from the mutant epithelium more rapidly, possibly due to shorter villus length in mutants, which would shorten their transit path to the villus tip (Fig. 2I,
Fig. 1. YY1 expression in the developing endoderm is required for villogenesis. (A) YY1 loss leads to an underdeveloped intestine at E18.5, with mutant intestine being distended, translucent and devoid of villi. Yy1 mutants have similar morphology compared to control littermates at E10.5 (B), E12.5 (C), E14.5 (D), subtle changes at E16.5 (E) and strikingly stunted villi at E18.5 (F) in the duodenum. YY1 immunostaining of duodenal sections shows specific and complete loss of YY1 immunoreactivity in the intestinal epithelium of Cre+ animals at, E10.5 (G), E12.5 (H), (E14.5 (I), E16.5 (J), and E18.5 (K). Dashed lines highlight the border between the epithelium and mesenchyme. (L) Villus height measurements are not dramatically altered in E16.5 Duodenum (p-value=0.125, n=4). However, Yy1 mutants have drastically reduced villus height at E18.5, a pattern observed from duodenum (*P-value=3E^-6, n=4) to ileum (**P-value=1E^-4, n=2). Error bars show standard error. Two-tailed t-tests were done based on the results of an F-test for sample variances. scale=50µm.
Fig. 2. YY1 loss compromises enterocyte differentiation but not cell proliferation. (A) Yy1 mutant enterocytes exhibit diminished alkaline phosphatase staining at E18.5, whereas goblet cell differentiation appears normal, as seen by PAS staining (B). Yy1 mutants have similar numbers of proliferating cells, as seen by Ki67 (C-E) and BrdU (F-H) staining. (I) BrdU pulse-chase labeling suggests a more rapid elimination of mutant cells from the epithelium (1hr control n=2; 1hr mutant n=2; 48hr control n=4; 48hr mutant n=3; 96hr control n=4; 96hr mutant n=3). All histological staining and quantification were done on duodenal regions. scale:50µm.

S2E). Taken together, our results indicate that YY1 is dispensable for proliferation in the developing intestinal epithelium, but clearly required for proper differentiation of enterocytes and elaboration of full villi.
Villogenesis initiates despite loss of YY1

To better understand why villi are stunted in YY1^{KO} animals, we evaluated whether initiation of villogenesis is disrupted in mutant animals. We stained for the presence of PDGFRα-expressing mesenchymal clusters, which form the signaling foci from which villus development proceeds (Walton et al., 2012). PDGFRα cluster formation was clearly present in the Yy1 mutants at E16.5 (Fig. 3A-D), suggesting that villogenesis initiates normally. Our results thereby highlight that villogenesis initiates properly in the absence of YY1, yet extension of the nascent villi into the lumen is subsequently compromised.

YY1 acts independently of mTOR signaling in villus development

A recent study inactivating mTOR signaling during intestinal development did not compromise duodenal villus length in the adult (Sampson et al., 2016), however villus formation was not investigated during early intestinal development. Since YY1 has been demonstrated to function downstream of mTOR (Cunningham, et. al., 2007), we queried whether disruption of mTOR signaling in the developing intestine would yield a similar villus elongation defect as observed upon YY1 loss. We inhibited mTOR signaling in the developing intestine using two approaches: pharmacologically, with the mTORC1 inhibitor Rapamycin, and genetically, using conditional ablation of Rptor. Rptor is an essential component of the mTOR complex 1 which is required for mTOR-mediated nutrient sensing, and Rapamycin destabilizes the mTOR-raptor complex (Kim et al., 2002). We confirmed mTOR signaling inhibition via both approaches at E18.5 using western blot staining of pS6, a canonical mTOR target, in whole gut tissues (Fig. S3A,B). However, villus
Fig. 3. YY1 loss is dispensable for the onset of villogenesis; YY1 acts independent of mTOR signaling in regulating villogenesis.
(A-D) Immunofluorescent staining of E16.5 YY1 KO intestine exhibits PDGFRα expression in mesenchymal clusters associated with villogenesis, similar to littermate controls. PECAM staining shows Yy1 mutants have similar vascularization as control. Villogenesis occurs normally upon inhibition of the mTOR pathway using genetic ablation of Rptor (F), or pharmacological inhibition using Rapamycin (G), as compared to controls (E) at E18.5. mTOR pathway inhibition by Rptor KO and Rapamycin treatment was confirmed by western blot using pS6 antibody (H-I). E18.5 Duodenal villi heights in mTOR-inhibited embryos were unchanged compared to controls (* P value= 0.20684, ** P value= 0.217758). Control or mTOR inhibited intestines were drastically longer when compared to YY1KO villi heights, as seen in Fig. 1H. Two-tailed t-tests were done based on the results of an F-test for sample variances. scale=50µm.

