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# A dynamic expression survey identifies transcription factors relevant in mouse digestive tract development

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Tissue-restricted transcription factors (TFs), which confer specialized cellular properties, are usually identified through sequence homology or cis-element analysis of lineage-specific genes; conventional modes of mRNA profiling often fail to report non-abundant TF transcripts. We evaluated the dynamic expression during mouse gut organogenesis of 1381 transcripts, covering nearly every known and predicted TF, and documented the expression of approximately 1000 TF genes in gastrointestinal development. Despite distinctive structures and functions, the stomach and intestine exhibit limited differences in TF genes. Among differentially expressed transcripts, a few are virtually restricted to the digestive tract, including Nr2e3, previously regarded as a photoreceptor-specific product. TFs that are enriched in digestive organs commonly serve essential tissue-specific functions, hence justifying a search for other tissue-restricted TFs. Computational data mining and experimental investigation focused interest on a novel homeobox TF, Isx, which appears selectively in gut epithelium and mirrors expression of the intestinal TF Cdx2. Isx-deficient mice carry a specific defect in intestinal gene expression: dysregulation of the high density lipoprotein (HDL) receptor and cholesterol transporter scavenger receptor class B, type I (Scarb1). Thus, integration of developmental gene expression with biological assessment, as described here for TFs, represents a powerful tool to investigate control of tissue differentiation.

**KEY WORDS: Transcription factor, Organogenesis, Differentiation, Isx, Stomach, Intestine, Mouse**

## INTRODUCTION

Cell differentiation is achieved in large measure through the actions of tissue-restricted transcription factors (TFs) (Davidson, 2001; Gilbert, 2000). Thus, an essential step in studying the molecular basis of differentiation is to identify TFs that are restricted in distribution and regulated during development. Historically, this goal has relied on evolutionary conservation of transcriptional regulatory pathways and enrichment of specific cis-elements in batteries of tissue-selective gene loci. Coupled with gain- and loss-of-function studies in model organisms, identification of crucial TFs has improved understanding of differentiation in tissues ranging in complexity from echinoderm and vertebrate endoderm to whole organs (Brent and Tabin, 2002; Cripps and Olson, 2002; Davidson et al., 2002; Inoue et al., 2005; Orkin, 2000; Shivdasani, 2002; Wilson et al., 2003; Zhu et al., 2005). We report a novel approach to identify developmentally regulated tissue-restricted TF genes, as applied in the mouse gastrointestinal (GI) tract.

The gut mucosa serves digestive, metabolic and barrier functions. It arises and operates in intimate contact with mesenchyme and provides a useful model to investigate tissue processes such as mesenchyme-epithelial interactions, establishment and renewal of stem-cell compartments, and specification of daughter lineages. Whereas gut development and homeostasis rely on many of the same pathways that regulate other organs (Radtke and Clevers, 2005), the basis for cell-specific responses to the limited signal repertoire and the range of tissue-restricted TFs remain unclear. Few such TFs are implicated directly in regulating gut epithelial stem cells (Tcf4) (Korinek et al., 1998) or differentiated lineages: for example, Math1 and Hes1 in secretory cells (Jensen et al., 2000; Yang et al., 2001), KLF4 in colonic goblet cells (Katz et al., 2002), and Cdx2 in enterocytes (Beck, 2004). Other GI-restricted TFs, particularly homeodomain (HD) proteins, define boundaries between discrete gut segments (Aubin et al., 2002; Kim et al., 2005; Offield et al., 1996; Roberts et al., 1998; Zakany and Duboule, 1999). However, current appreciation of TF activities and hierarchies in the mammalian GI tract lags behind that in other tissues (Cripps and Olson, 2002; Lee and Pfaff, 2001; Orkin, 2000; Zhu et al., 2005), in part because many key factors remain unknown.

We conducted an unbiased survey of the developing mouse gut for mRNA expression of all known and predicted DNA-binding proteins; transcript levels were evaluated over the period of greatest morphological change in fetal intestine and stomach. The ensuing analysis serves as a comprehensive assessment of TF gene expression in development of a mammalian organ. Some protein families are represented extensively and others sparingly, and we identified previously unknown gut expression of many known and novel TFs. Only a few dozen TF mRNAs reveal substantially different levels in the developing stomach and intestine or levels that increase with maturation of these organs. Such factors may be especially important for tissue differentiation, and we used various methods to isolate those expressed selectively in the gut, including a novel TF, intestine-specific homeobox (*Isx*). Targeted disruption of *Isx* in mice reveals its requirement in intestine-specific regulation of the high density lipoprotein (HDL) receptor and cholesterol transporter scavenger receptor class B, type I (Scarb1; previously SR-BI). Our approach thus uncovers promising candidates for transcriptional control of GI differentiation and outlines a general strategy to study a whole gene class in development or disorders of particular organs.

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## MATERIALS AND METHODS

### RNA harvest and RT-PCR

Stomach and small bowel were isolated from 10 (E17) to 80 (E11) CD1 mouse embryos. E11 stomach (1 μg) and E17 intestine (47 μg) total RNA was extracted using Trizol (Gibco), purified over RNeasy Columns (Qiagen), treated with DNaseI and reverse transcribed using oligo-dT and Superscript II reverse transcriptase (RT). Following RNase H treatment, cDNAs were normalized for equal loading by linear-range PCR for three genes (GAPDH, HPRT and β-actin) in the presence of trace α-[$^{32}$P] dCTP.

For the expression survey, solutions containing all components required for PCR, except primers, were distributed into 96-well plates by a pipetting robot that subsequently dispensed PCR primers so that pairs specific for each TF were applied to a complete set of eight samples. PCR reactions were run to 31 cycles using Hi-Fidelity Taq polymerase (Invitrogen) on a Tetrad thermal cycler (MJ Research) at 62°C annealing temperature. PCR products were resolved on pre-cast 1% agarose E-gels (Invitrogen) containing ethidium bromide and imaged under identical conditions for all gels. Pixel intensities for each band were summated using Quantity One software (BioRad) (data not shown, but see http://genome.dfci.harvard.edu/~mhu/GIFT). Because E-gels permit multiple PCR products to be compressed into a single band, we resolved the PCR products from 33 TF mRNAs by conventional electrophoresis in 1% agarose. Only three out of the 33 reactions returned more than 1 band, and in every case the dominant product was of the predicted size and sequence.

For conventional PCR, cycle numbers were adjusted to ensure linear amplification; products were resolved by agarose gel electrophoresis and detected by ethidium bromide staining. Real-time qPCR was carried out in an Applied Biosystems 7300 thermal cycler (94°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds) using SYBR-green and the results analyzed using software provided by the manufacturer. GAPDH mRNA controlled for equal sample loading in all these cases.

### Bioinformatics

TFs were selected for survey as described previously (Gray et al., 2004). Briefly, public and private gene databases were screened for nonredundant predictions of DNA-binding domains as defined in the Protein Families Database (Pfam; http://www.sanger.ac.uk). All annotations were confirmed manually, as reported (Gray et al., 2004). Primers were 20-22 bp in length, with ~40% GC content, and designed to amplify ~700 bp products.

