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RESEARCH ARTICLE

The novel enterochromaffin marker Lmx1a regulates serotonin biosynthesis in enteroendocrine cell lineages downstream of Nkx2.2

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ABSTRACT

Intestinal hormone-producing cells represent the largest endocrine system in the body, but remarkably little is known about enteroendocrine cell type specification in the embryo and adult. We analyzed stage- and cell type-specific deletions of *Nkx2.2* and its functional domains in order to characterize its role in the development and maintenance of enteroendocrine cell lineages in the mouse duodenum and colon. Although Nkx2.2 regulates enteroendocrine cell specification in the duodenum at all stages examined, it controls the differentiation of progressively fewer enteroendocrine cell populations when deleted from Ngn3^{+} progenitor cells or in the adult duodenum. During embryonic development Nkx2.2 regulates all enteroendocrine cell types, except gastrin and preproglucagon. In developing Ngn3^{+} enteroendocrine progenitor cells, Nkx2.2 is not required for the specification of neuropeptide Y and vasoactive intestinal polypeptide, indicating that a subset of these cell populations derive from an Nkx2.2-independent lineage. In adult duodenum, Nkx2.2 becomes dispensable for cholecystokinin and secretin production. In all stages and *Nkx2.2* mutant conditions, serotonin-producing enterochromaffin cells were the most severely reduced enteroendocrine lineage in the duodenum and colon. We determined that the transcription factor Lmx1a is expressed in enterochromaffin cells and functions downstream of Nkx2.2. Lmx1a-deficient mice have reduced expression of Tph1, the rate-limiting enzyme for serotonin biosynthesis. These data clarify the function of Nkx2.2 in the specification and homeostatic maintenance of enteroendocrine populations, and identify Lmx1a as a novel enterochromaffin cell marker that is also essential for the production of the serotonin biosynthetic enzyme Tph1.

KEY WORDS: Nkx2.2, Lmx1a, Enteroendocrine cells, Serotonin, Intestine, Enterochromaffin

INTRODUCTION

The intestinal epithelium comprises five terminally differentiated cell types: the absorptive enterocytes and the secretory Paneth cells, goblet cells, tuft cells and enteroendocrine cells. Enterocytes are the major cell population in the intestine and are important for nutrient absorption. Paneth cells produce antimicrobial peptides and lysozyme, and possibly provide the stem cell niche (Porter et al., 2002; Sato et al., 2011). Goblet cells secrete mucins and thereby establish and maintain the protective mucus layer (Kim and Ho, 2010). Tuft cells comprise a rare cell population marked by doublecortin-like kinase 1 (Dclk1) expression (Gerbe et al., 2011) and are implicated in chemoreception (Gerbe et al., 2012; Sato, 2007). Enteroendocrine cells are the hormone-producing cells in the intestine. Although they represent only 1% of the cells in the intestinal epithelium, they secrete at least fifteen different types of hormones (May and Kaestner, 2010; Rindi et al., 2004) and represent the largest endocrine system in the body. Enteroendocrine cells are found in the small and large intestine and are classified by their location and principal hormone and peptide product. However, most enteroendocrine cells express more than one hormone (Arnes et al., 2012a; Egerod et al., 2012; Habib et al., 2012; Sykaras et al., 2014) and can be identified by chromogranin A (Chga) expression. Since enteroendocrine cells secrete many different hormones, they control a variety of physiological functions in the intestine and body, including gut motility, glucose homeostasis, appetite and food intake.

The serotonin [5-hydroxytryptamine (5-HT)]-producing enterochromaffin cells are the largest enteroendocrine cell population in the intestine. Approximately 90% of the 5-HT in the body is synthesized in the gut, but it is also produced in the CNS. Biosynthesis of 5-HT is a two-step process. The first step involves the conversion of the essential amino acid tryptophan to 5-hydroxytryptophan by the rate-limiting enzyme tryptophan hydroxylase (Tph). Two Tph enzymes have been found to mediate this conversion; Tph1 is expressed in the enterochromaffin cells in the intestine, whereas Tph2 is only found in the brain (Walther et al., 2003). Subsequently, 5-hydroxytryptophan becomes decarboxylated by the enzyme 5-hydroxytryptophan decarboxylase to 5-HT (Manocha and Khan, 2012).

Several transcription factors are known to regulate the enteroendocrine cell lineages. The basic helix-loop-helix (bHLH) protein neurogenin 3 (Ngn3, or Neurog3) is expressed in enteroendocrine progenitor cells and is required for induction of the enteroendocrine cell lineage (Jenny et al., 2002; Lopez-Diaz et al., 2007; Schonhoff et al., 2004). *Ngn3*$^{-/-}$ mice do not develop enteroendocrine cells in the intestinal epithelium (Jenny et al., 2002). In addition, a number of transcription factors specify subpopulations of enteroendocrine cells downstream of Ngn3, including Arx (Beucher et al., 2012; Du et al., 2012), Foxa1/2 (Ye and Kaestner, 2009), Isl1 (Terry et al., 2014), Insm1 (Gierl et al.,

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2006), Neurod1 (Mutoh et al., 1997; Naya et al., 1997), Pax4 (Beucher et al., 2012; Larsson et al., 1998) and Pax6 (Larsson et al., 1998). The NK2 homeobox 2 (Nkx2.2) transcription factor also regulates cell fate decisions within the enteroendocrine cell lineage in the embryo (Desai et al., 2008; Wang et al., 2009); however, postnatal lethality of *Nkx2.2*$^{-/-}$ mice (Briscoe et al., 1999; Sussel et al., 1998) precludes functional analysis of Nkx2.2 in the adult intestine. Since the intestinal epithelium undergoes constant turnover in the adult, we sought to investigate whether Nkx2.2 is required for enteroendocrine cell subtype specification in the adult as well.

In this study, we demonstrate that deletion of *Nkx2.2* specifically in the intestinal epithelium in the embryo and the adult, and deletion of *Nkx2.2* in Ngn3^{+} enteroendocrine progenitor cells, results in loss of most enteroendocrine cell types and an increase in the ghrelin (Ghrl)$^{+}$ cell population within the duodenum. Deletion of *Nkx2.2* from the large intestine affects only a small number of enteroendocrine cell populations. Interestingly, Ghrl- and 5HT-producing cells are the most affected populations in the duodenum and colon. Overall, the intestine-specific *Nkx2.2* deletion displays a developmental phenotype that is similar to that of global *Nkx2.2* null mice (Desai et al., 2008; Wang et al., 2009), indicating that the misspecification of enteroendocrine cells is due to intestinal cell-intrinsic functions of Nkx2.2. Deletion of *Nkx2.2* from the adult intestinal epithelium did not affect the duodenal expression of cholecystokinin (*Cck*), gastrin (*Gast*), neuropeptide Y (*Npy*), secretin (*Sct*) and vasoactive intestinal polypeptide (*Vip*), indicating that Nkx2.2 is dispensable for the homeostatic maintenance of these enteroendocrine lineages in the adult. Additional analysis of the intestinal epithelium in *Nkx2.2* mutant mouse models carrying deletions of either the tinman (TN) domain or the NK2-specific domain (SD) revealed discrete functions of these Nkx2.2 regulatory domains in enteroendocrine cell specification. By determining gene changes that were common to the small and large intestine of all *Nkx2.2* mutant mice evaluated, we identified *Tph1* and the LIM homeobox transcription factor 1 alpha (*Lmx1a*) to be coordinately downregulated. Gene expression and deletion analyses also revealed Lmx1a to be a novel marker of 5-HT^{+} enterochromaffin cells, which is essential for 5-HT biosynthesis in the intestine.

RESULTS

Characterization of *Nkx2.2*$^{\Delta int}$ mice

Expression of the homeodomain transcription factor Nkx2.2 in the murine intestine begins at embryonic day (E) 15.5 and persists into adulthood (Desai et al., 2008; Wang et al., 2009). To analyze the function of Nkx2.2 in the adult intestine, we specifically deleted *Nkx2.2* in the intestinal epithelium using a conditional *Nkx2.2* allele (Mastracci et al., 2013) and the *Villin*$^{Cre/+}$ transgene (Madison et al., 2002). Intestine-specific deletion of *Nkx2.2* circumvents the early postnatal lethality of *Nkx2.2*$^{-/-}$ mice caused by the pancreatic defect (Sussel et al., 1998). *Nkx2.2*$^{flox/flox}$;*Villin*$^{Cre/+}$ or *Nkx2.2*$^{flox/lacZ}$;*Villin*$^{Cre/+}$ mice are referred to hereafter as *Nkx2.2*$^{\Delta int}$ mice. To verify that deletion of *Nkx2.2* is restricted to the intestine and does not occur in other organs, we performed PCR for the recombined *Nkx2.2* allele in several representative tissues. As expected, a recombined product was only detected in intestinal tissues (Fig. S1A). Furthermore, qPCR analysis of the duodenum and colon of 6-week-old adult *Nkx2.2*$^{\Delta int}$ mice showed significant ablation of *Nkx2.2* in the intestine (Fig. S1B).