length was unaffected in Rptor KO and Rapamycin-treated embryos compared to controls (Fig. 3E-H), and significantly longer than YY1 KO villi heights (Fig. 1L, 3H). Thus, while mTOR
may be involved in regulating other intestinal processes (Faller et al., 2015; Sampson et al., 2016), our findings suggest a dispensable role of mTOR in villus development. This also indicates that, unlike YY1’s function downstream of mTOR in other tissue (Blattler et al., 2012), YY1 is acting independently of mTOR in regulating villus growth.

Yy1 loss leads to mitochondrial dysfunction

To elucidate the underlying cause of deficient villus elongation in the absence of Yy1, we performed transcriptome analysis at E15.5, early in villus formation, immediately prior to when morphological phenotypes were discernable in the mutant. Small intestinal epithelial cells from E15.5 embryos were enriched by dissection and purified using antibodies to the epithelial cell specific marker EpCam (Fig. 4A). Transcriptome analysis identified 800 unique genes that were downregulated upon YY1 loss (log₂FC < -1 with Benjamini-Hochberg adjusted P value of < 0.05; Fig. 4B, and confirmed by qRT-PCR, Fig. S3C). Products of these genes were enriched for localization to the brush border, consistent with the deficient alkaline phosphatase activity ascribed to this structure (Fig. 2A, S2A), and supporting the idea that YY1 promotes maturation of the developing intestinal epithelium.

Mitochondrial function was the most strongly enriched attribute of genes downregulated in YY1⁰⁰ epithelia (Fig. 4C), and many of these genes have been shown to bind YY1 via ChIP-seq (Table S3). We therefore examined mitochondrial ultrastructure using transmission electron microscopy (TEM), and revealed that Yy1 loss leads to defective mitochondrial morphology, with distended, electron-poor inner membrane
Fig. 4. YY1 loss compromises mitochondrial ultrastructure and gene expression

(A) Experimental workflow to capture the E15.5 epithelial transcriptomes from control and YY1\textsuperscript{KO} embryos. (B) 800 genes were identified as downregulated (log\textsubscript{2}FC >1 and adjusted Benjamini Hochberg p-value <0.05) upon YY1 loss, and (C) their enriched ontologies, demonstrating reduced expression of nuclear mitochondrial genes upon YY1 loss. (D) Ultrastructural analysis of enterocytes shows deformed mitochondria (arrows) and significantly stunted microvilli (red box) (* P value= 0.0467) (D-E). Error bars represent standard error. Two-tailed t-tests were done based on the results of an F-test for sample variances. scale:0.5µm.
space, and disrupted cristae (Fig. 4D). TEM also revealed that microvillus length was significantly reduced (Fig. 4E,D-red boxes), consistent with down regulation of genes associated with the brush border (Fig. 4C). Together, transcriptomic analysis of E15.5 epithelium points towards a role for Yy1 regulation of mitochondrial function, and suggests that mitochondrial dysfunction disrupts proper villus growth and maturation in the developing intestine.

