Transcription Factors GATA4 and HNF4A Control Distinct Aspects of Intestinal Homeostasis in Conjunction with Transcription Factor CDX2*§

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Background: Different transcription factor combinations may control distinct or overlapping cellular functions.

Results: Intestines lacking tissue-restricted CDX2 and broadly expressed GATA4 or HNF4A show unique defects.

Conclusion: Combined with CDX2, GATA4 controls crypt cell replication, whereas HNF4A regulates enterocyte maturation and a cohort of functional enterocyte genes.

Significance: Combinatorial mechanisms for intestine-specific gene regulation may apply generally to other tissues.

Distinct groups of transcription factors (TFs) assemble at tissue-specific cis-regulatory sites, implying that different TF combinations may control different genes and cellular functions. Within such combinations, TFs that specify or maintain a lineage and are therefore considered master regulators may play a key role. Gene enhancers often attract these tissue-restricted TFs, as well as TFs that are expressed more broadly. However, the contributions of the individual TFs to combinatorial regulatory activity have not been examined critically in many cases in vivo. We address this question using a genetic approach in mice to inactivate the intestine-specifying and intestine-restricted factor CDX2 alone or in combination with its more broadly expressed partner factors, GATA4 and HNF4A. Compared with single mutants, each combination produced significantly greater defects and rapid lethality through distinct anomalies. Intestines lacking Gata4 and Cdx2 were deficient in crypt cell replication, whereas combined loss of Hnf4a and Cdx2 specifically impaired viability and maturation of villus enterocytes. Integrated analysis of TF binding and of transcripts affected in Hnf4a;Cdx2 compound-mutant intestines indicated that this TF pair controls genes required to construct the apical brush border and absorb nutrients, including dietary lipids. This study thus defines combinatorial TF activities, their specific requirements during tissue homeostasis, and modules of transcriptional targets in intestinal epithelial cells in vivo.

Tissue-specific gene expression reflects the coordinated activities of transcription factors (TFs) that are restricted to one or a few cell types and others that are expressed more broadly. In tissues of endodermal origin, TFs such as CDX2, PTF1, and PDX1 are restricted to individual organs (1–3), whereas others such as HNF4A and GATA are expressed in many endoderm-derived tissues. It is unclear if the latter TFs control separate and distinct programs and cellular function or merely support the activity of master regulators, such as the intestine-restricted CDX2.

In adult mammals, the small intestine contains abundant proliferative cells in submucosal crypts and mature post-mitotic cells along the villi. The homeodomain TF CDX2 is expressed exclusively in the epithelium of the small intestine and colon in both replicating crypt cells and differentiated villus cells (4–7). CDX2 is required for proper specification of the intestine during development (8) and is considered a master regulator of intestinal identity because its ectopic expression in the stomach or esophagus activates intestine-restricted genes (9, 10). In adult mice, absence of CDX2 dysregulates genes involved in terminal cell differentiation, and fatal malnutrition ensues over ~3 weeks (11–13). Simultaneous inactivation of its homolog CDX1 results in nearly complete arrest of intestinal crypt cell proliferation (14). Together, these findings demon-
strate diverse requirements for CDX2 in embryonic tissue spec-
ification, cell differentiation, and maintenance of the adult
intestinal epithelium. It is unclear how CDX2 partners with
various TFs to mediate these diverse functions.

One way to identify the TFs important in any tissue is
through DNA sequence motifs that are highly enriched among
active cis-regulatory sites. Enhancers active in the intestinal
epithelium show few recurring sequence motifs, including the
one preferred by CDX2; the GATA motif, especially in replicat-
ing cells; and the consensus motif for HNF4A, mainly in differ-
etiated villus cells (11). This select group of TFs thus seems
particularly important in intestinal gene regulation. Indeed,
knock-out mice lacking single factors show diverse, subtle,
and nonletal defects. GATA4 and GATA6 show regional
(proximal 4/5) and global intestinal expression, respectively,
and the corresponding mutant mice have subtle defects in
crypt cell replication, secretory cell differentiation, and
control of selected enterocyte genes (15–17). Loss of HNF4A
perturbs colon development (18), but Hnf4a<sup>−/−</sup> adult mouse
intestines are overtly normal, with modestly perturbed gene
expression (19). Coupled with the frequent co-occurrence of
their specific sequence motifs near CDX2-binding sites, the
limited defects in Gata and Hnf4a mutant mice led us to
postulate that they may regulate intestinal genes in com-