Nkx2.2$^{\Delta int}$ mice at all ages were viable and indistinguishable from their littermate controls, with no significant change in body weight (Fig. S2A). Interestingly, we observed a small but significant increase in the length of the small but not large intestine of 6-week-old *Nkx2.2*$^{\Delta int}$ mice (Fig. S2B,C,J). Increases in both the villus and crypt lengths in the small intestine appeared to contribute to the overall change in length (Fig. S2D-I). Interestingly, the change in intestinal length occurred gradually and was transient: there were no length differences in neonatal animals and intestine length had normalized by 19-20 weeks (Fig. S2K,L). The transient manifestation of this phenotype at the post-weaning stage suggests that the change in length might be due to an adaptive effect.

Loss of most enteroendocrine cell populations in the duodenum of *Nkx2.2*$^{\Delta int}$ mice

Since the enteroendocrine lineages within the duodenum are well characterized and Nkx2.2 is expressed at the highest levels within this region of the intestine, we chose to focus on the duodenum to analyze the precise molecular changes in the intestinal epithelium of *Nkx2.2*$^{\Delta int}$ mice. RNA-Seq analysis of the duodenum of 6-week-old *Nkx2.2*$^{\Delta int}$ mice demonstrated that expression of 395 genes was significantly altered compared with controls, with a slightly larger proportion of genes upregulated than downregulated (Fig. 1A). Similar to E18.5 mice carrying a null mutation of *Nkx2.2* (*Nkx2.2*$^{-/-}$) (Desai et al., 2008; Wang et al., 2009), *Nkx2.2*$^{\Delta int}$ mice displayed altered enteroendocrine cell lineages. In the *Nkx2.2*$^{\Delta int}$ duodenum, the enteroendocrine cell marker *Chga*, as well as the hormones *Cck*, gastric inhibitory polypeptide (*Gip*), *Npy*, neurotensin (*Nts*), *Sct*, somatostatin (*Sst*), tachykinin 1 (*Tac1*), *Tph1* and *Vip* showed significantly decreased expression. The only hormone that demonstrated a higher expression level in the duodenum of *Nkx2.2*$^{\Delta int}$ mice was *Ghrl* (Table 1). Interestingly, the expression of *Gast* and *ppGcg* is altered in the small intestine of *Nkx2.2*$^{-/-}$ mice (Desai et al., 2008), but did not appear changed in the duodenum of *Nkx2.2*$^{\Delta int}$ mice (*Gast*, fold change 0.22, P=0.38; *ppGcg*, fold change 1.15, P=0.68), suggesting that these changes could be secondary to loss of *Nkx2.2* in the CNS or pancreas. In the intestinal epithelium, the *ppGcg* product is processed to glucagon-like peptide 1 (GLP-1), an incretin hormone that is important for glucose homeostasis. Consistent with unchanged *ppGcg* expression in the duodenum of *Nkx2.2*$^{\Delta int}$ mice, we could not detect a change in blood glucose levels in fed mice or after glucose challenge in an intraperitoneal glucose tolerance test (ipGTT) (Fig. S3).

We confirmed the transcriptome results by qPCR analysis of gene expression in the duodenum and immunofluorescence analysis of the small intestine. The *R26R*^{Tomato} reporter (Madisen et al., 2010) was used to identify recombined areas of the small intestine in *Nkx2.2*$^{\Delta int}$ mice (data not shown). Representative images of immunofluorescent stainings of the small intestine from *Nkx2.2*$^{\Delta int}$ and control mice were consistent with the changes in transcript levels. In particular, both analyses demonstrated the absence and/or decrease of Chga^{+} enteroendocrine cells (Fig. 1B,C), including the 5-HT^{+} and Sst^{+} subpopulations (Fig. 1D,E,H,I), whereas the Ghrl^{+} cell number was increased (Fig. 1F,G). With the exception of *Sst*, the hormonal gene expression changes observed in the duodenum of 6-week-old *Nkx2.2*$^{\Delta int}$ mice were also observed in the colon (Fig. 1C,E,G,I). Consistent with the change in Sst^{+} cells in the duodenum of 6-week-old *Nkx2.2*$^{\Delta int}$ mice (Fig. 1H,I), urocortin 3 (*Ucn3*) was also decreased (Table 2). A recent study demonstrated a reduction in the Sst^{+} cell number in the pancreas of *Ucn3*-deficient mice (van der Meulen et al., 2015), suggesting that there is a similar relationship between *Ucn3*$^{+}$ and Sst^{+} cells in both the pancreas and intestine.

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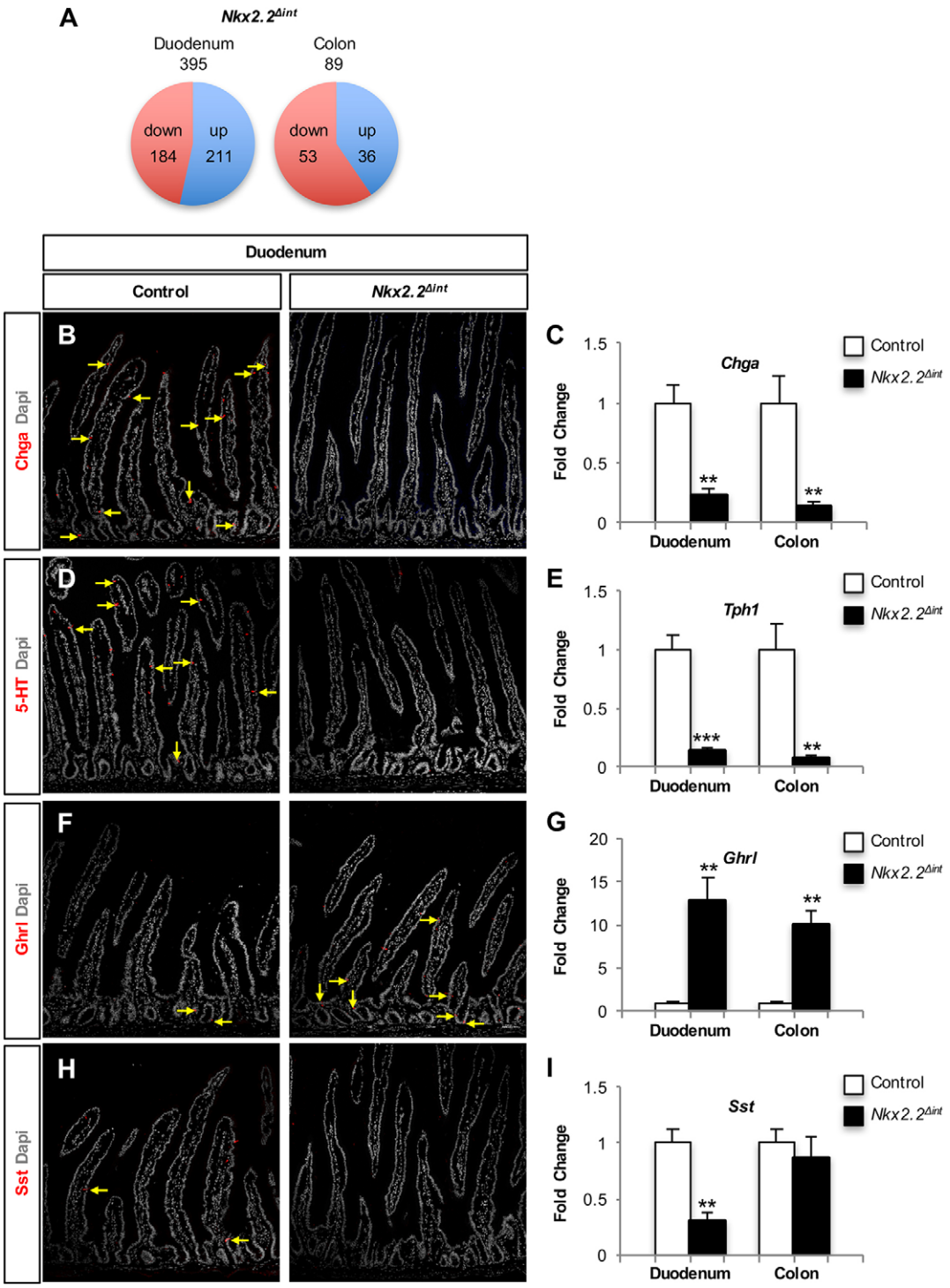

Fig. 1. Expression analysis of the duodenum and colon of *Nkx2.2*$^{\Delta int}$ mice. (A) RNA-Seq analysis of the duodenum and colon of 6-week-old *Nkx2.2*$^{\Delta int}$ mice revealed 211 significantly upregulated and 184 significantly downregulated genes in the duodenum and 36 significantly upregulated and 53 significantly downregulated genes in the colon (P<0.05). Among the significantly downregulated genes in the duodenum are most of the enteroendocrine cell hormones, except *Ghrl*, which is significantly upregulated (see Table 1). In the colon, few enteroendocrine hormones are changed. (B-I) Immunofluorescence of the duodenum (B,D,F,H) and qPCR analysis of the duodenum and colon (C,E,G,I; n=5) of 6-week-old *Nkx2.2*$^{\Delta int}$ mice, showing significant reduction in expression of the enteroendocrine marker *Chga* (B,C) and the rate-limiting enzyme for 5-HT biosynthesis *Tph1* (D,E). The expression of the hormone *Ghrl* is significantly higher in the duodenum, as well as in the colon, than in controls (F,G). Expression of the hormone *Sst* is significantly reduced in the duodenum of *Nkx2.2*$^{\Delta int}$ mice, but is unchanged in the colon (H,I). Arrows indicate hormone-positive cells. **P<0.01, ***P<0.001.