A metabolic shift to increased oxidative phosphorylation is required for villus development

While mitochondrial structure was clearly dependent upon Yy1 in the developing gut (Fig. 4D), it was surprising to find that a Yy1-loss phenotype only manifested itself in approximately 7 days after Shh-Cre mediated deletion at E9.5; especially considering that Yy1 transcripts (Fig. S4A) and protein levels (Fig. 1G-K) are fairly stable across developmental time. Consistent with mitochondrial localization, genes downregulated upon YY1 loss are also enriched for function in oxidative phosphorylation (OxPhos, ranked second after mitochondrial dysfunction in Ingenuity pathway analysis- canonical pathways; $P \text{ value}=1.02\times 10^{-10}$) and involved in all complexes of the ETC (Fig. 5C). To understand why reduction of OxPhos genes does not cause a YY1KO phenotype prior to E16.5, we analyzed expression of OxPhos genes along intestinal developmental stages (Kaiser et al., 2007). Interestingly, OxPhos genes show a marked increase in expression around E16.5 days in mouse intestine (Fig. 5A,B), coincident with the time of the villus elongation phenotype in YY1 mutants. Our analysis thus reveals a shift in metabolic gene
expression to OxPhos that coincides with villus elongation. We further corroborate our findings by showing that genes downregulated upon YY1 loss are destined to increase around E16.5 in the developing intestine (Fig. S4B,C) and are enriched for mitochondrial function (Fig. 4C).

To determine whether mitochondrial function is compromised in the Yy1 mutant epithelium, we performed assays of mitochondrial activity, in situ, to separately query the activities of mitochondrial complexes I and IV. In both cases, epithelial cells in the YY1 mutant were severely deficient in these activities compared to control epithelial cells, whereas cells in the developing muscle and stroma showed similar levels of mitochondrial complex activities (Figs. 5D, E and S4D, E). As an indicator of mitochondrial mass, we performed immunostaining and immunoblots for Tomm20. Consistent with the distended mitochondria we observed by TEM (Fig. 4D), we saw a dispersed immunoreactivity for Tomm20 in YY1 mutant epithelial cells (Fig. 5F). Additionally, via western blot we observed that Tomm20 protein levels are elevated in the mutant (Fig. 5G). Increased Tomm20 levels may be a response to the energy demand sensed by the cells as a result of compromised respiratory transport chain function. Together, these assays are consistent with the gene expression analysis, and indicate that OxPhos is compromised in the Yy1 mutant epithelium.

To test our hypothesis that OxPhos is indispensable to support the metabolic requirements of a budding villus structure, we utilized an explant culture system in which villus development can be observed ex vivo (Walton et al., 2012). Intestine segments of developing duodena, were cultured in the presence or absence of mitochondrial
Fig. 5. Oxidative phosphorylation genes normally gain expression at the onset of villigenesis, but fail to do so in the absence of YY1.

Developmental time course showing expression of oxidative phosphorylation genes in mouse intestine in CD1 (A) and C57BL6 genetic backgrounds(B). (C) Genes downregulated upon YY1 loss (log₂FC > 0.27 and adjusted Benjamini Hochberg p-value < 0.05, 2475 genes) were analyzed using IPA, and mitochondrial dysfunction canonical pathway (P value=1.02E-10) is enriched, highlighting YY1-dependent genes involved in all complexes (1-5) of the electron transport chain. Inhibitory targets of mitochondrial toxins are shown, with rotenone inhibiting complex I and oligomycin inhibiting complex V. Mitochondrial activity assays for respiratory chain complex I, (D, NADH-coenzyme Q reductase, blue) and complex IV (E, cytochrome C oxidase, brown) were dramatically reduced in the YY1 mutant epithelial cells. Non-epithelial cells exhibited similar staining; also see supplemental figure 4. Tom20 immunostaining (F) reveals increased but more diffuse immunoreactivity in the mutants. Elevated Tom20 protein levels were also observed by immunoblot (G). scale = 50µm.
Fig. 6. **Mitochondrial activity is required for villogenesis.**