EXPERIMENTAL PROCEDURES

Mice—Gata4<sup>Fl/Fl</sup>, Gata6<sup>Fl/Fl</sup>, Cdx2<sup>Fl/Fl</sup>, Hnf4a<sup>Fl/Fl</sup>, and trans-
genic Villin-Cre<sup>ERT2</sup> mice were described previously (11,
20–23). Gata4<sup>Fl/Fl</sup>, Gata6<sup>Fl/Fl</sup>, Cdx2<sup>Fl/Fl</sup>, and Villin-Cre<sup>ERT2</sup>
mice were crossed to generate conditional Gata4<sup>Fl/Fl</sup>,
Gata6<sup>Fl/Fl</sup>, Cdx2<sup>Fl/Fl</sup>, Villin-Cre<sup>ERT2</sup> compound-mutant mice.
Cdx2<sup>Fl/Fl</sup>, Hnf4a<sup>Fl/Fl</sup>, and Villin-Cre<sup>ERT2</sup> mice were mated to
generate Hnf4a<sup>−/−</sup>Cdx2<sup>−/−</sup>Villin-Cre<sup>ERT2</sup> mice. Genotypes
were verified using previously published protocols for each
mutant strain (11, 20–23). To activate Cre, mice received intra-
peritoneal injections of 1 mg of tamoxifen (Sigma) in sunflower
oil (Sigma) daily for 4–5 days. Mice were weighed daily and
euthanized when the first mouse of a particular genotype
became moribund (4 days for Gata4<sup>−/−</sup>Cdx2<sup>−/−</sup> and 7 days
for Hnf4a<sup>−/−</sup>Cdx2<sup>−/−</sup>). Controls were injected with tamox-
ifen but lacked Villin-Cre<sup>ERT2</sup>. The Animal Care and Use Com-
mittes at our institutions approved and monitored animal use.
RNA Expression Analysis—Mouse intestinal epithelial was harvested by incubating fresh jejunum in 5 ml EDTA solution for 45 min as described previously (24). RNA was isolated using TRIzol reagent (Invitrogen) and the RNaseasy kit (Qiagen). For quantitative RT-PCR, RNA was reverse-transcribed (Super-Script III, Invitrogen) and assessed using FastStart Universal SYBR Green Master Mix (Roche Applied Science) and specific primers for Cdx2 (5'-TCAACCAGTTAAGGAACCTTC-3' and 5'-GCAAGGAGGTCAAGAGGACTC-3'), Gata4 (5'-TTT-GAGCAGATGGG-3' and 5'-GAATGCGGGGTGTGC-3'), Gata6 (5'-CAGCAAGCTTGTAGTGTC-3' and 5'-GATCTGTG-TGATTCTCTCCGGG-3'), Hnf4a (5'-GGCTCAAGCTAGGAGACAGCTGC-3') or Hnf4a (5'-GGTCAAGCTAGGAGACAGCTGC-3') or HPRT (5'-ATGTACTTGGCCCACTCGAC-3' and 5'-TTGCCGCTCATCTAGGCTTT-3') mRNA and expressed relative to control tissues.

Global assessment of RNA levels was performed on isolated jejunal epithelia from two control and two Hnf4a-delCdx2-del mice. RNA was isolated using TRIzol reagent and the RNaseasy kit, followed by treatment with the Turbo DNA-free kit (Ambion) to remove genomic DNA. The RNA integrity number for each sample was >9.8. RNA-seq libraries were prepared with the TruSeq RNA sample preparation kit (Illumina) according to the manufacturer's instructions. 75-bp single-end reads were sequenced on an Illumina NextSeq 500 instrument. Sequence tags were aligned with the Mus musculus reference genome build 9 (mm9), and the Tuxedo software package was used to align reads, assemble transcripts, and determine differences in transcript levels using a false discovery rate of 0.05 (30). The Integrative Genomics Viewer was used to visualize aligned reads (31).

Association of TF Binding with Nearby Genes—Binding sites for Cdx2 and HNF4A from Chip-seq experiments (GEO accession number GSE34568) (24) were associated with the nearest gene within 30 kb using GREAT software (32). Genes with at least one binding site for each TF within this range were considered in our further analysis. BioVenn was used to generate Venn diagrams (33).

Gene Ontology Analysis—DAVID functional annotation clustering analysis was performed using medium classification stringency and default options (34). Clusters with significant enrichment scores (>1.3) were considered (34). When similar annotation clusters recurred in the list, we selected a representative gene ontology term from the cluster and listed it with the cluster enrichment score for that group of terms.

Electron Microscopy—Mouse ilea were flushed with PBS, fixed overnight or longer in EM fixative (2% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4)), and embedded in TAAB 812 resin (Marivac Ltd., Nova Scotia, Canada). 80-nm sections were cut, stained with 0.2% lead citrate, viewed, and imaged with a Philips Tecnai BioTwin Spirit electron microscope at an accelerating voltage of 80 kV.

Analysis of Intestinal Lipid Absorption—Mice were placed on a diet (D12331, Research Diets) containing 58% calories from fat (the regular diet, Prolab IsoPro RMH 3000, contains 14% calories from fat) starting 5 days before the first dose of tamoxifen until euthanasia. Intestines were fixed overnight at 4°C, equilibrated in 20% sucrose overnight at 4°C, embedded in O.C.T. compound (Tissue-Tek), and frozen on dry ice. Lipid accumulation was visualized in 10-μm tissue sections incubated in 0.6% Oil Red O (Sigma) in propylene glycol at 60 °C for 8 min.

RESULTS

cis-Element Features Identify Candidate Partner TFs in Intestinal Epithelial Cells—To assess the potential for coordinate regulation among intestine-specific and pan-endodermal TFs, we searched for overrepresented sequence motifs in Cdx2 Chip-seq data from mouse intestinal villus cells (24). Sequences corresponding to GATA4 and HNF4A were significantly enriched near Cdx2-bound sites (Fig. 1A), indicating that these endoderm-restricted TFs may recurrently join Cdx2 at intestinal enhancers in vivo. Indeed, Chip-seq for GATA4 and HNF4A in the intestinal villus indicate co-occupancy with Cdx2 at thousands of sites (24, 35). Interestingly, all three TFs are expressed in crypt and villus epithelial cells (Fig. 1B), suggesting the possibility of overlapping requirements that might be revealed in compound-mutant mice. If Cdx2 and these partner TFs largely overlap in function, then the compound-mutant mice should phenocopy one another. Alternatively, distinct phenotypes would indicate that TF pairs control distinct programs. Moreover, transcripts perturbed upon combined TF loss should identify genes that require more than one TF for optimal expression.