Although Nkx2.2 is expressed at lower levels in the colon, we sought to determine whether Nkx2.2 also regulates the enteroendocrine lineages in the large intestine. Comparative transcriptome analysis of the *Nkx2.2*$^{\Delta int}$ colon revealed a smaller number of gene changes but, similar to the duodenum, comprising both upregulated and downregulated genes (Fig. 1A). There was a

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Table 1. RNA-Seq analysis of enteroendocrine hormone gene expression in 6-week-old *Nkx2.2*$^{\Delta int}$ versus control mice

		Duodenum		Colon	
Gene symbol	Hormone	Fold change	*P*-value	Fold change	*P*-value
Cck	cholecystokinin	0.672	0.0081**	0.534	0.669
Chga	chromogranin A	0.249	0.0003***	0.130	0.0003***
Chgb	chromogranin B	0.316	0.0002***	0.097	9.39E–7***
Ghrl	ghrelin	16.37	3.01E–8***	6.930	0.0039**
Gip	gastric inhibitory polypeptide	0.135	7.11E–14***	1.383	1.000
Npy	neuropeptide Y	0.385	0.0016**	0.352	0.417
Nts	neurotensin	0.046	1.14E–7***	0.666	0.193
Pyy	peptide YY	0.147	8.79E–7***	2.622	0.0007***
Sct	secretin	0.530	0.0027**	0.900	0.812
Sst	somatostatin	0.336	6.74E–10***	0.911	0.677
Tac1	tachykinin 1	0.168	5.96E–20***	0.553	0.122
Tph1	tryptophan hydroxylase 1	0.117	1.57E–6***	0.064	3.39E–15***
Vip	vasoactive intestinal polypeptide	0.547	4.01E–5***	0.802	0.572

Most enteroendocrine hormone genes are significantly downregulated in the duodenum of *Nkx2.2*$^{\Delta int}$ mice, whereas in the colon few enteroendocrine hormones are affected. *Ghrl* is an exception, being significantly upregulated in both the duodenum and colon (see Fig. 1G). **P<0.01, ***P<0.001.

severe reduction in expression of the pan-endocrine genes *Chga* and *Chgb*, indicating that the absence of Nkx2.2 also affects enteroendocrine lineages of the colon (Table 1, Fig. 1C). The expression of *Tph1* and *Ghrl* was significantly altered (Table 1, Fig. 1E,G). However, the function of Nkx2.2 appears to be more limited in the colon as many of the hormones that were regulated by Nkx2.2 in the duodenum were not affected by the deletion of *Nkx2.2* in the colon (Table 1, Fig. 1I). This suggests that the regulation of these lineages in the colon is independent of Nkx2.2 or, as in the case of Gip and Cck, these hormones are not highly expressed in the colon.

Since the expression of the enteroendocrine progenitor marker *Ngn3* was not changed in the duodenum or colon of *Nkx2.2*$^{\Delta int}$ mice (Fig. 2A), it is unlikely that the change in enteroendocrine hormone expression is due to a loss of enteroendocrine progenitor cells or of the upstream progenitor populations that contribute to enteroendocrine cell lineages. For example, there is no change in atonal homolog 1 (*Atoh1*), which is essential for the production of all secretory cells, including enteroendocrine cells (Shroyer et al., 2007; Yang et al., 2001). Furthermore, hairy and enhancer of split 1 (*Hes1*), which functions upstream of *Atoh1* and *Ngn3* (Jensen et al., 2000; Kopinke et al., 2011), is also unchanged (Fig. 2A). Expression of even earlier genes, such as Kruppel-like factor 5 (*Klf5*), which marks early proliferative populations that contribute to proper cellular differentiation (Bell and Shroyer, 2015), and forkhead box A2 (*Foxa2*) and *Klf4*, which are expressed in most cells of the upper crypt and villus (Katz et al., 2002; Ye and Kaestner, 2009), is also unchanged (Fig. 2A). The lack of expression changes in these early markers of intestinal progenitor populations suggests that the defect in enteroendocrine cell specification in *Nkx2.2*$^{\Delta int}$ mice is likely to be specific to the enteroendocrine lineage and downstream of Ngn3^{+} progenitor formation.

In addition to the changes in enteroendocrine hormone expression in the duodenum, we detected differences in several transcription factors necessary for the development of specific enteroendocrine cell subtypes. *Neurod1* was significantly downregulated in the duodenum of 6-week-old *Nkx2.2*$^{\Delta int}$ mice, consistent with the decrease in *Cck* and *Sct* expression, the two cell populations regulated by Neurod1 (Naya et al., 1997), whereas aristaless related homeobox (*Arx*) and *Isl1* were highly upregulated (Table 2). Consistent with the increase in Ghrl^{+} cells, there was elevated expression of membrane bound O-acyltransferase domain containing 4 (*Mboat4*), the enzyme that is co-expressed with Ghrl and converts the Ghrl peptide to its biologically active, acylated form (Gutierrez et al., 2008, 2012; Kang et al., 2012) (Table 2).

Intriguingly, there was a striking upregulation of several duodenal genes that have antimicrobial and antiviral activity, and that have been implicated in the host immune response and/or inflammation in the intestine. Among these were resistin like alpha and beta (*Retnla* and *Retnlb*) (Artis et al., 2004; Munitz et al., 2009; Wang et al., 2005), defensin beta 1 (*Defb1*) (Morrison et al., 2002), mast cell protease 9 (*Mcpt9*) (Friend et al., 1998), interferon-induced protein 44 (*Ifi44*) (Hallen et al., 2007), vav 1 oncogene (*Vav1*) (Spurrell et al., 2009), TBC1 domain family, member 23 (*Tbc1d23*) (De Arras et al., 2012) and several genes encoding 2′-5′ oligoadenylate synthetases (*Oas1a*, *Oas1b*, *Oas1g*, *Oas2*, *Oas3*) (Mashimo et al., 2003). Given that expression of Nkx2.2 is restricted to the intestinal epithelium, these gene expression changes are likely to be secondary to the dysregulation of enteroendocrine populations, such as cells expressing 5-HT, Cck and Ghrl, and support the emerging concept that enteroendocrine hormones can play immunomodulatory roles in the gut (Worthington, 2015).

It is also interesting to note that the entire *gasdermin C* cluster (*Gsdmc*, *Gsdmc2*, *Gsdmc3*, *Gsdmc4*) was significantly upregulated in the duodenum and colon of 6-week-old *Nkx2.2*$^{\Delta int}$ mice (Table 2). Although it has been demonstrated that these genes are expressed in the intestinal epithelium, the function of this subfamily is relatively uncharacterized (Tamura et al., 2007).

Deletion of *Nkx2.2* in Ngn3-expressing enteroendocrine progenitor cells and in the adult intestine

To determine whether Nkx2.2 functions in enteroendocrine progenitor cells to regulate subsequent lineage decisions in the duodenum, we deleted *Nkx2.2* from Ngn3^{+} cells using the *Ngn3*$^{Cre/+}$ allele (Schonhoff et al., 2004). Since *Nkx2.2*$^{flox/flox}$; *Ngn3*$^{Cre/+}$ mice (referred to hereafter as *Nkx2.2*$^{\Delta progenitor}$) die shortly after birth with severe hyperglycemia due to the absence of insulin-producing cells in the pancreas (Sussel, et al., 1998; A. J. Churchill and L.S., unpublished), we examined the duodenum at postnatal day (P) 0 by qPCR. Although expression of *Nkx2.2* was only decreased by 50% in the duodenum of *Nkx2.2*$^{\Delta progenitor}$ mice, we observed reduced expression of *Chga*, *Cck*, *Gast*, *Gip*, *Nts*, peptide YY (*Pyy*), *Sct*, *Sst*, *Tac1* and *Tph1*. The expression of *ppGcg*, *Npy* and *Vip* was unchanged (Fig. 2B). Similar to *Nkx2.2*$^{\Delta int}$ mice, *Ghrl* expression was significantly upregulated, although to a lesser extent (Fig. 1G,

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Table 2. Selected differentially expressed transcripts in *Nkx2.2*$^{\Delta int}$ mice