We determined pixel densities for each RT-PCR product and, based on background levels in blank and control lanes, selected 100 units as the threshold for presence of the transcript. Signal strength ranged from 100 to 3400 units, with a large and apparently linear dynamic range, but varied between PCR primers. Absolute transcript levels may therefore only be compared across time points and not between TFs, whereas comparison of changes in TF levels are always valid. To qualify for a change between two developmental stages, we required a greater than twofold difference in signal strength of the RT-PCR product; the algorithm gave greater weight for changes of higher magnitude and those that occur over more than two consecutive stages.

To identify GI-restricted factors, we examined an expression dataset that profiled 42,000 mouse mRNAs across 55 tissues using customized 60-base oligonucleotide probes (Zhang et al., 2004) and referred to rigorous tissue isolation protocols. We extracted genes for which the intestine (small or large intestine, colon) showed normalized expression that was *k*-fold more than *m* of 51 adult tissues. Several combinations for *k* and *m* yielded restrictive results on known digestive markers when $k$=2.5 and $m$=40, thus identifying 1240 mRNAs with more than 2.5 fold levels in gut compared with 78% of tissues. We compared this pool with genes expressed in our TF survey using unique EntrezGene identifiers; 23 genes, representing less than 2.5% of TFs expressed in the developing gut, show GI-enriched expression postnatally. Manual review confirmed GI-enriched expression for 20 of these 23 genes in an independent profiling dataset (Su et al., 2004).

### Mice, tissue explants, and detection of RNA and proteins

Isx-deficient mice were generated by standard methods for targeted gene disruption by homologous recombination in 129/Sv strain-derived TC1 embryonic stem cells and maintained on a mixed 129/Sv-C57BL/6 background. Gene targeting was monitored by Southern analysis using the probes and restriction enzymes indicated in Fig. 6D. Organ culture and electroporation of fetal CD-1 mouse stomachs were performed as described previously for intestinal explants (Tou et al., 2004). Membranes blotted with adult mouse tissues or whole mouse embryos at sequential stages (Seegene, Seoul, South Korea) were hybridized with a ~700 bp probe corresponding to the mouse *Isx*-coding sequence. Rabbit Scarb1 antiserum was raised against a keyhole limpet hemocyanin-conjugated peptide corresponding to residues 495-509 (MSPAAKGTVLQEAKL) of murine Scarb1 and reacts with a single 82 kDa band on liver immunoblots from wild-type but not from *Scarb1*$^{-/-}$ mice. Northern blotting, immunochemistry and microarray comparison of wild-type and *Isx*$^{-/-}$ terminal ileum RNA were performed as described (Kim et al., 2005).

RT-PCR products were cloned into Topo II (Invitrogen), confirmed by DNA sequencing and amplified by PCR. The amplified fragments were transcribed to produce digoxigenin-labeled antisense and sense riboprobes. CD1 mouse embryos at E13, E15 and E17 were fixed in 4% paraformaldehyde at 4°C for 2, 3 and 12 hours, respectively; soaked overnight in 0.5 M sucrose at 4°C; and embedded in OCT compound (Sakura Finetech, Torrance, CA). Sections (12 μm) were fixed further in 4% paraformaldehyde for 20 minutes and then treated as described previously to detect mRNA localization, with hybridization temperatures of 58-62°C and washing stringency of 0.2× SSC. Images were captured using an Olympus BX41 compound microscope, CCD camera and Photoshop 7.0 software (Adobe).

## RESULTS

### Establishment and validation of parameters for a TF gene expression survey

Gut endoderm converts from a pseudostratified squamous to a columnar epithelium between embryonic days (E) 13 and 15 in the mouse. We performed reverse transcription (RT)-PCR on RNA isolated from the fetal stomach and small bowel at 2-day intervals between E11 and E17. From the set of all mouse genes, we extracted those that encode a DNA-binding motif (Gray et al., 2004). About 6% of all genes encode potential DNA-binding factors, and recent studies estimate nearly 1500 human TFs, including over 700 ZnB proteins (Messina et al., 2004). Our survey of 1381 genes covers the vast majority of known or predicted mammalian TFs, and Table 1 compares sub-groups of human TFs (Messina et al., 2004) with the mouse genes we examined.

Although variations in gene expression may be determined best by real-time quantitative (q) PCR, the ~100 bp amplicon length that is optimal for monitoring by qPCR is difficult to verify and limits other applications. We used conventional PCR to amplify ~700 bp fragments, so that products could be resolved by gel electrophoresis, isolated for sequencing and used to prepare probes for in situ hybridization. When possible, TF-specific PCR primers corresponded to coding sequences separated by introns. Non-reverse transcribed samples did not result in amplification. PCR products were quantified by the relative intensity of electrophoretic bands.

**Table 1. Representation of major TF protein families in our expression analysis**

| Domain | Number predicted (human)* | Number represented in this study |
|---|---|---|
| Homeodomain | 208 | 221 |
| bHLH | 117 | 116 |
| All ZnB | 762 | 642 |
| bZip | 72 | 57 |
| Nuc Rec | 49 | 47 |
| Forkhead | 40 | 40 |

*Messina et al. (2004).

To optimize quantitation, we assessed 16 genes known to be present at levels typical for TF transcripts in the developing gut, fewer than five copies per $10^5$ mRNA molecules (Lepourcelet et al., 2005). Fifteen of these 16 products could be detected in the linear range of amplification after 31 cycles of PCR (data not shown). In an interim analysis of the first 72 TFs, 21 primer pairs had failed to generate a PCR product; increasing the reaction to 36 cycles yielded weak signals for only two additional transcripts, whereas saturated PCR signals masked temporal changes in the levels of five factors. Conversely, among 31 genes that initially gave a saturated signal, reducing the reaction to 27 or 24 cycles uncovered subtle temporal change in just three transcripts. Another test midway through the analysis yielded similar results, and we completed the survey at 31 PCR cycles.

To judge the quality of the results, we interrogated a set of 213 TFs using two to five independent primer pairs. In small intestine, 42 (19.7%) of these transcripts never yielded a PCR product, whereas 29 (13.6%) gave weak signals with one primer pair and none with the others. These results agree roughly with our assessment that about 25% of all TF mRNAs are absent from the developing stomach or small bowel (Fig. 1A). One-hundred and thirty-nine of the remaining 142 mRNAs showed concordant patterns with multiple primer pairs. Second, using independent RNA isolates to examine 63 TFs that show developmental regulation of mRNA levels, we reproduced the results with high accuracy for 58; all but one of the failures were associated with weak and unreliable signals. The results are thus highly reproducible and strongly validated through redundancy among PCR primers. Third, 62% of reactions that failed to amplify a product from gut cDNA generated the correct product, verified by DNA sequence, when mouse brain served as the template (Gray et al., 2004), thus excluding primer design as the basis for PCR failure. Although failure to detect a PCR product may reflect low expression levels, some factors may be absent from both fetal brain and gut, as our method to detect TF transcripts appears to be highly sensitive. Fourth, DNA sequences of all 63 tested PCR products confirmed amplification of the expected product. Finally, where possible, we compared spatial and temporal variation revealed in our analysis with the results of published studies; expression of well-known TFs of the Hox, Cdx, Gata and Hnf groups were reproduced accurately. (The full dataset is not discussed here, but is available at http://genome.dfci.harvard.edu/~mhu/GIFT.)