Efficient Depletion of TFs in Mouse Intestinal Epithelium—As GATA4 and CDX2 are implicated in the control of selected villus cell genes in vivo (15, 36) and in replication of cultured progenitor cells (11), we first considered the intestinal GATA factors and crossed mice to obtain conditional Gata4floflo, Gata6floflo, Cdx2floflo, Villin-CreERT2 mutants. Tamoxifen treatment activated Cre recombinase and achieved intestinal loss of GATA4 and Cdx2 mRNA and protein (Fig. 1, C and D), although Gata4 loss was incomplete due to inefficient recombination at the Gata6 allele (Fig. 1, D and E) (17). Thus, we refer to these mice as Gata4flofloCdx2floflo and note that HNF4A expression was preserved (Fig. 1C).

Cdx2 and HNF4A co-occupy thousands of intestinal enhancers, where loss of Cdx2 perturbs chromatin structure, resulting in decreases in or loss of HNF4A binding (24). The absence of HNF4A alone has little consequence on intestinal function (19) or chromatin structure, but the combined absence of Cdx2 and HNF4A affects more transcripts than loss of either TF alone (24). To determine the functional consequences of combined TF loss, we produced Hnf4a-delCdx2-del intestines, which showed total or nearly total loss of Cdx2 and HNF4A mRNA and protein throughout (Fig. 1, F and G), without affecting GATA4 (Fig. 1F).

Loss of Cdx2 and Gata4 Impairs Intestinal Crypt Cell Replication—Gata4flofloCdx2floflo mice lost weight rapidly (Fig. 2A), became moribund, and required euthanasia within days of induced gene recombination. GATA4 is not expressed in the distal ileum, where Cdx2 levels are the highest and the defects in Cdx2floflo intestines are the most severe (37). In the duodenum and jejunum, where GATA4 is abundant in wild-type mice, villi
were slightly stunted in Gata4del mutants. In contrast, Gata4delCdx2del mice showed shallow crypts and short villi throughout the small intestine (Fig. 2, B–D). As the effects of the Cdx2del mice are most severe distally, we focused analysis on the most distal portion of the intestine that normally expresses Gata4, i.e., the jejunum. Here, significantly fewer crypt cells expressed the proliferation marker Ki67 (Fig. 3, A and B). In addition, the number of cells in S-phase, as marked by incorporation of BrdU during a 1-h pulse, was also reduced in Gata4delCdx2del mice (Fig. 3, A). The reduced crypt and villus heights did not reflect increased apoptosis in addition to the proliferation deficit (Fig. 3, C), but ectopic alkaline phosphatase expression (enterocytes) and Alcian blue staining (goblet cells) in Gata4delCdx2del crypts indicated precocious cell maturation (Fig. 3, D), probably reflecting the cell cycle arrest. Villus alkaline phosphatase expression and Alcian blue staining verified the presence of mature cells (Fig. 3, D), and although cells retained a columnar morphology, they varied in shape, size, and nuclear morphology. The fraction of goblet cells, but not of enterocytes or Paneth cells, was modestly increased over intestines lacking only GATA4 or CDX2 (Fig. 3, D and E). These data show that intestinal crypt cell replication is a prominent shared function for CDX2 and GATA4, with a significantly larger defect in the compound-mutant mouse than in either single-mutant mouse. The villus defects may reflect this poor crypt cell turnover or indicate additional joint functions in cell maturation.

**Combined Loss of CDX2 and HNF4A Compromises Enteroctye Differentiation without Affecting Crypt Cell Replication—Swift weight loss in Hnf4adelCdx2del mice (Fig. 4A) warranted euthanasia 2 weeks earlier than in Cdx2del littermates, with severe diarrhea and steatorrhea occurring during the course of tamoxifen administration. Duodenal and jejunal villus heights were similar to those in Cdx2del intestines, and enterocytes in these regions showed little cellular atypia (data not shown). By contrast, villi in the ileum were dysplastic and significantly stunted (Fig. 4, B and C), with many cells showing pyknosis, and total villus cell numbers were significantly lower compared...**

**FIGURE 1. Recurrence of DNA sequence motifs at CDX2-binding sites in mouse intestinal villus cells and efficient gene deletion in conditional mutant mice. A, motifs for CDX2, GATA, and HNF4A are enriched near the 5000 strongest CDX2-binding sites in wild-type mouse intestinal villus cells. Position weight matrices, with the corresponding Z-score and p value, are indicated for each motif. B, immunohistochemistry for each TF showed nuclear staining throughout the crypt-villus axis in wild-type mice. Dashed boxes and insets show crypt details. C, CDX2 (upper panels) and GATA4 (middle panels) immunohistochemistry in control and Gata4delCdx2del jejuna confirmed the absence of the targeted TF proteins. Immunohistochemistry for HNF4A (lower panels) revealed similar levels in Gata4delCdx2del and control mice. D, quantitative RT-PCR for Cdx2 and Gata4 revealed complete loss of these mRNAs in the Gata4delCdx2del jejunum compared with controls (n = 2 each). However, Gata6 mRNA levels were only mildly reduced (n = 4 each). E, GATA6 immunohistochemistry showed persistent protein in the Gata4delCdx2del jejunum after 4 days of tamoxifen. At the same time, mice with Gata6del alone recombined the allele efficiently and lost GATA6. F, immunohistochemistry for HNF4A (upper panels) and CDX2 (middle panels) verified the absence of both proteins in the ileum of compound-mutant intestines. Staining for GATA4 (lower panels) in the duodenum of Hnf4adelCdx2del mice (GATA4 is not expressed in the ileum) was similar to the control. G, quantitative RT-PCR for Cdx2 and Hnf4a showed complete and nearly complete loss of mRNA levels (n = 3 each), respectively. Scale bars = 30 μm. Graphs show means ± S.E. Transcription Factor Combinations in Intestinal Epithelium**