Gene symbol	Gene name	Fold change	*P*-value
Downregulated genes			
Transcription factors			
Lmx1a	LIM homeobox transcription factor 1 alpha	0.091	0.0002
Onecut3	one cut domain, family member 3	0.143	0.0036
Ptf1a	pancreas specific transcription factor, 1a	0.267	0.0025
Fev	FEV (ETS oncogene family)	0.355	0.0019
Rbpjl	recombination signal binding protein for immunoglobulin kappa J region-like	0.449	0.0022
Neurod1	neurogenic differentiation 1	0.620	0.0297
Isx	intestine specific homeobox	0.711	0.0114
Others			
Afp	alpha fetoprotein	0.058	1.25E–7
Trpc7	transient receptor potential cation channel, subfamily C, member 7	0.070	5.23E–5
Ucn3	urocortin 3	0.103	1.34E–10
Trpa1	transient receptor potential cation channel, subfamily A, member 1	0.156	6.15E–5
Ahcy	S-adenosylhomocysteine hydrolase, SAHH	0.158	1.54E–26
Reg3d	regenerating islet-derived 3 delta	0.327	4.34E–11
Tpbg	trophoblast glycoprotein	0.371	2.72E–5
Ctrb1	chymotrypsinogen B1	0.385	1.76E–5
Amigo2	adhesion molecule with Ig like domain 2	0.417	3.38E–6
Cckar	cholecystokinin A receptor	0.433	0.006
Tle6	transducin-like enhancer of split 6, homolog of *Drosophila* E(spl)	0.607	0.0389
Grem1	gremlin 1	0.672	0.013
Grem2	gremlin 2	0.681	0.0143
Upregulated genes			
Transcription factors			
Hmx2	H6 homeobox 2	5.308	0.0429
Hmx3	H6 homeobox 3	4.793	0.0119
Arx	aristaless related homeobox	4.715	6.28E–7
Gfi1b	growth factor independent 1B	4.370	0.0269
Zfp791	zinc finger protein 791	2.241	0.0008
Isl1	ISL1 transcription factor, LIM/homeodomain	2.076	0.0084
Sp5	trans-acting transcription factor 5	1.424	0.0122
Others			
Retnlb	resistin like beta	81.111	5.84E–24
Gsdmc2	gasdermin C2	27.574	0.0012
Gsdmc4	gasdermin C4	24.514	0.003
Defb1	defensin beta 1	21.641	0.0123
Gsdmc3	gasdermin C3	21.164	0.0054
Mcpt9	mast cell protease 9	11.708	0.0001
Mboat4	membrane bound O-acyltransferase domain containing 4	10.811	0.001
Plb1	phospholipase B1	10.469	2.78E–9
Retnla	resistin like alpha	9.180	0.0046
Irs4	insulin receptor substrate 4	7.654	1.40E–7
Nmu	neuromedin U	6.145	0.0014
Oas2	2′-5′ oligoadenylate synthetase 2	5.360	0.0139
Oas3	2′-5′ oligoadenylate synthetase 3	4.102	0.0282
Dclk1	doublecortin-like kinase 1	3.286	0.0499
Oas1b	2′-5′ oligoadenylate synthetase 1B	3.091	0.0149
Ifi44	interferon-induced protein 44	2.874	9.63E–5
Oas1g	2′-5′ oligoadenylate synthetase 1G	2.767	0.0415
Mt1	metallothionein 1	2.620	0.0015
Vav1	vav 1 oncogene	2.478	0.0154
Oas1a	2′-5′ oligoadenylate synthetase 1A	2.436	0.033
Gchfr	GTP cyclohydrolase I feedback regulator	2.083	6.78E–5
Nmur1	neuromedin U receptor 1	2.069	0.0122
Gm6086	predicted gene 6086	2.011	0.0423
Asb11	ankyrin repeat and SOCS box-containing 11	1.905	2.11E–5
Slc5a9	solute carrier family 5 (sodium/glucose cotransporter), member 9	1.750	8.19E–5
Tbc1d23	TBC1 domain family, member 23	1.730	6.32E–5
Tgfbi	transforming growth factor, beta induced	1.582	0.0152
Igf1	insulin-like growth factor 1	1.556	0.0027

Fig. 2B). Compared with *Nkx2.2*$^{\Delta int}$ mice, the significantly downregulated hormones were less drastically affected in *Nkx2.2*$^{\Delta progenitor}$ mice. However, this could be a consequence of lower Ngn3-Cre recombination efficiency; alternatively, it could be due to the fact that Nkx2.2 is expressed in only ∼80% of Ngn3^{+} cells (Wang et al., 2009).

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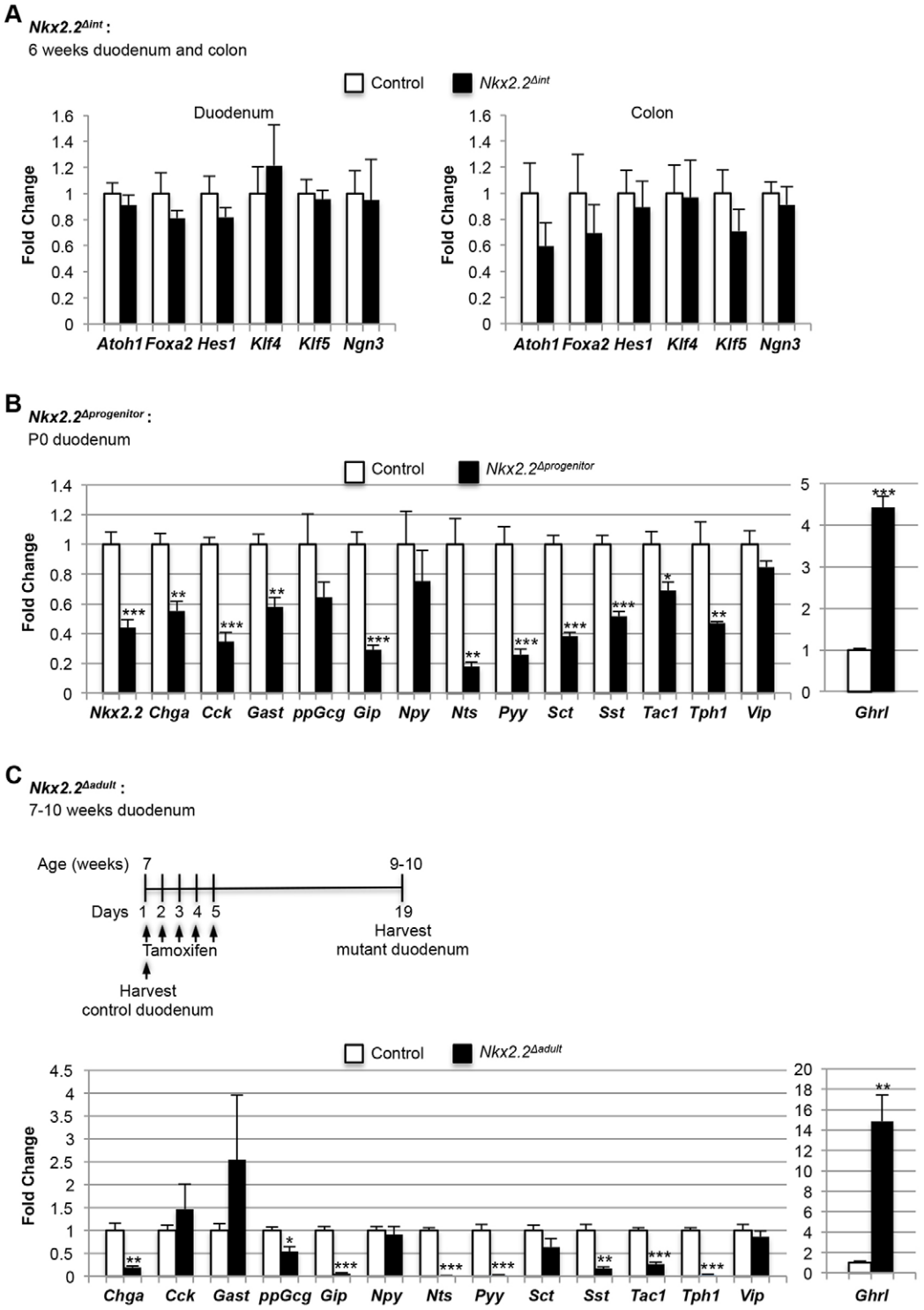

Fig. 2. qPCR expression analysis of the duodenum or colon of *Nkx2.2*$^{\Delta int}$, *Nkx2.2*$^{\Delta progenitor}$ and *Nkx2.2*$^{\Delta adult}$ mice. (A) Analysis of the duodenum and colon of 6-week-old *Nkx2.2*$^{\Delta int}$ mice (*n*=5) shows no changes in *Atoh1*, *Foxa2*, *Hes1*, *Klf4*, *Klf5* and *Ngn3*. (B) The P0 *Nkx2.2*$^{\Delta progenitor}$ duodenum (*n*=5) revealed significantly decreased expression of most hormones analyzed, except for higher expression of *Ghrl* and no change in expression for *ppGcg*, *Npy* and *Vip*. (C) *Nkx2.2*$^{\Delta adult}$ mice were tamoxifen injected at 7 weeks of age for 5 consecutive days (days 1-5) and the duodenum harvested 14 days after the last injection (day 19; *n*=4). The duodenum of 7-week-old control mice was harvested at day 1 (*n*=3). qPCR analysis showed a significant reduction in expression of *Chga*, *ppGcg*, *Gip*, *Nts*, *Pyy*, *Sst*, *Tac1* and *Tph1*, but an increase in *Ghrl*, in the duodenum of the 7- to 10-week-old *Nkx2.2*$^{\Delta adult}$ mice. Expression of the hormones *Cck*, *Gast*, *Npy*, *Sct* and *Vip* was unchanged. **P*<0.05, ***P*<0.01, ****P*<0.001.