## Spatial and temporal variation in TF gene expression in the developing gut

About 78% of all TF mRNAs are expressed at one or more stages and sites in the developing gut, similar to the fraction detected by in situ hybridization in the nervous system (Gray et al., 2004). Although we cannot judge how this fraction compares with TF gene expression in other organs, expression of such a large number of TFs in the GI tract implies a significant overlap in TF use among tissues. Furthermore, temporal modulation of TF genes during a period of substantial morphogenesis is modest (Fig. 1A). Among expressed transcripts, most (48% in small intestine, 50% in stomach) show minimal modulation over time (e.g. Fig. 1B) or similar variation (5% of transcripts) in the two organs (e.g. Fig. 1C); the extent of temporal variation for 7% (intestine) to 10% (stomach) of transcripts fell within margins of error. Gut development and maturation thus occur with limited specific changes in TF gene expression, which emphasizes the likely importance of those TFs that are temporally regulated: according to 'relaxed' criteria, up to 253 in the intestine

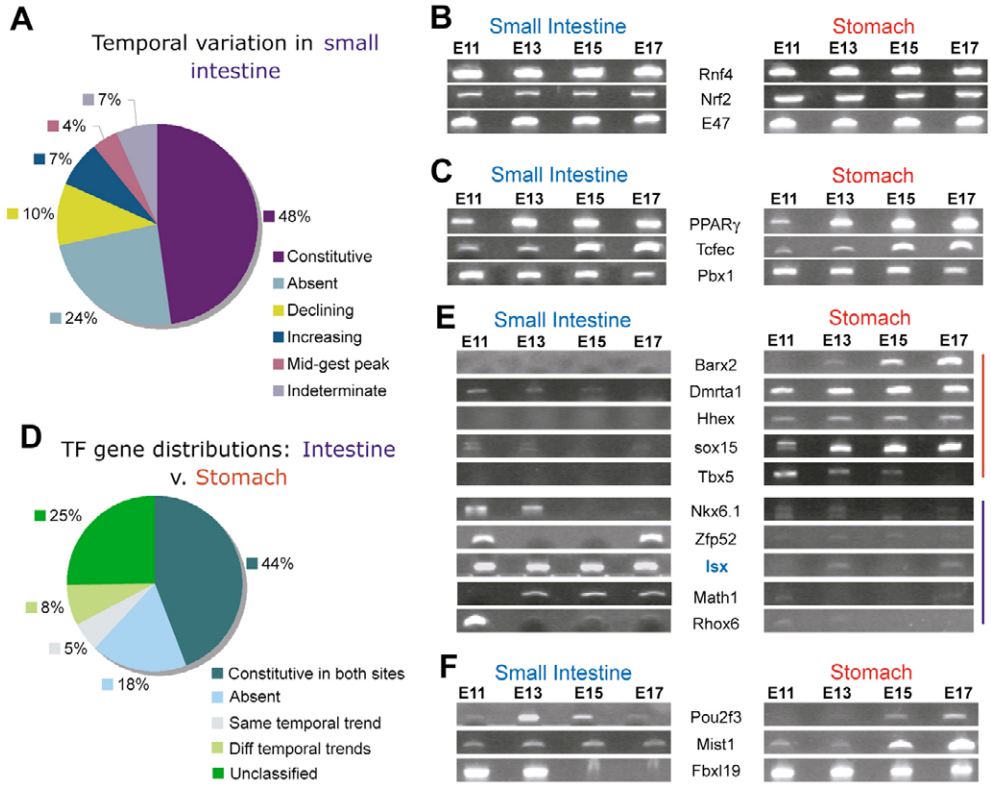


**Fig. 1. Analysis and representative results from the GIfT expression survey.** (**A**) Group breakdown of TFs according to temporal variation in the developing intestine; the fetal stomach showed virtually identical grouping. All RT-PCR data were quantified; classifications were derived computationally and verified manually. (**B**) Arbitrary examples from hundreds of TF mRNAs with constant levels during GI organogenesis. The data represent electrophoretic bands of RT-PCR products from our TF survey. (**C**) RT-PCR results of temporal modulation of TF transcripts during GI organogenesis, highlighting the similar trends observed for most TFs in the intestine and stomach. (**D**) Statistical analysis of differential gene expression. Most TF mRNAs are expressed either constitutively or with the same temporal variation in both stomach and intestine, and more than 18% of transcripts are not expressed in either site. Only ~8% (103 TFs) meet criteria for differential expression between intestine and stomach, including the examples shown in E and F. (**E**) Selected examples from ~25 TF mRNAs that are restricted to the developing stomach (grouped beside the red bar) or intestine (grouped by the blue bar). (**F**) Examples of TF transcripts that differ between stomach and intestine in pattern but not in absolute expression. The full set of original data can be analyzed at

and 191 in the stomach (e.g. Fig. 1C). Slightly fewer TF transcripts increase in expression over the E11-E17 period than decrease in that interval. In small bowel, for example, 114 transcripts decrease in abundance compared with 90 that increase steadily, whereas 49 TF genes show peak expression at E13 or E15. Such TFs probably serve stage-specific functions.

The stomach and intestine, derivatives of a common primordium, develop vastly different features. Over the embryonic stages we examined, 103 TFs (~8%) are expressed differentially between the two organs (Fig. 1D). Although our survey readily identified known intestine-specific genes such as *Cdx2* (data not shown), fewer than 25 TF mRNAs appear to be expressed exclusively in the fetal stomach or intestine (e.g. Fig. 1E). Most spatial differences occur at the level of signal strength or of temporal variation in mRNA levels (e.g. Fig. 1F). Based solely on differential gene expression, we recently proposed and demonstrated a vital role for the homeobox TF Barx1 in stomach epithelial specification (Kim et al., 2005). The limited extent to which TF genes are expressed only in one organ or the other implies that such examples are rare. Nevertheless, spatially restricted TF expression (Fig. 1D) is probably a good predictor of tissue-specific functions.

## Expression of TF families in gut development

There is wide variation in the extent to which the members of well-characterized TF families are expressed during gut development (Fig. 2A). About 90% of all ZnB factors and basic-leucine zipper (bZip) proteins are expressed, whereas the smallest fractions are detected among forkhead (32%) and HD (45%) proteins (Fig. 2A). The two TF families with highest proportional representation (ZnB and bZip) also have the largest fraction of genes expressed constitutively from E11 to E17. By contrast, the HD family shows an especially high degree of regulated expression, with over 60% of genes showing differential expression in space or time (Fig. 2A, Table 2; data not shown). Two families, HD and nuclear receptors (NR), account for the bulk of differential TF gene expression between the stomach and intestine (Fig. 2A).

Of the 47 known NRs, 33 are expressed during GI organogenesis, 22 with similar trends in stomach and intestine (Fig. 2C and data not shown). Of the 103 TF transcripts that differ notably between stomach and intestine, 11 belong to the NR family. Moreover, in the small intestine, 53% of expressed NR genes change in levels during development and 13 transcripts, representing the highest proportion of any TF family, increase in relative abundance. At least four such factors, PPAR-γ, Nr2a1 (HNF-4α), Nr1f3 (ROR-γ) and *Nr5a2* (LRH-1) are known to regulate region- and stage-specific intestinal genes or crypt functions (Drori et al., 2005; Kan et al., 2004; Ladias et al., 1992; Raspe et al., 2001; Sauvaget et al., 2002; Botrugno et al., 2004; Schoonjans et al., 2005). Biological functions correlate precisely with spatiotemporally restricted expression in each of these cases, and our survey has the potential to identify other factors with limited expression. Indeed, late in intestine development we observed high levels of *Nr2e3* (Fig. 2C,D), a transcript previously believed to be present only in the retina (Chen et al., 2005; Kobayashi et al., 1999). We confirmed presence of *Nr2e3* mRNA in fetal gut by northern analysis and cloning of the PCR product (data not shown); in situ hybridization localized the transcript in epithelial cells (Fig. 2F). *Nr2e3* expression is confined to the period between E15 and 3 weeks after birth and to the proximal bowel (Fig. 2E). These findings suggest the possibility of regional or stage-specific functions, and highlight the potential for gene discovery through the GIfT resource.