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with Hnf4ad el or Cdx2del villi (Fig. 4D). Importantly and in contrast to Gata4del Cdx2del mice, this was not a result of reduced crypt cell proliferation (Fig. 4, E and F) or increased apoptosis (Fig. 4G) but rather of aberrant epithelial cell differentiation. A total absence of alkaline phosphatase in Cdx2del and Gata4del Cdx2del intestines compared with loss of either TF alone. Scale bars = 30 μm. C and D, quantitation of crypt (C) and villus (D) length in the Gata4del Cdx2del jejunum compared with single mutants and controls (n ≥ 3). Graphs show means ± S.E. Statistical significance was assessed using Student’s t test; p values are indicated when significant and are color-coded to match the sample with which Gata4del Cdx2del samples were compared.

Identification of Genes Co-regulated by CDX2 and HNF4A—To identify likely direct transcriptional targets, we selected genes showing CDX2 and HNF4A co-occupancy in jejunal villus epithelial cells (<30 kb from the nearest gene). More than half (52.9%) of the genes reduced in Hnf4ad el Cdx2del intestines were bound by both TFs, compared with 16.4% of genes with increased levels (Fig. 5B), consistent with a role for these factors primarily in gene activation. Among the 310 genes down-regulated in Hnf4ad el Cdx2del mice compared with controls and showing TF co-occupancy, k-means clustering identified three prominent patterns with roughly equal frequency (Fig. 5C and supplemental Table 1): genes equally reduced compared with controls in Cdx2del and Hnf4ad el Cdx2del intestines, but not in Hnf4ad el intestines (cluster C); genes modestly perturbed in each single mutant and further dysregulated in the compound mutant (cluster B); and transcripts that barely changed in either single mutant compared with controls, but were significantly reduced in Hnf4ad el Cdx2del intestines (cluster A). Selected examples of genes from clusters A (Synpo) and C (Abp1/Aoc1) (Fig. 5D) showed binding of both CDX2 and HNF4A. This analysis indicates that CDX2 alone is required for proper expression of genes in cluster C, whereas additional loss of HNF4A is necessary to affect genes in clusters A and B.

To examine these changes in gene expression with greater confidence and quantitative accuracy, we performed RNA-seq on epithelial cells isolated from control and Hnf4ad el Cdx2del jejuna. We confirmed disruption of Cdx2 and Hnf4a by RNA-seq, noting an absence of transcript reads from exon 2 in Cdx2
and a nearly complete absence of transcript reads from exons 4 and 5 in Hnf4a (data not shown).

RNA-seq results corroborated our microarray data and expanded the number of candidate direct transcriptional targets, identifying 840 down-regulated and 642 up-regulated genes \( (Q < 0.05) \) in Hnf4a\(^{del}\)Cdx2\(^{del}\) intestines. Integration of RNA-seq and ChIP-seq data indicated that both CDX2 and HNF4A were bound near genes that respond to loss of both TFs is a good indication of co-regulation.

CDX2 and HNF4A Co-regulate Genes for Specific Aspects of Digestive Physiology—To determine the physiologic consequences of gene co-regulation, we performed gene ontology analysis on the 368 genes that RNA-seq identified as reduced in Hnf4a\(^{del}\)Cdx2\(^{del}\) intestines and for which ChIP-seq showed binding of both TFs (supplemental Table 2). Clustering of significantly enriched gene ontology terms highlighted genes central to enterocyte functions, including oxidation-reduction; transport of ions, carbohydrates, and lipids; and components of the plasma membrane (Fig. 6C). Thus, combined loss of CDX2 and HNF4A in vivo produced attrition of enterocytes in the ileum and significantly altered gene expression in jejunal enterocytes, albeit with few overt histologic manifestations. Together, these anomalies can account for the rapid uniform lethality in Hnf4a\(^{del}\)Cdx2\(^{del}\) mice.