(A,B) E14.5 explants cultured for 96 hours in control and rotenone-supplemented media exhibit a villogenesis defect. Mitochondrial inhibitors did not affect YY1 levels (C-E) or proliferation (F-H), similar to the YY1^KO^ phenotype (Fig. 1-2). (I) Quantification of villogenesis in the explants. (J) qRT-PCR shows reduced levels of enterocyte marker transcripts upon treatment of explants with mitochondrial inhibitors. For statistical analysis, inhibitor treatments were grouped (n=4) and tested against the control (n=3) (Epcam, \( P \) value=0.0564; Villin, \( P \) value=0.0866; Alpi, \( P \) value=0.00329). (K) Explants treated with rotenone also exhibited decreased levels of alkaline phosphatase activity compared to controls. Two-tailed t-tests were done based on the results of an F-test for sample variances. **scale=50µm.**

inhibitors rotenone (inhibiting ETC complex-1) or oligomycin (inhibiting complex-5) (Fig. 5C). Targeting these complexes reduced formation of villus structures and revealed that the ETC function is required for villus elongation (Fig. 6A-I), akin to Yy1 activity in vivo.
These results suggest that increased aerobic respiration is critical to intestinal epithelial development, specifically during late gestation and villus development. Notably, the concentrations of ETC inhibitors used in this experiment did not compromise cell proliferation (Fig. 6F-H) or cause significant apoptosis (Fig. S5A-C), suggesting that the effects of the rotenone and oligomycin treatments were not generally toxic, but were more specifically disruptive in the manifestation of mature villi (Fig. S5D). Indeed, RT-qPCR on explant tissues reveals that inhibition of the ETC reduces expression of villus maturation genes, but does not affect proliferating cell markers (Fig. 6J). This is corroborated by reduced alkaline phosphatase staining upon ETC inhibition (Fig. 6K). Together, these results indicate that increased aerobic respiration is indispensable for villus elongation and maturation of intestinal cells.

Necrotizing enterocolitis patients exhibit a transcriptome deficient in YY1-dependent genes and diminished in OxPhos gene expression.

Underdeveloped intestines are suspected to be a primary cause of NEC (Neu and Walker, 2011; Zani and Pierro, 2015), but the molecular etiologies of NEC remain unclear. Given that loss of YY1 in the developing gut leads to a phenotype reminiscent of NEC patient bowels, we explored the possibility that NEC patients may exhibit deficiencies in the YY1-dependent transcriptome. We analyzed expression data from necrotizing enterocolitis patients compared to surgical-control patients (Chan et al., 2014) using Gene Set Enrichment Analysis. A strong correlation was observed between genes downregulated in NEC patients and genes downregulated upon YY1 loss (Fig. 7A). Genes enriched at the
leading edge of this correlation between NEC patients and YY1 loss were enriched in mitochondrial and brush border gene ontologies (Fig. 7B), consistent with altered metabolism and poorly matured intestine in both systems. To corroborate our findings, we analyzed whether OxPhos genes were under-expressed in NEC patient samples and indeed observed that oxidative phosphorylation genes are significantly reduced in NEC patients (Fig. 7C, \( P = 2.20 \times 10^{-10} \), Wilcoxon test). While YY1 target genes were under-expressed in the NEC patient group, YY1 itself was unaffected (average expression value of 10.41 ± 0.04 in controls vs. 10.40 ± 0.05 in patients, ± s.d.), suggesting there are multiple pathways responsible for diminished OxPhos gene expression in these patients. Our study thus highlights deficiencies in aerobic metabolism as a possible underlying etiology of NEC.

**Figure 7**

![Figure 7](image)

**Fig. 7.** Necrotizing enterocolitis patients exhibit transcriptomes similar to Yy1 mutants. (A) GSEA analysis reveals that genes downregulated upon YY1 loss strongly correlates with genes that have reduced expression in NEC patients. (B) Genes at the leading edge of the GSEA, representing genes downregulated in both NEC patients and YY1\(^{\text{KO}}\) transcriptomes are enriched in the indicated gene ontologies. (C) Box plot shows that NEC patients exhibit an overall reduction in oxidative phosphorylation gene expression (Wilcoxon test, *\( P = 2.20 \times 10^{-10} \)), suggesting a potential contributor to this disease etiology. Data in (C) are derived from 4 NEC patients and 4 surgical controls, GSE46619.
Discussion