**Table 2. Expression of selected HD gene families in GI development**

| HD sub-family | Number expressed in the developing gut | Number differentially expressed in space or time |
|---|---|---|
| POU family | 5/15 | 3/5 |
| LIM family | 2/12 | 1/2 |
| Paired (including Isx) | 6/28 | 5/6 |
| Nkx group | 6/16 | 5/6 |
| Msx-Dlx group | 9/13 | 8/9 |
| HOX cluster | 16/36 | 14/16 |

Authors frequently comment on a special role for HD proteins, including the products of Hox-cluster genes, in gut development (Beck et al., 2000; Grapin-Botton and Melton, 2000). Although HD subfamilies are unequally represented in the fetal gut, ranging from five out of 28 Paired-class proteins to nine out of 13 factors from the Msx-Dlx group (Table 2), every sub-family is developmentally regulated, either in changes over time or in differences between stomach and intestine. Levels of most HD TFs, including the products of Hox clusters, decline late in fetal gut development, which suggests that their functions may concentrate at early stages. Our data extend previous work that investigated a limited number of Hox-cluster genes and developmental stages or relied on methods less sensitive than RT-PCR (Kawazoe et al., 2002; Pitera et al., 1999), and we detect several Hox mRNAs previously reported to be absent from the gut. All Hox gene transcription in developing gut occurs from genes located near the 3′ end of Hox clusters, with strongest expression from paralogous sub-groups 5, 6 and 7 (Fig. 2B); mRNAs derived from subgroups 9-13 are absent. With the exception of HoxC4, HoxB6, HoxA7 and HoxC8, expression dynamics are remarkably concordant in the developing stomach and intestine. Thus, whereas genes from paralogous subgroups 6, 7 and 8 may serve in regional specification, Hox gene expression is unlikely to act alone to distinguish the two organs from one another.

## Identification of TFs expressed in a restricted distribution

TFs that are confined to the gut or differentially expressed within the GI tract are especially likely to regulate tissue-specific genes, and we adopted three methods to reveal such TFs. The first approach sought to identify TFs that are highly enriched in mature intestine and reinforced the idea of tissue-restricted functions for selectively expressed genes. We mined an expression database of 51 adult mouse tissues (Zhang et al., 2004) and found all mRNAs expressed at more than 2.5-fold higher levels in intestine compared with at least 40 other sites. For these 1240 transcripts (partially represented in Fig. 3A), the commonest sites of additional expression are the stomach, liver and pancreas, organs that share embryonic origin and metabolic or digestive functions with intestine. Thirty-four intestine-enriched genes encode TFs (http://www.geneontology.org). The GIfT survey identified 23 of them in the developing gut (Fig. 3B) and we confirmed restricted expression for 20 TFs in another database of 61 organ profiles (http://symatlas.gnf.org). Detailed review of published information on knockout mice (Fig. 3C; see Table S1 in the supplementary material) revealed that the principal abnormalities uniformly lie in restricted or dominant sites of gene expression. Whereas mice deficient in these TFs occasionally show anatomic GI defects, a common factor is defective metabolism of bile acids, lipids, glucose or xenobiotics, which reflects the metabolic function of endoderm-derived tissues. These observations support the idea of organ-specific functions for GI-restricted TFs and drew attention to an