**FIGURE 3. Effects of combined loss of Gata4 and Cdx2 on proliferating and differentiated intestinal cells.** A, immunohistochemistry of proliferative markers Ki67 and BrdU (administered 1 h before euthanasia) confirmed significantly reduced proliferation in Gata4\(^{del}\)Cdx2\(^{del}\) jejunum compared with single-mutant and control littermates (quantified in B). Dashed boxes outline the areas magnified to the right. Scale bars = 50 \( \mu \)m. C, cleaved caspase-3 staining revealed that apoptosis was not increased in Gata4\(^{del}\)Cdx2\(^{del}\) intestines compared with control villi or crypts (areas in dashed boxes are magnified below). Arrows indicate caspase-3-positive cells (an example is magnified in the dashed box on the upper left). D, histochemistry and immunohistochemistry of the jejuna for alkaline phosphatase and CRS4C1 in Gata4\(^{del}\)Cdx2\(^{del}\) and control mice showed no differences in mature enterocytes and Paneth cells, respectively. Alcian blue staining revealed an increased fraction of villus goblet cells in Gata4\(^{del}\)Cdx2\(^{del}\) intestines (quantified in E). Of note, Gata4\(^{del}\)Cdx2\(^{del}\) mice had ectopic alkaline phosphatase and Alcian blue staining in crypts. Graphs show means ± S.E. Significant changes between Gata4\(^{del}\)Cdx2\(^{del}\) and other genotypes were calculated using Student’s \( t \) test. Results are color-coded by genotype; insignificant differences are not marked. Scale bars = 50 \( \mu \)m (A) and 30 \( \mu \)m (C).
Because defects in cell membranes and in nutrient absorption should be objectively observable, we focused efforts on identifying these deficits in Hnf4a<sup>del</sup>Cdx2<sup>del</sup> mice. Apical microvilli in Hnf4a<sup>del</sup> mice are grossly intact (19). In contrast, microvilli on the apical membranes of Cdx2<sup>del</sup> enterocytes were moderately scant and stunted, whereas those in Hnf4a<sup>del</sup>Cdx2<sup>del</sup> cells were sparse, shortened, and disarrayed (Fig. 7A). Beyond this significant brush-border defect, the Hnf4a<sup>del</sup>Cdx2<sup>del</sup> enterocytes showed additional intracellular anomalies, including an accumulation of light droplets or vacuoles near the cell apex and dark, possibly membrane-bound vesicles within mitochondria. The contribution of these defects to cell attrition is not presently clear. However, together with reduced expression of peptidases, other hydrolases, and genes involved in symporter activity and intestinal absorption, the brush-border anomalies undoubtedly contribute to the severe malnutrition.

In addition, we made particular note of lipid metabolism because intestinal absorption of dietary lipids is highly relevant to human health, and HNF4A is known to regulate hepatic lipid metabolism (23). To test the hypothesis that co-regulation of genes related to lipid absorption might also underlie the pro-
found rapid malnutrition in Hnf4a\textsuperscript{del}Cdx2\textsuperscript{del} mice, we placed mice on a high-fat diet starting 5 days before TF gene disruption (Fig. 7B) and stained intestines 12 days later with Oil Red O to detect lipid deposits. Enterocytes accumulate lipids as a result of fatty acid and cholesterol transport as well as \textit{de novo} biosynthesis (38). In the presence of bile salts and pancreatic lipase, enterocytes in the proximal intestine (duodenum) absorb dietary lipids efficiently, leaving no luminal lipid residue for absorption by distal (ileal) enterocytes (39). Accordingly, Cre\textsuperscript{-} and Hnf4a\textsuperscript{del} control mice showed prominent Oil Red O staining in duodenal cells and virtually none in the ileum (Fig. 7C). In contrast, Cdx2\textsuperscript{del} single-mutant mice showed reduced lipid in duodenal enterocytes and significant levels in the ileum (Fig. 7C). In contrast, Cdx2\textsuperscript{del} single-mutant mice showed reduced lipid in duodenal enterocytes and significant levels in the ileum, indicating that CDX2-null cells absorb dietary lipids inefficiently, leaving a residual amount for ileal absorption. Hnf4a\textsuperscript{del}Cdx2\textsuperscript{del} intestines showed minimal Oil Red O staining in any region (Fig. 7C), revealing a global and profound defect in lipid absorption. Importantly, this was not a trivial consequence of enterocyte depletion because the duodenum, where enterocytes were plentiful, showed no lipid uptake. Moreover, we observed no lipid uptake in ileal villi that contained both goblet cells and a few enterocytes. Taken together, these findings reveal that CDX2 and HNF4A co-regulate genes necessary for dietary lipid metabolism, which is more severely impaired when both TFs are absent than when either TF alone is lost. This striking defect likely also contributes to rapid weight loss in Hnf4a\textsuperscript{del}Cdx2\textsuperscript{del} mice.

**DISCUSSION**

Selected lineage-restricted TFs exert considerable control over each cell type's unique transcriptional program. For example, GATA1, TAL1, EKLF, and NF-E2 together regulate most erythroid blood cell genes (40), and a few basic helix-loop-helix TFs control much of the muscle cell-specific transcriptome (41). Several lines of evidence implicate CDX2, HNF4A, and GATA4/6 in control of intestinal genes. First, functional \textit{cis}-elements for individual intestinal genes repeatedly reveal these and few other TF activities (42, 43). Second, the corresponding sequence motifs recur frequently at intestine-active enhancers identified by histone marks and nucleosome depletion (11, 44) or differential DNA methylation (45). Here, we have shown that additional loss of CDX2 dramatically unmasks GATA4 and HNF4A requirements in knock-out mice. Defects in both compound-mutant intestines are rapidly lethal, include distinct components, and help delineate functional TF hierarchies in the intestine.