To determine whether Nkx2.2 is also required for maintenance of the enteroendocrine cell lineages during normal cellular turnover in the adult, we deleted *Nkx2.2* from the duodenum of 9- to 10-week-old tamoxifen-injected *Nkx2.2*$^{flox/flox}$;*Villin*$^{CreERT2/+}$ and *Nkx2.2*$^{flox/lacZ}$;*Villin*$^{CreERT2/+}$ mice (referred to hereafter as *Nkx2.2*$^{\Delta adult}$), and compared them with 7-week-old *Nkx2.2*$^{\Delta adult}$

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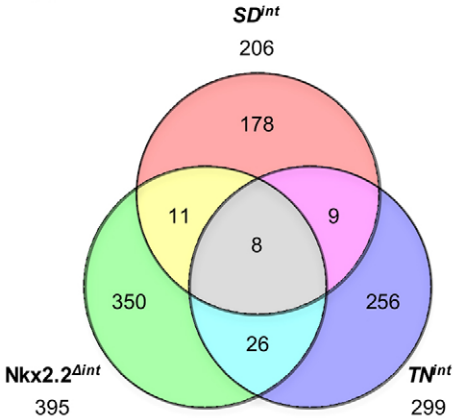

B

Common (8)	Nkx2.2$^{\Delta int}$ + SD^{int} (11)	Nkx2.2$^{\Delta int}$ + TN^{int} (26)		SD^{int} + TN^{int} (9)
Afp	Ahcy	Amigo2	4930581F22Rik	A430107O13Rik
Chga	Cartpt	Ano1	Car4	Galr2
Lmx1a	Fam83c	Chgb	Cck	Serpini1
Nts	Npy	Grem2	Enpp5	Tmtc3
Tph1	Fcer1a	Pyy	Gm5771	Lars2
Trpa1	Ghrl	Rab3b	Isx	Qdpr
Mt1	Ly6k	Rab3c	Ltb	Rbp4
Gm6086	Mbl2	Scn3a	Sct	Tnni1
	Ces1g	Tac1	Socs2	Klri1
	Mx2	Tpbg		
	Sst	Arx		
		Ifi44		
		Kcnk16		
		Ndufa3		
		Oas1a		
		Oas3		
		Prdx5		

Down-regulated Up-regulated Up- and Down-regulated

Fig. 3. Comparison of gene expression changes in the duodenum of *Nkx2.2*$^{\Delta int}$, *SD*^{int} and *TN*^{int} mice. (A) Venn diagram summarizing gene expression changes in the duodenum of *Nkx2.2*$^{\Delta int}$, *SD*^{int} and *TN*^{int} mice identified by RNA-Seq. (B) List of the eight genes with significantly changed expression in the duodenum of *Nkx2.2*$^{\Delta int}$, *SD*^{int} and *TN*^{int} mice; the 11 genes significantly changed in *Nkx2.2*$^{\Delta int}$ and *SD*^{int} mice; the 26 genes that are changed in both *Nkx2.2*$^{\Delta int}$ and *TN*^{int} mice; and the nine genes significantly differentially expressed in *SD*^{int} and *TN*^{int}.

mice that were not tamoxifen injected. Analysis of the intestine from *Nkx2.2*^{floxflox};*Villin*$^{CreERT2/+}$ mice showed that *Nkx2.2* was efficiently deleted after tamoxifen injection (Fig. S4A). In addition, PCR analysis exclusively for the recombined *Nkx2.2*$^{flox/+}$ allele, but not the *Nkx2.2*$^{lacZ/+}$ allele, confirmed successful tamoxifen-induced deletion of exon 2 of *Nkx2.2* specifically in the duodenum (Fig. S4B). *Chga*, *Ghrl*, *Gip*, *Nts*, *Pyy*, *Sst*, *Tac1* and *Tph1* displayed a similar expression change as in the developing duodenum of *Nkx2.2*$^{\Delta int}$ mice (Table 1, Fig. 2C). *Ghrl* was similarly upregulated, regardless of the timing of *Nkx2.2* deletion from the intestine (Fig. 1G, Fig. 2C; Fig. S5A,B). Interestingly, the expression of *Cck*, *Gast*, *Npy*, *Sct* and *Vip* was unchanged in tamoxifen-injected *Nkx2.2*$^{\Delta adult}$ mice, suggesting that Nkx2.2 might not be required for the continued production of these enteroendocrine subtypes in the adult. Furthermore, *ppGcg* was expressed at significantly lower levels in tamoxifen-injected *Nkx2.2*$^{\Delta adult}$ mice (Fig. 2C). We conclude that Nkx2.2 is necessary for the maintenance of only a subset of enteroendocrine cell populations in the adult.

Changes in hormone expression after mutating the SD or TN domain of Nkx2.2

Previous studies in the ventral neural tube and pancreas have shown that the TN domain of Nkx2.2 is important for interaction with the transducin-like enhancer of split (Tle) proteins (Muhr et al., 2001; Papizan et al., 2011) to regulate gene expression. The function of the SD domain of Nkx2.2 is unknown but appears to be important for endocrine cell differentiation in the pancreas (J. Levine and L.S., unpublished). To determine whether the TN or SD domains contribute to the distinct functional activities of Nkx2.2 in regulating the various enteroendocrine lineages, we generated *Villin*$^{Cre/+}$;*Nkx2.2*$^{flox/TN}$ or *Nkx2.2*$^{flox/SD}$ mice. These mice, hereafter referred to as *TN*^{int} or *SD*^{int} mice, express the respective mutant allele of *Nkx2.2* in the intestine and are wild type for *Nkx2.2* in other tissues.

Transcriptome analysis of the duodenum of 6-week-old *TN*^{int} or *SD*^{int} mice revealed significant gene expression changes: 299 genes were significantly altered in expression in the *TN*^{int} duodenum versus 206 genes in the duodenum of *SD*^{int} mice. By comparing the datasets from *Nkx2.2*$^{\Delta int}$, *TN*^{int} and *SD*^{int} mice, we found that only eight genes were significantly changed in the duodenum of all three mutant mouse strains (Fig. 3A). Six genes were significantly downregulated (Fig. 3B), including the enteroendocrine cell marker *Chga*, the enteroendocrine hormone *Nts*, *Tph1*, alpha fetoprotein (*Afp*) and *transient receptor potential cation channel, subfamily A, member 1* (*Trpa1*). All of these genes have been shown to be expressed in enteroendocrine cells (Cho et al., 2014; Rindi et al., 2004; Tyner et al., 1990), suggesting that the TN and SD domains in Nkx2.2 are important for enteroendocrine cell specification, especially for the Nts^{+} and 5-HT^{+} cell subtypes (Fig. 3B; Fig. S6D-G). In addition, Ghrl^{+} cell numbers were also increased in both mutants (Fig. S6A-C,G). However, *Ghrl* expression was significantly increased in the *SD*^{int} mice (Fig. 3B) but only trended up in the *TN*^{int} mutant (fold change 1.78, P=0.08). Interestingly, metallothionein 1 (*Mt1*) is the only gene that was highly upregulated in all three mutants. Since Mt1 functions as an antioxidant, its upregulation might be due to a secondary response to altered gut hormone ratios. Comparison of *Nkx2.2*$^{\Delta int}$ with the *TN*^{int} or *SD*^{int} mice revealed that the TN domain alone is important for *Pyy*, *Sct* and *Tac1* expression, whereas the SD domain alone is necessary for *Npy* and *Sst* expression (Fig. 3B).