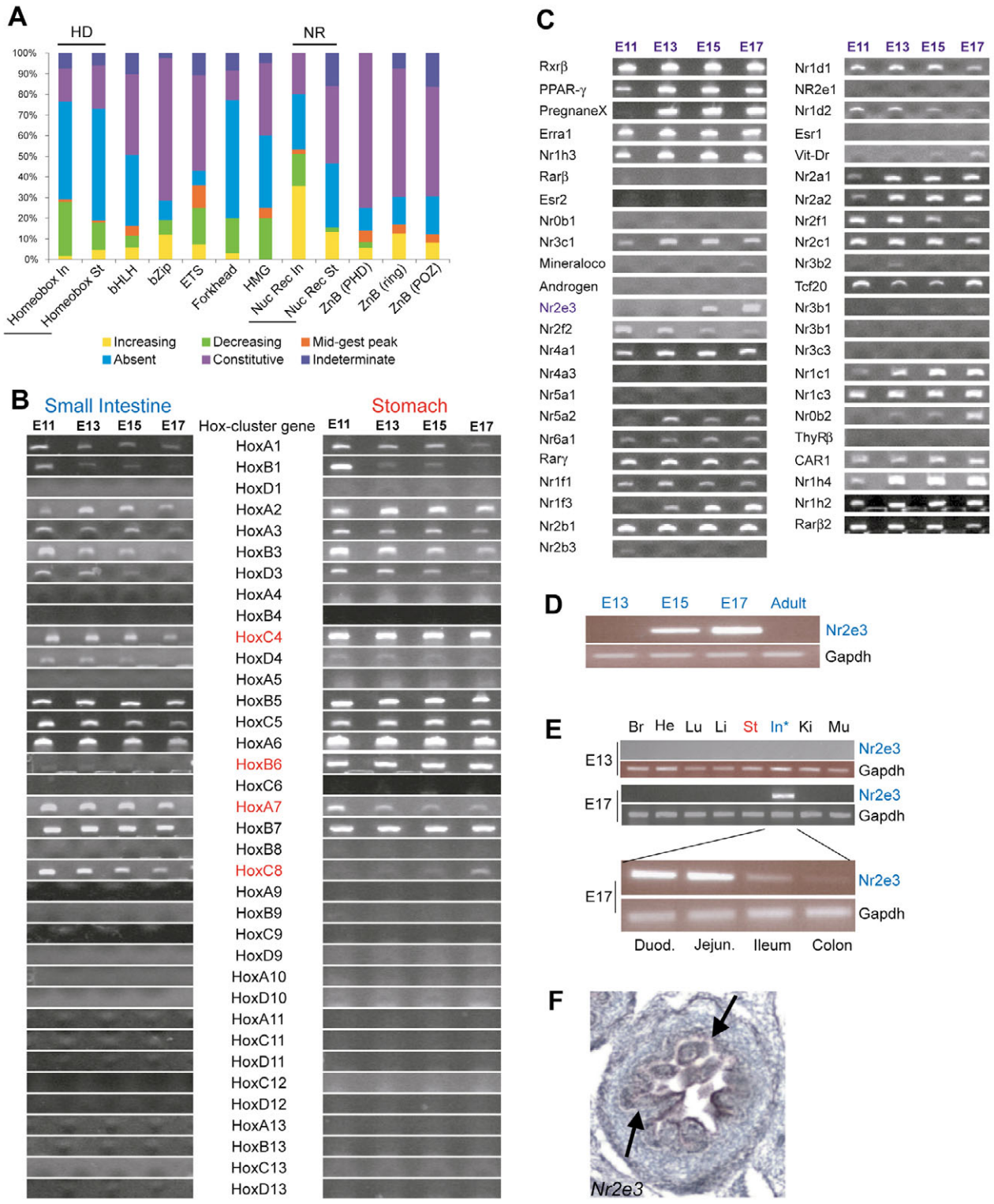


**Fig. 2. Analysis of TF gene expression in the developing GI tract according to protein families.** (**A**) TF gene families show divergent patterns of expression and temporal modulation during intestine development, with proportionally higher representation of basic-leucine zipper (bZip) and zinc-binding (ZnB) factors. Nuclear receptor (NR), homeodomain (HD) and high-mobility group (HMG) factors have the highest degree of temporal modulation. Except for HD and NR, most families are distributed similarly in intestine (In, shown here) and stomach (St, data are shown only for NR and HD). (**B**) Comparative gene expression for a single TF subfamily, Hox-cluster genes, commonly proposed as candidates for anteroposterior gut patterning. Expression in the developing gut is limited to genes at the 3′ ends of collinear clusters and, with the few exceptions marked in red type, is very similar in extent and modulation in fetal stomach and small intestine. (**C**) As a group, NRs showed the greatest increase during intestine development, mostly from E11 to E13. One factor expressed with these dynamics is Nr2e3, previously regarded as a photoreceptor-specific product. (**D**) *Nr2e3* mRNA, expressed in the developing gut, is abrogated in adult mice. (**E**) Fetal expression of Nr2e3 is restricted to the proximal small bowel. (**F**) In situ hybridization (E15 intestine) reveals Nr2e3 expression in the epithelial compartment (arrows).

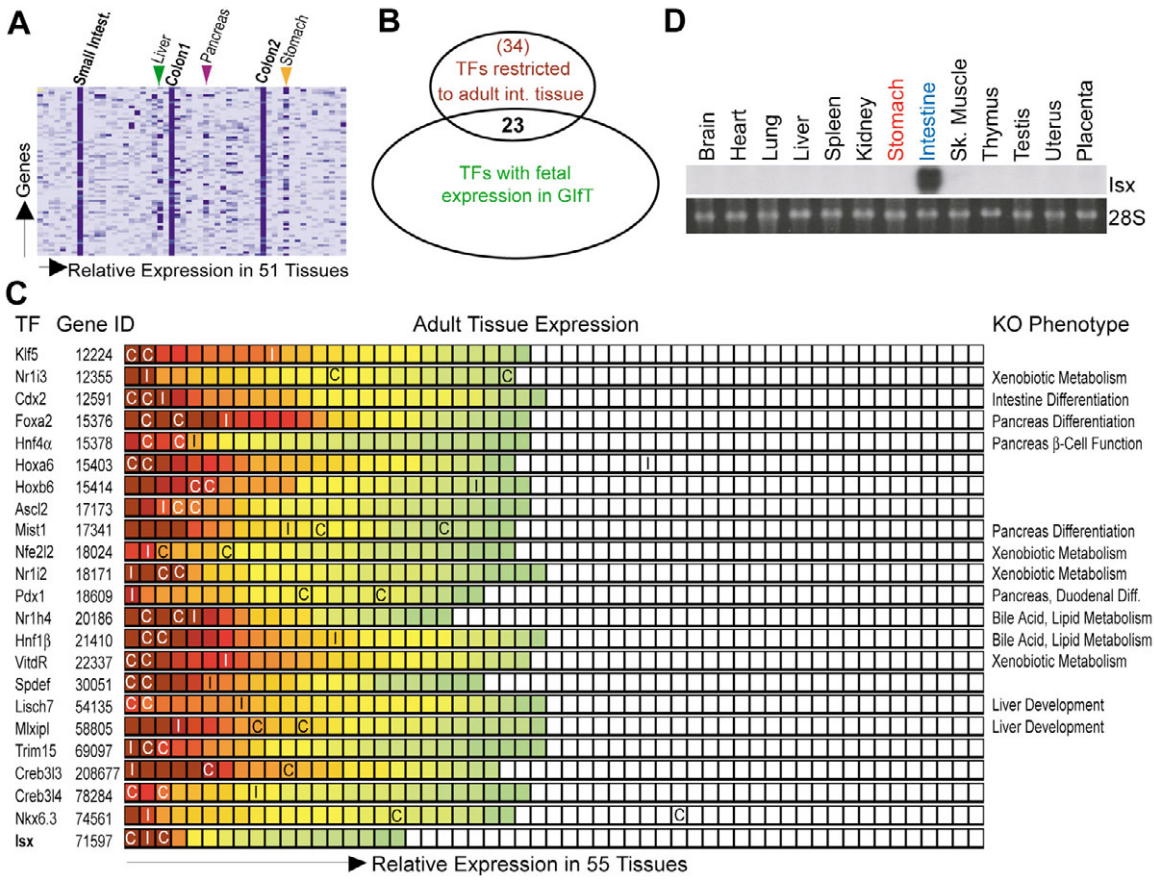


**Fig. 3. Identification of novel GI-restricted TFs.** (**A**) Schematic representation of relative expression levels, in light (lowest expression) to dark (highest) purple, of 161 sample transcripts out of 1240 that are enriched in the small and large intestine; colon was analyzed twice in the study from which the data are extracted (Zhang et al., 2004). The most common extra-intestinal sites of expression were stomach, liver and pancreas; most mRNAs are expressed sparingly in other tissues. (**B**) Intersection of these gene expression data (34 TFs) with the GIfT survey, revealing developmental representation of 23 TFs that are highly enriched in GI expression. (**C**) Detailed expression profile of these 23 TF genes in 55 adult and embryonic mouse tissues; again, data are taken from Zhang et al. (Zhang et al., 2004). In each row, the TF gene is listed towards the left, every square represents an organ and expression levels are represented by a color scale (red-orange, high; yellow-green, low; white, absent). Expression levels in adult small intestine and colon are marked with 'I' and 'C', respectively. Predominant phenotypes reported in knockout mice are indicated on the right. (**D**) Northern analysis of adult mouse tissues, showing significant intestine enrichment (and possibly exclusive expression) of the novel TF transcript corresponding to Gene ID 71597 (last row in panel C), for which we propose the name *Isx*.

uncharacterized gene within the group. Northern analysis confirmed that this mRNA (Gene ID 71597) is highly restricted to the intestine (Fig. 3C, bottom; Fig. 3D). It encodes a novel HD protein and entered our survey based on ESTs derived from a cecum library. As its mRNA expression is highly restricted, we propose the name Isx (intestine-specific homeobo*x*).