Arrested crypt cell replication in Gata4\textsuperscript{del}Cdx2\textsuperscript{del} intestines indicates collaboration of these TFs in intestinal crypts and contrasts sharply with the lack of replication deficits in Hnf4a\textsuperscript{del}Cdx2\textsuperscript{del} intestines. Although our data do not address
whether CDX2 and GATA control the same genes additively or different genes, the GATA sequence motif and binding of a closely related factor, GATA6, are highly enriched near CDX2-binding sites in replicating human intestinal cells in culture (11), suggesting possible regulation of the same genes. CDX2 and GATA proteins likely have additional, joint roles in villus cell maturation, as evidenced by a large overlap of their respective binding sites in the intestinal villus (35). It was difficult to address this point unequivocally in our analysis because the deficit in crypt cell replication may explain the reduced villus cell height whether cell maturation is intact or defective. In the future, identification of GATA4-binding sites and GATA4-dependent genes in intestinal crypt cells may uncover co-regulated genes in this compartment, much like this study uncovered CDX2/HNF4A-co-regulated genes in villus cells.

Suppression of crypt cell replication and the increase in goblet cell numbers in Gata4delCdx2del intestines may reflect reduced Notch signaling or an effect on some other shared pathway. Whether the phenotype is caused by defects in stem cells or transit-amplifying progenitors also warrants further investigation. By contrast, because Hnf4a(del)Cdx2(del) crypt cells proliferate normally, we can categorically attribute these phenotypes to failures in cell maturation and in enterocytes, in particular.

CDX2 loss disrupts chromatin structure and binding of other TFs such as HNF4A (24), implying that CDX2 maintains chromatin access in intestinal cells. In a simple hierarchy, where other TFs depend on CDX2 wholly, compound-mutant and Cdx2del intestines should largely phenocopy one another, but our studies on HNF4A indicated otherwise. Hnf4a(del)Cdx2(del) mice fared profoundly worse than Cdx2(del) littermates, with accelerated demise, near absence of ileal enterocytes, significant gene dysregulation in jejunal villus cells, and lack of dietary fat absorption. Many more genes are dysregulated in Hnf4a(del)Cdx2(del) than in either single mutant. One likely reason is that HNF4A regulates genes in addition to those where CDX2 enables chromatin access, but CDX2 loss is necessary to expose that dependence. A second reason is that HNF4A provides necessary additive activity at regulatory sites that both TFs co-occupy. Indeed, the genes most affected in Hnf4a(del)Cdx2(del) intestines are enriched for such co-occupancy, indicating that loss of single TFs may preserve some enhancer function, but the

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**FIGURE 6.** RNA-seq analysis further identifies CDX2- and HNF4A-co-regulated intestinal genes. A and B, integration of CDX2 and HNF4A binding data from ChIP-seq with mRNA expression data from RNA-seq analysis of control and Hnf4a(del)Cdx2(del) intestines. A, genes significantly down- or up-regulated (Q < 0.05) are shown in relation to those not expressed (fragments/kb of transcript/million mapped reads (FPKM) < 1) in either sample. The fraction of genes in each group bound by CDX2 (blue), HNF4A (red), and both TFs (purple) indicates again that these TFs activate mainly target genes. B, FPKM graphs showing RNA-seq expression of significantly up- and down-regulated genes are overlaid with binding for CDX2 (left panel, yellow), HNF4A (middle panel, aqua), and both (right panel, green), demonstrating a higher distribution of bound down-regulated genes. C, gene ontology annotation clusters from DAVID analysis of the 362 genes significantly down-regulated in Hnf4a(del)Cdx2(del) intestines and bound by both TFs.

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absence of both TFs is necessary to abrogate transcription completely.

In summary, combinatorial control allows TFs to elicit diverse transcriptional outcomes in both embryos and adult tissues, and our data highlight this aspect of gene control in vivo. The intestinal “master regulator” CDX2 participates in various TF combinations. Specific pairings with partners such as GATA proteins drive cell replication, whereas separate pairings with HNF4A and other TFs regulate genes in terminally differentiated cells. The contributions of each TF no doubt vary at different cis-regulatory sites. At some sites, the absence of a single factor has profound effects, as occurs commonly with loss of CDX2, but not GATA4 or HNF4A. At other sites, gene expression suffers only when more than one TF is absent. Our studies reveal many examples of such cooperativity, and the scope of gene dysregulation in Hnf4a<sup>del</sup>Cdx2<sup>del</sup> (compared with Cdx2<sup>del</sup>) intestines matched the greater severity of tissue defects and malnutrition. Particularly at enterocyte genes necessary to construct the microvillus brush border or absorb nutrients, including dietary fat, both CDX2 and HNF4A are necessary. These TFs co-occupy the corresponding enhancers, and loss of both TFs affects mRNA levels more than the absence of either factor alone. These findings illuminate the transcriptional basis for vital intestinal functions.