Lmx1a is expressed downstream of Nkx2.2 and regulates 5-HT production

A notably downregulated gene in the duodenum of *Nkx2.2*$^{\Delta int}$, *SD*^{int} and *TN*^{int} mice encoded the transcription factor Lmx1a. Since expression of *Tph1*, the rate-limiting enzyme for 5-HT biosynthesis, was decreased to a similar degree in mice carrying each of the three mutant *Nkx2.2* alleles (Fig. 3B), we hypothesized that *Lmx1a* might be expressed in 5-HT^{+} cells in the intestine. Furthermore, *Lmx1a* expression was significantly downregulated in the duodenum of *Nkx2.2*$^{\Delta progenitor}$ and *Nkx2.2*$^{\Delta adult}$ mice, corresponding to the observed decrease in *Tph1* expression (Fig. 2B,C, Fig. 4A). Interestingly, Lmx1b, a paralog of Lmx1a, regulates serotonergic neuron development in the brain downstream of Nkx2.2 (Cheng et al., 2003; Ding et al., 2003), suggesting that Lmx1a could be

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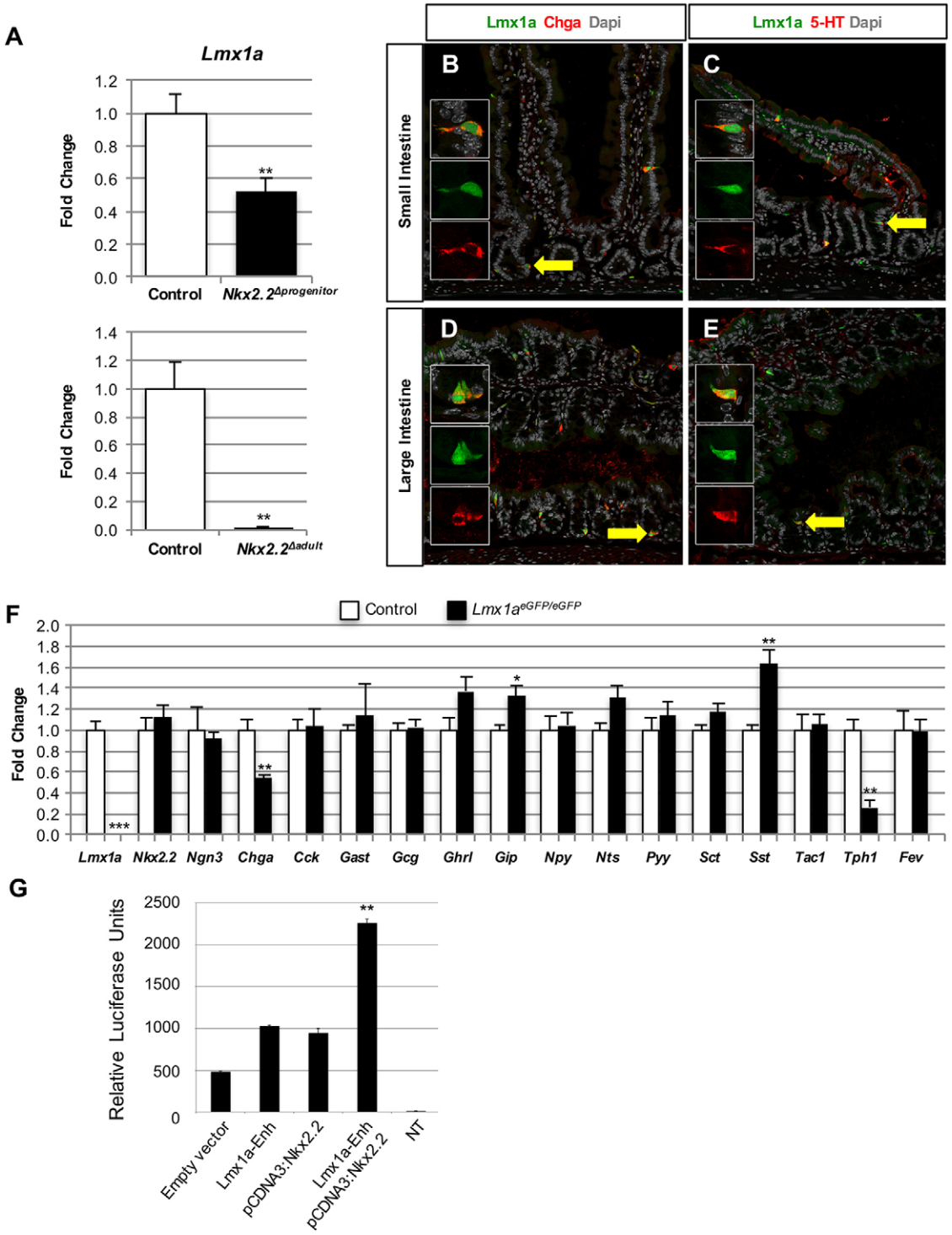

Fig. 4. Lmx1a is expressed in 5-HT-expressing cells. (A) qPCR analysis of the duodenum of *Nkx2.2*$^{\Delta progenitor}$ (n=5) and *Nkx2.2*$^{\Delta adult}$ (control, n=3; mutant, n=4) mice reveals a significant reduction in *Lmx1a* in *Nkx2.2*$^{\Delta progenitor}$ mice and absence of *Lmx1a* expression in *Nkx2.2*$^{\Delta adult}$ mice compared with controls. (B-E) Immunofluorescence analysis of the duodenum of 6-week-old *Lmx1a*$^{eGFP/+}$ mice shows that Lmx1a is expressed in Chga^{+} enteroendocrine cells (B,D) and in 5-HT^{+} cells (C,E) in the small (B,C) and large (D,E) intestine. Arrows indicate co-expressing cells that are shown at higher magnification in the insets. (F) qPCR analysis of the small intestine of P0 *Lmx1a*$^{eGFP/EGFP}$ mice shows that *Chga* and *Tph1* expression is significantly reduced, whereas *Gip* and *Sst* are upregulated (n=4). (G) Luciferase reporter assays in MIN6 cells. The pGL4.27:Lmx1a enhancer element (Lmx1a-Enh) and pcDNA3:myc-Nkx2.2 expression plasmid were co-transfected into MIN6 cells. Luciferase values were normalized to Renilla activity to account for transfection efficiencies (n=3). *P<0.05, **P<0.01, ***P<0.001.

important for the specification of 5-HT^{+} cells. We performed immunofluorescence staining on the duodenum of 6-week-old *Lmx1a*$^{eGFP/+}$ mice and confirmed that Lmx1a is expressed in Chga^{+} enteroendocrine cells in the epithelium of both the small and large intestine, and is specifically expressed in 5-HT^{+} cells (Fig. 4B-E). To investigate whether Lmx1a is important for the differentiation of 5-HT^{+} cells, we assessed the intestinal phenotype of homozygous *Lmx1a*$^{eGFP/eGFP}$ null mice (Deng et al., 2011). Since these mice die

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shortly after birth, we analyzed the small intestine of newborn *Lmx1a*$^{eGFP/eGFP}$ mice. *Chga* and *Tph1* expression was significantly reduced (Fig. 4F). It has been demonstrated that Fev (Pet1) $^{+}$ cells can lineage label 5-HT^{+} cells; however, deletion of *Fev* does not affect the formation of the enterochromaffin population (Wang et al., 2010). Interestingly, *Fev* expression, as well as that of *Nkx2.2*, was unchanged in the small intestine of newborn *Lmx1a*$^{eGFP/eGFP}$ mice (Fig. 4F), suggesting that Lmx1a functions independently of Fev and downstream of Nkx2.2 to regulate *Tph1* and 5-HT biosynthesis in the gut.

To determine whether Nkx2.2 directly activates *Lmx1a*, we identified an active *Lmx1a* enhancer element (mm9; Chr1:169730978-169733204) in the ENCODE dataset that contained two Nkx2.2 consensus binding sites. Since an enterochromaffin cell line is not available, we tested the ability of Nkx2.2 to activate the *Lmx1a* enhancer in the closely related pancreatic MIN6 cell line (Ishihara et al., 1993). In this cellular context, Nkx2.2 was able to activate the *Lmx1a* enhancer in a luciferase assay (Fig. 4G). Future studies in a more relevant cellular context will be necessary to confirm whether endogenous *Lmx1a* is a direct target of Nkx2.2 in the intestinal enterochromaffin cells.

DISCUSSION

Previous studies have analyzed the function of the homeodomain transcription factor Nkx2.2 in the brain and pancreas (Briscoe et al., 1999; Sussel et al., 1998). Its role in the intestine has only been analyzed in a global knockout during embryonic development (Desai et al., 2008; Wang et al., 2009). In this study, we analyzed the intrinsic function of Nkx2.2 in the duodenum by deleting *Nkx2.2* specifically in the intestinal epithelium using *Villin*$^{Cre/+}$ mice (Madison et al., 2002). *Nkx2.2*$^{\Delta int}$ mice are viable, but display a transiently elongated small intestine and a reduction in most enteroendocrine hormones. Despite the dramatic changes in gut hormones, glucose homeostasis remains normal. Interestingly, mice with an intestine-specific deletion of the enteroendocrine progenitor marker *Ngn3*, which display a loss of all enteroendocrine cells, have a different metabolic phenotype. *Ngn3*$^{\Delta int}$ mice are severely growth retarded, frequently die in the first week after birth, have a smaller intestine and have improved glucose clearance (Mellitzer et al., 2010). We postulate that the differences in metabolic phenotypes between these two mouse models might be attributed to the hormone Ghrl, which is highly upregulated in the *Nkx2.2*$^{\Delta int}$ mice (this study), but downregulated in the *Ngn3*$^{\Delta int}$ mutant (Mellitzer et al., 2010).