Second, to identify TFs that enriched in the developing gut, we tested six fetal non-GI tissues from E13 and E17 mice for expression of 66 TF mRNAs that either increase during intestine development or differ notably between stomach and intestine. Twelve of these 66 factors, including Nr2e3 and Isx, appear selectively in the GI tract and hardly at all in the other fetal organs (Fig. 4A). Another nine factors are expressed in a limited distribution, including the gut (data not shown), whereas 45 TFs are expressed widely. In situ hybridization for the 66 genes on mouse embryo sections allowed us to localize developmentally regulated TFs and to verify tissue-specific expression (Fig. 4B,C). Fifty-six percent of the probes yielded satisfactory results and assigned epithelial distribution to 23 (35%) of the TF transcripts, and mesenchymal or mesenteric expression to 4 (6%); eight TF genes (14%) are expressed in multiple compartments (Fig. 4C). *Isx*, *Nr2e3* and *Nr1i3* stood out for selective expression in intestinal epithelium, although trace *Isx* signals also appeared in the fetal stomach in a PCR assay (Fig. 4A).

Finally, Cdx2, the best characterized TF in intestinal epithelium, is implicated in control of intestine-specific genes (Beck, 2004). Cdx2 haploinsufficiency promotes limited stomach-type differentiation in the colon (Beck et al., 1999; Chawengsaksophak et al., 1997) and transgenic mice with forced Cdx2 expression in the stomach mucosa display intestinal features in that tissue (Mutoh et al., 2002; Silberg et al., 2002). Intestinal TFs that are normally excluded from the stomach but are present within Cdx2-induced intestinal metaplasia may be especially important in intestinal gene regulation. We therefore examined stomachs derived from FoxA3-Cdx2 transgenic mice (Silberg et al., 2002) for presence of 15 TF mRNAs that met stringent criteria for selective expression late in normal intestine development. Consistent with reports that Cdx2 induces partial intestine differentiation in this animal model (Silberg et al., 2002), only one TF, Isx, was induced by the transgene to appreciable levels (Fig. 5A). Thus, three independent approaches

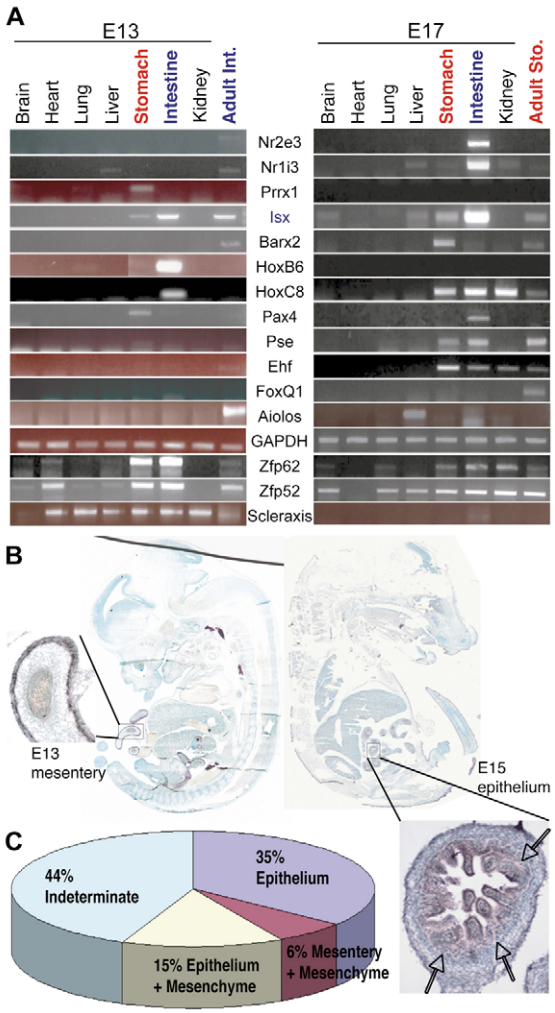


**Fig. 4. Tissue-enriched and compartmentalized expression of TF mRNAs.** (**A**) Partial results from comparative expression of 66 TF mRNAs in multiple fetal mouse tissues at E13 (left) and E17 (right), determined by RT-PCR. Twelve factors are considerably enriched in the developing stomach and/or intestine, with persistent expression of some TFs in the adult GI tract. GAPDH provides a mRNA loading control, and three examples of TFs with broad tissue distribution are included. (**B**) Illustrative examples of two TFs, an uncharacterized ZnB protein (left) and Isx (right) revealed by in situ hybridization to be expressed in the E13 mesentery (left) and E15 mucosa (right, arrows), respectively. (**C**) Pie-chart representation of mRNA localization of the 66 tested TFs, which either increase during intestine development or differ notably between stomach and intestine.

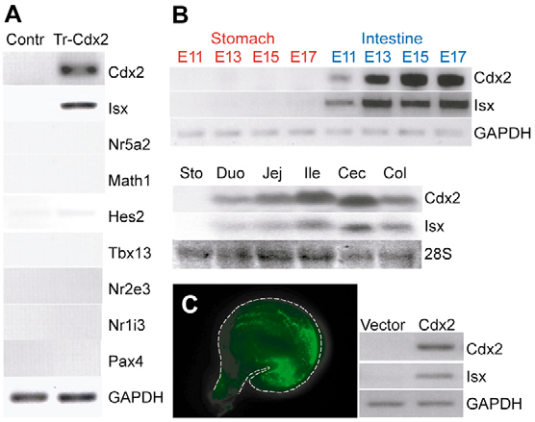


**Fig. 5. Relation between Isx and the gut homeotic regulator Cdx2.** (**A**) Representative RT-PCR analysis of 15 late-activated, intestine-restricted TFs in the stomach of FoxA3-Cdx2 transgenic mice. *Isx* is the only tested transcript present in the metaplastic stomach. (**B**) *Isx* mRNA shadows that of the intestine regulator Cdx2 in time and space. Both TFs are virtually absent from stomach and are activated simultaneously between E11 and E13 in developing mouse intestine (top, RT-PCR analysis); concentration of both mRNAs is highest in adult ileum and cecum (bottom, northern analysis). GAPDH or 28S RNA serve as loading controls. (**C**) Forced expression of GFP-tagged Cdx2 in fetal mouse stomach explants (left, organ margin outlined in white) consistently induces ectopic expression of *Isx* mRNA (right, RT-PCR analysis).

converged on Isx as a novel intestine-restricted TF, and its unique appearance in the Foxa3-Cdx2 transgenic stomach may particularly reflect a role in intestinal gene regulation.

## Characterization of the developmentally regulated, intestine-restricted TF Isx

*Cdx2* and *Isx* transcripts appear coordinately in the fetal intestine, just before the endoderm transitions into a columnar epithelium, and both TFs vary similarly in levels along the adult duodenum-colon axis (Fig. 5B). Moreover, forced expression of green fluorescent protein (GFP)-tagged Cdx2 by electroporation into fetal mouse stomach explants led to rapid and robust induction of *Isx* mRNA in treated but not in mock-transduced explants (Fig. 5C). Thus, both normal and transgenic mice reveal striking concordance between expression of *Isx* mRNA and that of the key intestinal TF Cdx2.

Unlike Cdx2, which shows a limited wave of early expression at peri-implantation stages (Strumpf et al., 2005), *Isx* mRNA appears late in development (Fig. 6A), just before the villus transition in intestine morphogenesis. *Isx* transcripts are restricted to the epithelial compartment in both adult (Fig. 6B) and fetal (Fig. 4B) intestine. Phylogenetic comparison of the Isx HD with those of nearly 200 other HD proteins places Isx within the Paired family; homology is closest to Pax3, Pax7 and Prrx1 (Fig. 6C), which serve organ-specific functions. To test Isx functions in vivo, we generated mutant mice. Our strategy for targeted gene disruption replaced the first exon with a neomycin-resistance cassette (Fig. 6D) and resulted in elimination of Isx transcripts, as judged by northern analysis (Fig. 6F). Isx-null mice are born in the expected numbers and appear healthy for at least 1 year. Under standard housing conditions, they gain weight at the same rate as their littermates and display normal histological features in the gut (data not shown).