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REFERENCES

1. James, R., Erler, T., and Kaezenedel, J. (1994) Structure of the murine homeobox gene cdx-2. Expression in embryonic and adult intestinal epithelium. J. Biol. Chem. 269, 15229–15237
2. Krapp, A., Knöfler, M., Frutiger, S., Hughes, G. J., Hagenbüchle, O., and Wellauer, P. K. (1996) The p48 DNA-binding subunit of transcription factor PTF1 is a new exocrine pancreas-specific basic helix-loop-helix protein. EMBO J. 15, 4317–4329
3. Ohlsson, H., Karlsson, K. and Edlund, T. (1993) IPF1, a homeodomain-containing transactivator of the insulin gene. EMBO J. 12, 4251–4259
4. German, M. S., Wang, J., Chadwick, R. B., and Rutter, W. J. (1992) Synergistic activation of the insulin gene by a LIM-homoe domain protein and a basic helix-loop-helix protein: building a functional insulin minihancer complex. Genes Dev. 6, 2165–2176
5. James, R., and Kaezenedel, J. (1991) Homeobox gene expression in the intestinal epithelium of adult mice. J. Biol. Chem. 266, 3246–3251
6. Suh, E., Chen, L., Taylor, J., and Traber, P. G. (1994) A homeodomain protein related to caudal regulates intestine-specific gene transcription. Mol. Cell. Biol. 14, 7340–7351
7. Jin, T., and Drucker, D. J. (1996) Activation of proglucagon gene transcription through a novel promoter element by the caudal-related homeodomain protein Cdx-2. Mol. Cell. Biol. 16, 19–28
8. Gao, N., White, P., and Kaestner, K. H. (2009) Establishment of intestinal identity and epithelial-mesenchymal signaling by Cdx2. Dev. Cell 16, 588–599
9. Liu, T., Zhang, X., So, C. K., Wang, S., Wang, P., Yan, L., Myers, R., Chen, Z., Patterson, A. P., Yang, C. S., and Chen, X. (2007) Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2 transfection on morphology and gene expression of human esophageal epithelial cells. Carcinogenesis 28, 488–496
10. Silberg, D. G., Sullivan, J., Kang, E., Swain, G. P., Moffett, J., Sund, N. J., Sackett, S. D., and Kaestner, K. H. (2002) Cdx2 ectopic expression induces gastric intestinal metaplasia in transgenic mice. Gastroenterology 122, 689–696
11. Verzi, M. P., Shin, H., He, H. H., Sulahian, R., Meyer, C. A., Montgomery, R. K., Fleet, J. C., Brown, M., Liu, X. S., and Shivdasani, R. A. (2010) Differentiation-specific histone modifications reveal dynamic chromatin interactions and partners for the intestinal transcription factor Cdx2. Dev. Cell 19, 713–726
12. Hryniuk, A., Grainger, S., Savory, J. G., and Lohnes, D. (2012) Cdx2 function is required for maintenance of intestinal identity in the adult. Dev. Biol. 363, 426–437
13. Stringer, E. J., Duluc, I., Saandi, T., Davidson, I., Bialecka, M., Sato, T., Barker, N., Clevers, H., Pritchard, C. A., Winton, D. J., Wright, N. A., Freud, J. N., Deschamps, J., and Beck, F. (2012) Cdx2 determines the fate of postnatal intestinal endoderm. Development 139, 465–474
14. Verzi, M. P., Shin, H., Ho, L. L., Liu, X. S., and Shivdasani, R. A. (2011) Essential and redundant functions of caudal family proteins in activating adult intestinal genes. Mol. Cell. Biol. 31, 2026–2039
15. Bosse, T., Piatekcy, C. M., Burghard, E., Fialkovich, J. J., Rajagopal, S., Pu, W. T., and Krasinski, S. D. (2006) Gata4 is essential for the maintenance of
Transcription Factor Combinations in Intestinal Epithelium

jejunal-ileal identities in the adult mouse small intestine. Mol. Cell. Biol. 26, 9060–9070

16. Beuling, E., Baffour-Awuah, N. Y., Stapleton, K. A., Aronson, B. E., Noah, T. K., Shroyer, N. F., Duncan, S. A., Fleet, I. C., and Krasinski, S. D. (2011) GATA factors regulate proliferation, differentiation, and gene expression in small intestine of mature mice. Gastroenterology 140, 1219.e1–2; 1229.e1–2

17. Beuling, E., Aronson, B. E., Tran, L. M., Stapleton, K. A., ter Horst, E. N., Vissers, I. A., Verzi, M. P., and Krasinski, S. D. (2012) GATA6 is required for proliferation, migration, secretory cell maturation, and gene expression in the mature mouse colon. Mol. Cell. Biol. 32, 3392–3402

18. Garrison, W. D., Battle, M. A., Yang, C., Kaestner, K. H., Sladek, F. M., and Duncan, S. A. (2006) Hepatocyte nuclear factor 4α is essential for embryonic development of the mouse colon. Gastroenterology 130, 1207–1220

19. Babeu, J.-P., Darsigny, M., Lussier, C. R., and Boudreau, F. (2009) Hepatocyte nuclear factor 4α contributes to an intestinal epithelial phenotype in vitro and plays a partial role in mouse intestinal epithelium differentiation. Am. J. Physiol. Gastroint. Liver Physiol. 297, G124–G134

20. Sodhi, C. P., Li, J., and Duncan, S. A. (2006) Generation of mice harbouring a conditional loss-of-function allele of Gata6. BMC Dev. Biol. 6, 19

21. Pu, W. T., Ishiwata, T., Juraszek, A. L., Ma, Q., and Izumo, S. (2004) Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis 39, 186–193

22. Hayhurst, G. P., Lee, Y. H., Lombert, G., Ward, J. M., and Gonzalez, F. J. (2001) Hepatocyte nuclear factor 4α (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. Mol. Cell. Biol. 21, 1393–1403

23. Verzi, M. P., Shin, H., San Roman, A. K., Liu, X. S., and Shivdasani, R. A. (2013) Intestinal master transcription factor CDX2 controls chromatin access for partner transcription factor binding. Mol. Cell. Biol. 33, 281–292