There has been a renewed interest in cellular metabolism in the recent years, particularly in the cancer field, where the observation of Warburg metabolism has undergone a renaissance. Warburg observed that cancer cells ferment sugar (an inefficient source of 2 ATP per molecule of glucose), even in the presence of oxygen (conditions in which differentiated cells can produce 36 molecules of ATP by metabolizing sugar to CO₂ through oxidative phosphorylation) (Warburg O., 1924). Recent hypotheses to explain the Warburg effect have centered upon the fact that biomass is a key requirement for a growing tissue, possibly more important than ATP. Glycolysis provides glycolytic intermediates which are the building blocks for amino acids and nucleotides, and acetyl-CoA for fatty acids (Vander Heiden et al., 2009). Thus a growing tissue, be it in a tumor or in a developing embryo, benefits from the fermentation of sugar to acquire biomass. Our study identifies an increase in OxPhos genes as the intestine takes on a more differentiated role. We propose that glycolysis is the preferred metabolism from E9.5 through E16.5, to increase biomass as the gut is rapidly expanding and as nearly all cells are proliferative. Then, as cells begin to differentiate on elongated villi at E17.5, we believe that a shift to oxidative metabolism is favored, increasing ATP availability for transporters and enzymes associated with digestion. Interestingly, we find that enterocyte differentiation appears more affected than goblet cell differentiation; this finding might suggest different metabolic demands for these epithelial cell lineages.

Several recent studies have observed an intermingling between metabolic state and developmental processes, including in hematopoietic, mesenchymal, and neural
lineages (Shyh-Chang et al., 2013; Ufer and Wang, 2011). In the mature intestine, where rapid growth in intestinal crypts feeds differentiated cells onto the villi, it was recently observed that the proliferating progenitor cells exhibit a more glycolytic metabolism compared to their differentiated counterparts (Stringari et al., 2012; Xiong et al., 2015).

A key role of future studies will be to determine whether metabolic states are dependent on developmental signaling processes, whether developmental signaling processes depend upon metabolic states, or most likely an interdependence of the two processes. Along these lines, interesting recent analyses have put metabolic control under the important intestinal WNT signaling pathway (Pate et al., 2014). We have previously shown that the WNT-dependent adult intestinal stem cells rely upon YY1 for renewal; whether there is a relationship between WNT and YY1 in mitochondrial regulation has not been explored, but YY1-dependent control of stem cell markers in the developing gut is less clear (Fig. S5E). Another metabolic regulator, PPARGC1A, has been shown to work as a co-factor with YY1 in other contexts (Cunningham et al., 2007); PPARGC1A and its family members are downregulated in the YY1 embryonic intestinal mutant (Fig. S5F), and could play a role in mitochondria-dependent villus growth as well.

NEC is a major cause of neonatal mortality and the etiologies behind NEC are unknown (Neu and Walker, 2011; Zani and Pierro, 2015). Our study found a strong correlation between the transcriptomes of YY1 mutants and NEC patients, broaching the possibility that metabolic dysfunction could contribute to NEC. We also observed that oxidative phosphorylation genes are downregulated in NEC patients. The reduction of Ox-Phos genes in NEC patients could reflect the often-observed microvascular defects
observed in NEC intestines, could reflect the underdeveloped state of NEC guts, or could be a primary deficit leading to the NEC phenotype. Our work, showing that intestinal maturation is compromised when either disrupting a regulator of Ox-Phos genes or directly inhibiting the ETC, demonstrates that a primary disruption of a metabolic shift can lead to a NEC-like phenotype. Additionally, our study highlights the potential impact of environmental toxins on NEC, as we demonstrate that mitochondrial toxins commonly used as pesticides can elicit a NEC phenotype.

Future examinations could explore whether there is a link between environmental use of pesticides and NEC incidence. We can use our identified NEC readout of reduced aerobic metabolism, to develop in-utero diagnostic techniques for detecting NEC onset. For better understanding the mechanisms linking reduced aerobic metabolism and NEC, further investigations can be conducted using mouse intestinal explant cultures and human fetal enteroid cultures, which will enable us to investigate underlying mechanisms. Culture systems are also suitable for conducting drug screens to identify curative agents against this fatal neonatal disorder, including the repurposing of drugs used to treat metabolic syndromes.