The transient increase in length of the small intestine of 6-week-old *Nkx2.2*$^{\Delta int}$ mice, as opposed to the decrease seen in the *Ngn3*$^{\Delta int}$ mice (Mellitzer et al., 2010), is also of interest. The increased length could be due to compensatory intestinal growth as an adaptive response to the alteration of gut hormone expression. For example, the altered ratios of hormones that either stimulate or inhibit proliferation, such as Gast and Sst (Thomas et al., 2003), could favor excess growth. Alternatively, we observed increased expression of insulin-like growth factor 1 (*Igf1*) in the *Nkx2.2*$^{\Delta int}$ mice at 6 weeks of age (Table 2). Igf1 is positively regulated by luminal nutrients and is able to promote growth of the epithelium of the small intestine. Furthermore, transgenic mice expressing human IGF1 exhibit a longer small intestine as well as increased villus length and crypt depth similar to *Nkx2.2*$^{\Delta int}$ mice (Ohneda et al., 1997). A study in rats also showed that Ghrl administration increases serum levels of IGF1, thereby stimulating duodenal growth (Warzecha et al., 2006), suggesting that the increase in Ghrl contributes to the increase in intestinal length in *Nkx2.2*$^{\Delta int}$ mice. It is possible that these compensatory responses are not triggered in the *Ngn3*$^{\Delta int}$ mice because they have a more uniform lack of all hormones.

Similar to the *Nkx2.2* null mice, we demonstrated that most enteroendocrine hormones are significantly reduced in 6-week-old *Nkx2.2*$^{\Delta int}$ mice, with the exception of Ghrl, which is highly increased, and Gast and ppGcg, which are unchanged (Fig. S7A). However, in contrast to deletion of *Nkx2.2* throughout the intestinal epithelium, *Npy* and *Vip* expression was unchanged in the *Nkx2.2*$^{\Delta progenitor}$ mice, suggesting that the differentiation of these two subtypes is independent of Nkx2.2 function in the Ngn3^{+} progenitor cells (Fig. S7B). Deletion of *Nkx2.2* in the adult intestine (*Nkx2.2*$^{\Delta adult}$) also showed that Nkx2.2 plays an essential role postnatally in maintaining enteroendocrine specification during the normal turnover of enteroendocrine cells. However, during normal turnover, Nkx2.2 does not appear to be required for maintaining the *Cck*$^{+}$, *Gast*$^{+}$, *Npy*$^{+}$, *Sct*$^{+}$ and *Vip*$^{+}$ enteroendocrine cell populations, suggesting that although these cell lineages are specified by Nkx2.2 in the embryo they are maintained in the adult by alternative mechanisms. Alternatively, *ppGcg* was specifically downregulated in *Nkx2.2*$^{\Delta adult}$ mice (Fig. S7C), suggesting there might be distinct regulatory programs for GLP-1^{+} cells in the adult versus the developing duodenum. Currently, it is not well understood why some enteroendocrine cell populations should be differentially regulated.

Our studies also begin to clarify the position of Nkx2.2 within the known enteroendocrine regulatory pathways. For example, the expression of Neurod1, a transcription factor that is essential for Cck^{+} and Sct^{+} cell development (Naya et al., 1997), was severely reduced in *Nkx2.2*$^{\Delta int}$ mice, suggesting that Nkx2.2 regulates the Cck^{+} and Sct^{+} cell lineages through the regulation of *Neurod1*. Furthermore, there is a correlation between Nkx2.2 and Arx regulation of *Sst*, in that *Sst* is upregulated in Arx-deficient intestine (Beucher et al., 2012; Du et al., 2012) and downregulated when *Arx* expression is increased in *Nkx2.2*$^{\Delta int}$ mice, suggesting that *Sst* might be regulated by Arx downstream of Nkx2.2.

In addition to elucidating the relationship between known intestinal regulatory proteins, our studies have identified Lmx1a as a novel regulator of the 5-HT signaling pathway in the gut. Enterochromaffin cells are the major source of 5-HT in the body, regulating a variety of processes, including gut motility (Gershon, 2013). Although *Lmx1a* RNA expression has been reported in the intestine, the function of Lmx1a was not investigated (Makarev and Gorivodsky, 2014). We have demonstrated that Lmx1a is co-expressed with Chga and 5-HT in enterochromaffin cells and is essential for the expression of Tph1, the gut-specific 5HT-synthesizing gene. Interestingly, the Lmx1a paralog Lmx1b regulates *Tph2* (Song et al., 2011) and serotonergic neuron development downstream of Nkx2.2 in the brain (Cheng et al., 2003; Song et al., 2011). Since *Lmx1b* is only expressed at extremely low levels in the intestine (Makarev and Gorivodsky, 2014), it is likely that Lmx1a performs analogous functions downstream of Nkx2.2 for intestinal 5-HT^{+} cell development. Interestingly, other regulators of 5-HT^{+} cell development do not appear to be conserved between the intestine and CNS. For example, Fev functions downstream of Nkx2.2 in the brain to specify 5-HT neurons (Cheng et al., 2003; Hendricks et al., 2003). In the intestine, however, expression of *Fev* was unchanged in *Nkx2.2*$^{\Delta int}$ mice. Furthermore, although Fev^{+} cells lineage-label 5-HT^{+} cells, *Fev*-deficient mice do not show a change in *Tph1* or *Chga* expression, or in 5-HT^{+} cell numbers (Wang et al., 2010). In addition, Ascl1 – another key regulator of hindbrain 5-HT^{+} cells (Tsarovina et al., 2004) – is not expressed in the intestine.

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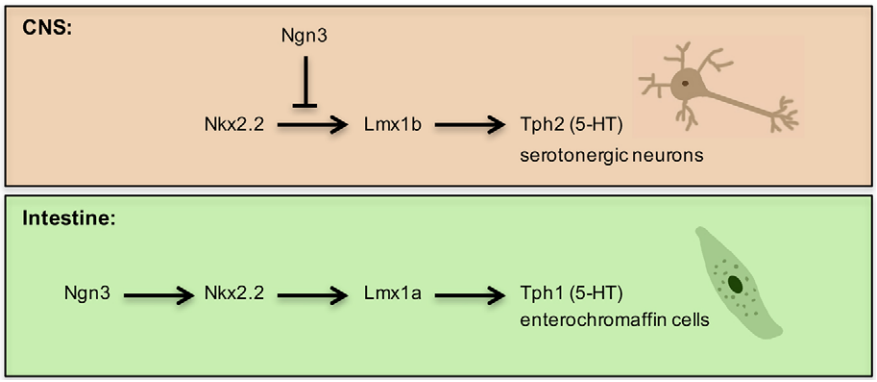

Fig. 5. Regulation of 5-HT biosynthesis in the CNS and intestine by Nkx2.2. In the CNS, Lmx1b is downstream of Nkx2.2 and is required for 5-HT biosynthesis by regulating *Tph2* expression. Ngn3 represses the serotonergic fate. In the intestine, Nkx2.2 is downstream of the enteroendocrine progenitor marker Ngn3. We identified Lmx1a, a paralog of Lmx1b, downstream of Nkx2.2 as a regulator of *Tph1* expression and thereby controls 5-HT biosynthesis in enterochromaffin cells.

Furthermore, in the brain Ngn3 represses the serotonergic neuron fate (Carcagno et al., 2014), whereas in the intestine Ngn3 is expressed in enteroendocrine progenitor cells and is required for all enteroendocrine lineages, including 5-HT$^+$ enterochromaffin cells. These findings suggest that, although Nkx2.2 and Lmx1a/b may represent conserved essential components of the transcriptional pathway regulating 5-HT$^+$ lineages, other constituents of these pathways in the intestine and CNS have diverged to provide important tissue-specific 5-HT$^+$ cell identities (Fig. 5).

In conclusion, our data show that Nkx2.2 is required for the specification of enteroendocrine cells during development and that it is necessary for the maintenance of most enteroendocrine lineages in the adult (Fig. S7). In addition, we have identified Lmx1a as a novel marker for 5-HT$^+$ cells that is expressed downstream of Nkx2.2 to regulate *Tph1* expression, which is analogous to the role of Lmx1b in CNS 5-HT$^+$ cells (Fig. 5). Further investigation of the shared and distinct regulatory pathways of 5-HT$^+$ cells in the intestine and CNS will help elucidate the important regulatory mechanisms that regulate superficially similar cell types in two different tissues.