24. Liu, T., Ortiz, J. A., Tsang, L., Meyer, C. A., Lee, B., Zhang, Y., Shin, H., Wong, S. S., Ma, J., Lei, Y., Pape, U. J., Poidinger, M., Chen, Y., Yeung, K., Brown, M., Turpaz, Y., and Liu, X. S. (2011) Cistrome: an integrative platform for transcriptional regulation studies. Genome Biol. 12, R83

25. Sanges, R., Cordero, F., and Calogero, R. A. (2007) oneChannelGUI: a graphical interface to Bioconductor tools, designed for life scientists who are not familiar with R language. Bioinformatics 23, 3406–3408

26. Irizarry, R. A., Hobbs, B., Collin, F., Beazer-Barclay, Y. D., Antonellis, K. J., Scherf, U., and Speed, T. P. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4, 249–264

27. Smyth, G. K. (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3, Article3

28. Benjamini, Y., and Hochberg, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B Stat. Methodol. 57, 289–300

29. Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., Pimentel, H., Salzberg, S. L., Rinn, J. L., and Pachter, L. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. 7, 562–578

30. Robinson, J. T., Thorvaldsdóttir, H., Winckler, W., Gutmann, M., Lander, E. S., Getz, G., and Mesirov, J. P. (2011) Integrative genomics viewer. Nat. Biotechnol. 29, 24–26

31. McLean, C. Y., Bristor, D., Hiller, M., Clarke, S. L., Schara, B. T., Lowe, C. B., Wenger, A. M., and Bejerano, G. (2010) GREAT improves functional interpretation of cis-regulatory regions. Nat. Biotechnol. 28, 495–501

32. McLean, C. Y., Bristor, D., Hiller, M., Clarke, S. L., Schara, B. T., Lowe, C. B., Wenger, A. M., and Bejerano, G. (2010) GREAT improves functional interpretation of cis-regulatory regions. Nat. Biotechnol. 28, 495–501

33. Hulsen, T., de Vlieg, J., and Alkema, W. (2008) BioVenn—a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. BMC Genomics 9, 488

34. Huang, da, W., Sherman, B. T., and Lempicki, R. A. (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57

35. Aronson, B. E., Rabello Aronson, S., Berkhourt, R. P., Chavoussi, S. F., He, A., Pu, W. T., Verzi, M. P., and Krasinski, S. D. (2014) GATA4 represses an ideal program of gene expression in the proximal small intestine by inhibiting the acetylation of histone H3, lysine 27. Biochem. Biophys. Acta 1839, 1273–1282

36. Benoit, Y. D., Paré, F., Francoeur, C., Jean, D., Tremblay, E., Boudreau, F., Escafti, F., and Beaulieu, J. F. (2010) Cooperation between HNF-1α, Cdx2, and GATA-4 in initiating an enterocyte differentiation program in a normal human intestinal epithelial progenitor cell line. Am. J. Physiol. Gastrointest. Liver Physiol. 298, G504–G517

37. van Wering, H. M., Bosse, T., Musters, A., de Jong, E., de Jong, N., Hogen Esch, C. E., Boudreau, F., Swain, G. P., Dowling, L. N., Montgomery, R. K., Grand, R. J., and Krasinski, S. D. (2004) Complex regulation of the lactase-phlorizin hydrolase promoter by GATA-4. Am. J. Physiol. Gastrointest. Liver Physiol. 287, G899–G909

38. Hurley, T. L., and Hasson, M. M. (2012) Lipid droplet formation on opposing sides of the endoplasmic reticulum. J. Lipid Res. 53, 1800–1810

39. Yamada, T., and Alpers, D. H. (2009) Textbook of Gastroenterology, 5th Ed., Wiley-Blackwell, Hoboken, NY

40. Orkin, S. H., and Zon, L. I. (2008) Hematopoiesis: an evolving paradigm for stem cell biology. Cell 132, 631–644

41. Tappcott, S. J. (2005) The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. Development 132, 2685–2695

42. Gregory, P. A., Lewinsky, R. H., Gardner-Stephen, D. A., and Mackenzie, P. I. (2004) Coordinate regulation of the human UDP-glucuronosyltransferase 1A8, 1A9, and 1A10 genes by hepatocyte nuclear factor 1α and the caudal-related homeodomain protein 2. Mol. Pharmacol. 65, 953–963

43. Mitchelmore, C., Troelsen, J. T., Spodsberg, N., Sjöström, H., and Norén, E. S., Getz, G., and Mesirov, J. P. (2011) Integrative genomics viewer. Nat. Biotechnol. 29, 24–26

44. Zhu, J., Adli, M., Zou, J. Y., Verstappen, G., Coyne, M., Zhang, X., Durham, T., Mi, M., Deshpande, V., De Jager, P. L., Bennett, D. A., Houmard, J. A., Muioio, D. M., Onder, T. T., Camahorta, R., Cowan, C. A., Meissner, A., Epstein, C. B., Shoresh, N., and Bernstein, B. E. (2013) Genome-wide chromatin state transitions associated with developmental and environmental cues. Cell 152, 642–654

45. Sheaffer, K. L., Kim, R., Aoki, R., Elliott, E. N., Schug, J., Burger, L., Schubeler, D., and Kaestner, K. H. (2014) DNA methylation is required for the control of stem cell differentiation in the small intestine. Genes Dev. 28, 652–664