To investigate the role of other candidate genes involved in metabolic regulation of embryonic intestinal development, the Shh-cre mouse model system can be used. The effect of metabolism on adult tissue health is still under-explored, and evidence from our research opens several paths for future studies. The effect of reduced aerobic respiration on adult intestinal tissue health can be evaluated using the Villin-cre mouse model to induce temporal ablation of YY1 and possibly other candidate genes. Understanding the
Impact of altered metabolism is also of great importance, as many sports training routines commonly include intermittent hypoxic training. There has also been a rise in usage of drugs like FG-4592 and sporting gear like hypoxic masks, which induce hypoxic conditions to increase red blood cell counts, however these training regimens might be detrimental for vital tissues thus necessitating future studies.

Supplemental Figure 1

Fig. S1 YY1 mutants have a villus development defect in the developing ileum
Fig. S2 YY1 mutants do not have significant cell death, but have compromised enterocyte differentiation in ileum. (A) Yy1 mutant enterocytes exhibit diminished alkaline phosphatase staining in developing ileum. (B) YY1 mutants have similar goblet cell differentiation in ileum compared to littermate controls, as seen by PAS staining. (C) Ki67 immunohistochemistry shows no significant difference in proliferation at E10.5 and
E12.5 (D) YY1 mutants do not have drastically higher cell death compared to littermate controls, as seen by CC3 staining. (E) Representative BrdU pulse chase immunostaining as documented in figure 2l. (F) Epithelial cell number is substantially reduced in the YY1 mutants at E18.5 (**P value=2.78E-4, n=4) but no difference is seen at E14.5 (P value=0.95, n=4). (G) Similar numbers of BrdU positive cells were observed between controls and mutants in duodenum at E14.5 and E18.5. Error bars show standard error** indicates P value < 0.01

**Fig. S3 YY1 acts independently of mTOR signaling in villogenesis**

(A) Immunoblot showing diminished pS6 levels in isolated epithelial cells from mice treated with rapamycin. (B) pS6 levels were also diminished in whole tissue extracts from epithelial cell specific Rptor knockout. Levels of pS6 decrease do not appear as robust due to inclusion of non-epithelial cells in the protein lysate. (C) A set of genes was selected to validate the microarray findings by qRT-PCR. Shown is the relative transcript level compared to the matched control. For qRT-PCR, data were normalized to actin transcript levels, n=3 control and 5 knockout isolated epithelia from e18.5 intestine; bars indicate standard error.
Fig. S4 Genes downregulated upon YY1 loss are destined to increase expression, coincident with the onset of villogenesis. (A) Yy1 RNA levels don’t change appreciably during murine intestinal development. Relative Yy1 levels were calculated based upon the average readings at 3 different microarray probes, across 12 replicate samples for each timepoint (GSE5204). Bars represent the SEM between the probes. Developmental time course showing expression of YY1 downregulated genes in mouse intestine in CD1 (B) and C57BL6 genetic
backgrounds (C). Genes include those downregulated $> \log_2$ fold change $> 1.5$ and with p-value $< 0.05$ that are included in the developmental timecourse microarray data. (D-E) Electron transport chain complex activities are diminished in the YY1 mutant epithelium, 2 additional biological replicates to accompany those in figure 5D-E. scale = 50 µm.

Fig S5. **Mitochondrial inhibitor restricts villogenesis, but does not Increase cell death.** (A-C) cleaved caspase 3 immunostaining on tissue explants from the indicated treatments. (D) pAMPK levels were elevated upon treatment with mitochondrial inhibitors, confirming expected activity of these compounds in compromising electron transport chain function and reducing cellular ATP levels. (E) Transcript levels of markers of the Lgr5+, crypt base columnar cell population were assayed in control versus Yy1 knockout E18.5 intestinal epithelium. Data were normalized to actin transcript levels, n=3 control and 5 knockout; bars indicate standard error. (F) Regulatory changes in PPAR and PPARGC1 family members upon Yy1 loss in E15.5 epithelium, detected by microarray, showing that several of these known mitochondrial regulators are diminished upon Yy1 loss.
References