MATERIALS AND METHODS

Animals

Mice were housed and treated in accordance with the animal care protocol (AAAG3206) approved by Columbia University's Institutional Animal Care and Use Committee (IACUC). Mice were maintained on a C57BL/6J background (The Jackson Laboratory). *Villin*$^{Cre/+}$ [B6.SJL-Tg(Vil-cre) 997Gum/J] (Madison et al., 2002) and *R26RTomato* [B6.Cg-*Gt(ROSA)26-Sor*$^{tm14(CAG-tdTomato)Hze}$/J] (Madisen et al., 2010) mice were obtained from The Jackson Laboratory. *Nkx2.2*$^{flox/+}$, *Nkx2.2*$^{lacZ/+}$, *Nkx2.2*$^{TN/+}$, *Ngn3*$^{Cre/+}$, *Villin*$^{CreERT2/+}$ and *Lmx1a*$^{eGFP/+}$ mice were described previously (Arnes et al., 2012b; Deng et al., 2011; el Marjou et al., 2004; Mastracci et al., 2013; Papizan et al., 2011; Schonhoff et al., 2004). *Nkx2.2*$^{SD/+}$ mice (J. Levine and L.S., unpublished) were genotyped with the following PCR primers: 5′-GCGGCAGCACCGGCAGCCGCA-3′ and 5′-GACAACGTTAACGTTG GGATG-3′. To analyze recombination of the *Nkx2.2*$^{flox/+}$ allele in different tissues, the following primers were used, resulting in a 464 bp PCR product: 5′-TCCTTTTAAAAATCTGCCCACGTCT-3′ and 5′-GAGGTCAACTA GGCCTCAACTTGGT-3′. Unless otherwise indicated, adult mice were analyzed at 6 weeks of age. In all experiments, *Nkx2.2*$^{flox/+}$, *Nkx2.2*$^{lacZ/+}$, *Nkx2.2*$^{flox/flox}$ or wild-type mice were used as controls.

To delete *Nkx2.2* in adult mice, 7-week-old *Nkx2.2*$^{flox/flox}$;*Villin*$^{CreERT2/+}$ and *Nkx2.2*$^{flox/lacZ}$;*Villin*$^{CreERT2/+}$ mice were injected intraperitoneally with 100 µl tamoxifen (100 mg/ml; Sigma, T5648) for 5 consecutive days (days 1-5) and analyzed 2 weeks after the last injection (day 19; Fig. 2C). These mice are referred to as *Nkx2.2*$^{\Delta adult}$ mice. Seven-week-old *Nkx2.2*$^{\Delta adult}$ mice that were not injected with tamoxifen served as controls for the qPCR analysis and were dissected at day 1 (Fig. 2C). Tamoxifen was prepared in corn oil (Sigma, C8267).

Metabolic analysis

To analyze blood glucose levels in the fed state, measurements were obtained at the same time of day while mice were kept on a regular chow diet. Intraperitoneal glucose tolerance tests (ipGTTs) were performed after a 16 h overnight fast, followed by an intraperitoneal glucose injection (2 g/kg body weight). Blood glucose was measured at 0, 15, 30, 45, 60, 90, 120 and 150 min after the glucose injection. Blood glucose measurements were taken with an Accu-Check Compact Plus glucose monitor (Model GT; Roche).

Histology and immunofluorescence

Intestines were cut longitudinally, washed with cold PBS and rolled into 'swiss rolls' (Moolenbeek and Ruitenberg, 1981). After overnight fixation in 4% paraformaldehyde at 4°C, samples were cryopreserved with 30% sucrose and cryo-embedded in Tissue-Tek O.C.T. (Fisher Scientific, 14-373-65). Sections were cut to 5 µm thickness.

For immunofluorescence staining, sections were incubated for 15 min in 0.3% H_2O_2, washed in PBS and blocked for 30 min at room temperature with 10% donkey serum (Fisher Scientific, NC9624464) in PBT (PBS with 0.3% Triton X-100). Primary antibodies were diluted in 5% donkey serum in PBT and incubated on the sections overnight at 4°C. The following primary antibodies were used: rabbit anti-chromogranin A (1:500-1000; ImmunoStar, 20085), goat anti-ghrelin (1:200; Santa Cruz Biotechnology, sc-10368), rabbit anti-5-HT (1:200; ImmunoStar, 20079), rabbit anti-Sst (1:200; Phoenix Pharmaceuticals, H-060-03) and rat anti-Sst (1:500; Abcam, ab30788). The GFP signal was detected by direct fluorescence of the protein. After washing with PBT, sections were incubated with appropriate secondary antibodies diluted in 5% donkey serum in PBT for 2 h at room temperature. Secondary antibodies were conjugated with Alexa 488 or Alexa 647 (1:200; Jackson ImmunoResearch). Nuclei were stained with DAPI (1:1000; Invitrogen) for 15 min at room temperature. Sections were mounted with fluorescence mounting medium (Dako, S3023). Images were acquired with either a Zeiss LSM710 confocal microscope (Zen 2012 software) or a Leica DM5500B upright microscope (LAS AF version 2.6.0.7266 software).

To analyze tissue morphology, sections were stained with Alcian Blue (pH 2.5; Sigma, A-3157) to visualize goblet cells and counterstained with Nuclear Fast Red (Vector Laboratories, NC9483816).

Intestine measurements

To analyze the length of villi and the depth of crypts, 35 well-sectioned villi or crypts in the outermost layer of the 'swiss roll' of the adult small intestine (Moolenbeek and Ruitenberg, 1981) were measured using ImageJ v1.48 (http://imagej.nih.gov/ij/).

Gene expression analysis

The duodenum or colon of adult mice (2 cm, measured from the stomach or caecum), 1 cm of the duodenum or the whole small intestine of newborn mice was dissected and stored at −20°C in RNAlater (Ambion, AM7021) until total RNA was extracted using the RNeasy Mini or Midi Kit (Qiagen, 74106 or

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75144). cDNA was prepared with random hexamer primers and the SuperScript III First-Strand Synthesis System (Invitrogen, 18080-051). Quantitative real-time PCR (qPCR) was performed with TaqMan assays (Applied Biosystems; Table S1) and qPCR MasterMix (AnaSpec, RT-QP2X-03-15+) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). A standard two-step real-time PCR program was used with an annealing temperature of 61°C and 40 cycles of amplification. All gene expression values were normalized to cyclophilin B (*Ppib*) and the fold change between wild-type and mutant samples calculated. All samples were analyzed in triplicate.

RNA sequencing was performed by the Columbia Genome Center (Columbia University) on the duodenum (n=3) and colon (n=2) of 6-week-old mice. Libraries were prepared from total RNA [RNA integrity number (RIN)>8] with the TruSeq RNA Preparation Kit (Illumina). Libraries were then sequenced using the HiSeq2000 instrument (Illumina). More than 20 million reads were mapped to the mouse genome (UCSC/mm9) using TopHat (Trapnell et al., 2009) (v2.0.4) with four mismatches and ten maximum multiple hits. Significantly differentially expressed genes were calculated using DEseq (Anders and Huber, 2010). RNA-Seq data have been deposited at GEO under series accession numbers GSE72761, GSE72762, GSE72764 and GSE78902.

Luciferase assays

A 2.226 kb fragment containing an active *Lmx1a* enhancer element (mm9; Chr1:169730978-169733204) was cloned into the pGL4.27 luciferase vector (Promega). 1 µg of the experimental vector pGL4.27:Lmx1a enhancer region (Lmx1a-Enh) was co-transfected with 0.1 µg Renilla luciferase vector pRL into MIN6 cells (Ishihara et al., 1993) in triplicate. The MIN6 cells were recently validated in our laboratory by RNA-Seq and tested for contamination. Luciferase activity was measured after 48 h using the Dual Luciferase Assay System (Promega). pcDNA3:myc-Nkx2.2 (pcDNA3:Nkx2.2) has been described previously (Anderson et al., 2009; Raum et al., 2006). Luciferase values were normalized to Renilla activity to account for transfection efficiencies and expressed as fold increase over the empty vector.

Data analysis

Results are expressed as mean±s.e.m. Statistical analysis on qPCR data and measurements was performed using a two-tailed unpaired Student's t-test. P<0.05 was considered significant. Fig. 3A was made with the help of Venn diagram generator (http://www.bioinformatics.lu/venn.php).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.G. designed and performed experiments, analyzed data and wrote the paper. D.C.G. assisted with the immunostaining and cell counting experiments. D.A.B. performed the colon RNA-Seq studies and the luciferase assays. J.M.D. provided *Lmx1a* mutant mice and edited the manuscript. T.L.M. assisted with generation of the *Nkx2.2* floxed allele and edited the manuscript. T.P. and J.E. made the *Lmx1a^{eGFP}* mice and edited the manuscript. L.S. oversaw the entire project, designed experiments, analyzed data and wrote the paper.

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Data availability

The RNA-Seq data discussed in this publication have been deposited in NCBI Gene Expression Omnibus and are accessible through GEO series accession numbers GSE72761 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72761), GSE72762 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72762), GSE72764 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72764) and GSE78902 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE78902).

Supplementary information

Supplementary information available online at
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