## Intestinal gene expression defect in Isx-null mice

Intestinal genes tend to express in anteroposterior (AP) gradients, with few transcripts exclusive to a given segment (Bates et al., 2002). To determine if Isx might control regionally restricted gene expression, we used qPCR to measure mRNA levels of *Upa*, *Adh1*, *Ephx2*, *Slc2a2* (all enriched in the duodenum), *Cdx1*, *Pap*, cubilin and guanylin (ileum-enriched transcripts) (Bates et al., 2002). No consistent differences were evident between control and mutant samples (data not shown). Thus, Isx appears to be dispensable for gross intestinal development and function or to establish the AP axis.

We hypothesized that, like other gut-restricted TFs (Fig. 3C), Isx may control some activities relevant to metabolism. To identify such a role, we performed RNA microarray analysis of adult terminal

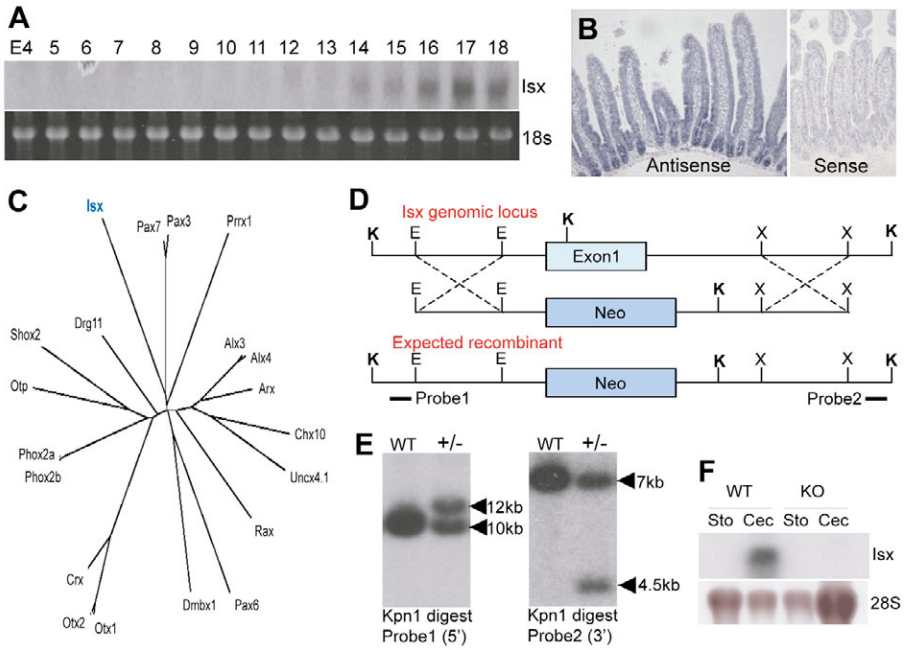


**Fig. 6. Characterization of the novel, intestine-restricted homeobox gene *Isx*.** (**A**) Northern analysis of whole mouse embryos at the indicated post-fertilization (E) days shows absence of *Isx* mRNA expression before E14. This feature distinguishes Isx from Cdx2, which is also expressed at peri-implantation stages. (**B**) In situ hybridization reveals epithelium-restricted expression of *Isx* transcripts in adult intestine; right panel shows results with a sense probe. (**C**) The Isx HD is most closely related to that found in the Paired family. Unrooted phylogenetic analysis using the CLUSTAL_W algorithm (20 of 28 Paired proteins are shown) reveals closest homology to Pax3, Pax7 and Prrx1. (**D**) *Isx* gene targeting strategy. 3.5 kb *Eco*RI (E) and 2.5 kb *Xba*I (X) genomic fragments were isolated from a 129/Sv BAC clone and used to flank a PGK-Neo$^{R}$ cassette, positioned in reverse orientation, in the targeting construct. Positions of *Kpn*I (K) sites enabled confirmation of gene targeting. (**E**) Correct targeting, with deletion of exon 1, was determined by Southern analysis of *Kpn*I-digested DNA probed with the genomic fragments indicated in D. Homologous recombination produced the expected 12 kb and 4.5 kb bands with the 5′ and 3′ probes, respectively. (**F**) Northern blots confirmed loss of Isx expression after targeted gene disruption. RNA isolated from the cecum (Cec) and stomach (Sto) of nullizygous mutant (KO) mice and littermate controls (WT) was probed with *Isx* cDNA. Ethidium bromide staining of 28S RNA shows equal or excess loading of mutant samples.

ileum, the bowel segment with highest Isx expression (Fig. 5B). Although levels of very few mRNAs differed significantly between Isx$^{+/-}$ and Isx$^{-/-}$ samples, transcripts for the scavenger receptor, class B, type I (*Scarb1*), an HDL receptor and lipid transporter (Rigotti et al., 2003), are increased ~10-fold in *Isx*$^{-/-}$ gut. We detected this anomaly with three independent probe sets (e.g. Fig. 7A) and verified the results by qPCR analysis on multiple independent samples (Fig. 7B). *Scarb1* is expressed more strongly in the liver and in adrenal steroidogenic cells than in the gut (Acton et al., 1996; Rigotti et al., 2003). In *Isx*$^{-/-}$ mice, however, changes in Scarb1 expression are not observed in the liver or adrenal glands but are confined to the gut (Fig. 7B,C).

Scarb1 protein is normally found on the apical surfaces of absorptive epithelial cells, and the levels decrease from duodenum to ileum (Cai et al., 2001; Hauser et al., 1998; Voshol et al., 2001), opposite to the increasing duodenum-ileum gradient for Isx (Fig. 5B). *Scarb1* mRNA levels in *Isx*$^{-/-}$ mice are higher in both duodenum and ileum (Fig. 7B), and immunoblot analysis confirms that ileal protein levels are substantially (more than fivefold) elevated (Fig. 7C). As in the duodenum of wild-type animals (Hauser et al., 1998; Voshol et al., 2001), Scarb1 protein in the mutant ileum resides in the epithelial brush border (Fig. 7E), where low endogenous levels are undetectable in the normal ileum (Fig. 7D). Mice with forced Scarb1 expression in the gut absorb dietary cholesterol and triglycerides more efficiently (Bietrix et al., 2006); although Scarb1-null mice are not deficient in these functions (Mardones et al., 2001), they show reduced absorption of other lipids such as carotenoids, lutein and vitamin E (Reboul et al., 2006; van Bennekum et al., 2005). Thus, regulation of the location and extent of intestinal *Scarb1* expression by Isx probably reflects an underlying function in lipid absorption or homeostasis in this organ, similar to the essential metabolic roles of other gut-restricted TFs (Fig. 3C).

## DISCUSSION

Precise spatial and temporal control of gene expression during development and cell differentiation serves to establish and maintain tissue-specific properties. The approach outlined in this report provides a means to explore the contributions of regulated TFs in GI development and our results demonstrate the value of combining this approach with identification and functional characterization of tissue-restricted factors. These studies, and others that build on the GIfT database, should advance appreciation of molecular mechanisms of gut differentiation, morphogenesis and function. Our rigorous validation of the TF expression data indicates both that observed spatiotemporal variation accurately reflects developmental modulation and that expression is probably absent or negligible when a transcript went undetected. The quality of the data hence inspires confidence in exploiting the GIfT resource to identify important TFs on the basis of their stage- or organ-specific expression during GI development.

Our study reveals some surprising features in the TF landscape during gut morphogenesis. A remarkably high fraction (78%) of all TF mRNAs is expressed, similar to the high representation of TF transcripts in the developing mouse nervous system (Gray et al., 2004). These data highlight the likely redundant manner in which tissues deploy a finite TF repertoire. Second, despite many functional and morphological differences between the stomach and intestine, at the resolution of our analysis, expression of only a few dozen TFs differs appreciably between these tissues. Thus, to the extent that differential TF expression is important, as strongly implied in the literature, a few TFs might suffice to help achieve complex developmental differences. Of course, differences other than total mRNA expression are also important, including the cellular context and post-transcriptional modulation of TF activities, localization and protein interactions.

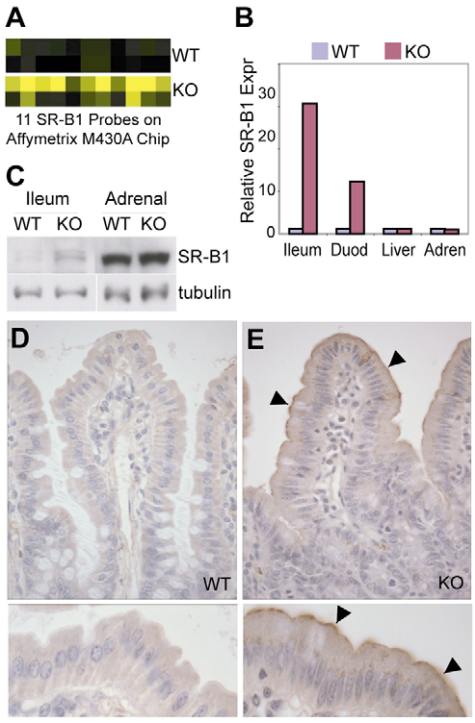


**Fig. 7. Altered intestinal gene expression in mice lacking Isx function.** (**A**) Microarray data for increased *Scarb1* mRNA levels in Isx$^{-/-}$ (KO) ileum compared with control (WT) littermates. Dark signals represent absence and yellow the presence of hybridization; in each set, the top row shows probes that perfectly match the target transcript and the bottom row shows probes with single-base mismatches. Similar results were obtained for two independent Scarb1-specific probe sets. (**B**) qPCR confirmation of significant elevations in *Scarb1* mRNA in *Isx*$^{-/-}$ ileum and duodenum (Duod) but not in the other sites of Scarb1 expression, adrenal gland and liver, where Isx is absent. All mutant (KO) values are expressed in relation to the control (WT, assigned a value of 1.0) for that tissue. *Scarb1* mRNA levels in liver and adrenal glands are higher than in intestine, but increases in *Isx*$^{-/-}$ mice are confined to the gut. (**C**) Immunoblot confirmation of elevated Scarb1 protein levels in *Isx*$^{-/-}$ intestine but not liver (data not shown) or adrenal glands. (**D**,**E**) Immunohistochemistry with Scarb1 antiserum. Wild-type ileum (D) reveals no specific signal as Scarb1 is primarily a duodenal product, whereas Scarb1 localizes (arrowheads) in the mutant (KO) ileal apical brush border (E).

Nevertheless, TFs that are restricted in distribution are especially likely to regulate tissue-specific genes, and our analysis of the reported requirements for gut-restricted TFs (Fig. 3) underscores the high frequency at which tissue distribution predicts gene functions. Together, these results point to the limited pool of spatiotemporally regulated TFs as an enriched source of factors that are especially pertinent in tissue differentiation. Thus, a particular value of GIfT lies in its examination of all TFs on a common methodological platform, whereas the experimental approach outlined in this report highlights the ability to generate cogent functional hypotheses based initially on differential mRNA expression. Indeed, intestinal expression of TFs such as Nr2e3 and Isx may not have been revealed as readily by methods other than a global survey.

TFs restricted to the GI tract may be expected to fall into two functional classes. Some factors, known and unknown, directly influence cell lineages or tissue architecture. Conventional laboratory methods favor detection of such effects, and, as a result, current understanding of TF functions in gut development relies heavily on the study of genes that overtly influence tissue morphology. The second functional class of TFs, which is equally important for the physiological duties of an organ, may exert discrete effects on tissue-specific gene expression without producing morphological correlates. In the gut, which serves digestive and metabolic functions, tissue-specific TFs probably serve a variety of evolutionarily adaptive roles, and the novel, GI-restricted homeobox TF Isx seems to represent such a factor. Although mRNA profiling of *Isx*$^{-/-}$ ileum reveals dysregulation of certain α-defensin-related genes (M.Y.C. and R.A.S., unpublished) normally expressed in intestinal Paneth cells, our comparison of *Isx*$^{+/-}$ and *Isx*$^{-/-}$ ileum emphasizes deregulation of the epithelial HDL receptor and lipid transporter Scarb1. Consistent with GI-restricted expression of Isx, Scarb1 levels in *Isx*$^{-/-}$ mice are altered only in the gut, to comparable degrees in proximal (duodenum) and distal (ileum) segments. *Scarb1* transcription is activated by C/EBP family TFs and sterol regulatory element binding protein 1 (Malerod et al., 2002) and suppressed post-transcriptionally by treatments that reduce intestinal bile delivery, such as bile duct ligation or surgical diversion (Voshol et al., 2001). Our data suggest that Isx serves specifically to refine the extent and domain of intestinal Scarb1, which is normally confined to the duodenum, the principal site for uptake of dietary lipids and most nutrients.

Absorption of dietary lipids is an essential intestinal function mediated by brush border membrane receptors, including the class B scavenger receptors Scarb1 and CD36. Scarb1 is required to absorb β-carotene and vitamin E in mice (Reboul et al., 2006; van Bennekum et al., 2005), and displays high affinity for cholesterol in vitro (Acton et al., 1996). Although forced Scarb1 expression in heterologous cells confers lipid uptake properties that resemble those of enterocytes (van Bennekum et al., 2005), cholesterol absorption is intact in *Scarb1*$^{-/-}$ mice (Mardones et al., 2001), possibly because CD36 or other factors compensate for its absence. As a multiligand receptor with diverse functions, it is probably important that Scarb1 expression is restricted. Indeed, *Villin* promoter-driven Scarb1 overexpression, mainly in the intestine but also to a lesser degree in the liver, accelerates absorption of dietary cholesterol and triglycerides (Bietrix et al., 2006). Ileal expression, which is normally absent, may be especially detrimental as this distal intestinal region has important functions in absorption of bile salts and integrity of enterohepatic bile acid circulation. Thus, Isx, among other possible activities, tailors gene expression to meet physiological needs in the intestine. It is one of a few dozen TFs our study identified, by virtue of regional and restricted expression, as a candidate for specific gut functions. The approach we have taken with TFs can readily be extended to other protein classes.

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**Supplementary material**

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/20/4119/DC1

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