Corrections

**GENETICS**

The authors note that, due to a printer’s error, the second affiliation for Anders Molven should instead appear as “Department of Pathology, Haukeland University Hospital, N-5021 Bergen, Norway.” The corrected affiliation line appears below. The online version has been corrected.

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www.pnas.org/cgi/doi/10.1073/pnas.1101890108

**BIOCHEMISTRY**

The authors note that in the Materials and Methods under the section “Actin Pelleting Assay,” the 10x stock concentration was listed instead of the 1x final working concentration for the polymerization and reaction buffers. The correct concentrations are as follows: polymerization buffer (20 mM Imidazole pH 7.0, 100 mM KCl, 2 mM MgCl₂, 0.5 mM ATP, 1 mM EGTA) and reaction buffer (20 mM Imidazole pH 7.0, 150 mM NaCl, 2 mM MgCl₂, 0.5 mM ATP, 1 mM EGTA, 1 mM DTT).

www.pnas.org/cgi/doi/10.1073/pnas.1102194108

**MEDICAL SCIENCES**
Correction for “Prolonged duration local anesthesia with minimal toxicity,” by Hila Epstein-Barash, Iris Shichor, Albert H. Kwon, Sherwood Hall, Michael W. Lawlor, Robert Langer, and Daniel S. Kohane, which appeared in issue 17, April 28, 2009, of Proc Natl Acad Sci USA (106:7125–7130; first published April 13, 2009; 10.1073/pnas.0900598106).

The authors note that on page 7129, left column, fourth full paragraph, sentences 3 through 5, “DSPC:DSPG:cholesterol or DMPC:DMPG-cholesterol (molar ratio 3:1:2) were dissolved in t-butanol. Dexamethasone was added in some samples before lyophilization. The lyophilized cake was hydrated with 250 mM ammonium sulfate or, in some groups, with 0.1 mg STX, at 55–60 °C.” should instead appear as “DSPC:DSPG:cholesterol or DMPC:DMPG-cholesterol (molar ratio 3:1:2) were dissolved in 9:1 vol/vol chloroform:methanol solution. Dexamethasone was added in some samples. A thin lipid film was obtained by drying the lipid mixture in a rotary evaporator at 60 °C. The pressure in the evaporator was maintained at 480 torr for 20 minutes and then gradually decreased to 20 torr over a period of 30 min. The lipid film was reconstituted in t-butanol at 55–60 °C and immediately cooled by immersing in liquid nitrogen. The sample was then transferred to a lyophilizer for 24 h. The lyophilized cake was hydrated with 250 mM ammonium sulfate or, in some groups, with 1.0 mg STX, at 55–60 °C.”

www.pnas.org/cgi/doi/10.1073/pnas.1102194108
Long-range gene regulation links genomic type 2 diabetes and obesity risk regions to HHEX, SOX4, and IRX3

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Communicated by Michael S. Levine, University of California, Berkeley, CA, November 10, 2009 (received for review January 15, 2009)

Genome-wide association studies identified noncoding SNPs associated with type 2 diabetes and obesity in linkage disequilibrium (LD) blocks encompassing HHEX-IDE and introns of CDKAL1 and FTO (Sladek R, et al. 2007) Nature 445:881–885; Steinhorsdottir V, et al. (2007) Nat. Genet 39:770–775; Frayling TM, et al. (2007) Science 316:889–894). We show that these LD blocks contain highly conserved noncoding elements and overlap with the genomic regulatory blocks of the transcription factor genes HHEX, SOX4, and IRX3. We report that human highly conserved noncoding elements in LD with the risk SNPs drive expression in endoderm or pancreas in transgenic mice and zebrafish. Both HHEX and SOX4 have recently been implicated in pancreas development and the regulation of insulin secretion, but IRX3 has had no prior association with pancreatic function or development. Knockdown of its orthologue in zebrafish, irx3a, increased the number of pancreatic ghrelin-producing epsilon cells and decreased the number of insulin-producing beta cells and glucagon-producing alpha cells, thereby suggesting a direct link of pancreatic IRX3 function to both obesity and type 2 diabetes.

Results

Risk SNPs Identify Regions Regulating HHEX and SOX4. For the HHEX-IDE region, the associated SNPs lie in a 295-kb block of LD that includes three genes, HHEX, KIF1, and IDE, encoding a transcriptional regulator involved in pancreatic development (13), a kinesin interacting factor, and an insulin-degrading enzyme, respectively (14). The risk allele has been associated with decreased pancreatic beta-cell function (15). Throughout vertebrates, HHEX is in conserved synteny with the neighboring EXOC6 gene. The HHEX conserved synteny block overlaps with a small part of the risk allele–containing LD block. This part of the LD block contains the SNPs with the highest association scores, as well as the HHEX gene (Fig. L4 and Table 1). In contrast, KIF1 and IDE are located outside the conserved synteny block, strongly suggesting

http://www.pnas.org/cgi/doi/10.1073/pnas.0911591107
Table 1. T2D/obesity risk SNPs fall into GRBs

<table>
<thead>
<tr>
<th>SNP</th>
<th>GRB/target gene</th>
<th>Bystander gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1111875*</td>
<td>HHEX</td>
<td>-</td>
<td>1-4</td>
</tr>
<tr>
<td>rs7754840 rs7756992 rs10946398</td>
<td>SOX4</td>
<td>CDKAL1†</td>
<td>2, 3, 5</td>
</tr>
<tr>
<td>rs8050136* rs9939608* rs1421085* rs17817449*</td>
<td>IRX3</td>
<td>FTO†</td>
<td>6, 7</td>
</tr>
</tbody>
</table>

*SNPs located within HCNEs.
†Gene reported as disease-associated.

that any cis-regulatory elements within this part of the LD block regulate HHEX, and not KIF1 or IDE. The second risk region is a 200-kb LD block that includes the proximal promoter and exons and introns 1 to 5 of the gene CDKAL1. The SNP rs10946398 in this block is associated with decreased insulin secretion (15) but, in contrast to HHEX, CDKAL1 had no prior connection to either pancreatic development or β-cell function. Comparative genomic analysis revealed CDKAL1 to lie within a mammal:zebrafish synten block containing CDKAL1 and SOX4 in tetrapods and cdkal1 and sox4b in zebrafish (Fig. 1B). Of these genes, SOX4/sox4b has been linked to both pancreatic development (16, 17) and insulin secretion in the mouse (18), and as such represents a plausible candidate for T2D, whereas cdkal1 in zebrafish is expressed ubiquitously and at low levels (Fig. S1). The other teleost fish with sequenced genomes (e.g., stickleback, medaka, fugu) retained only one copy of sox4 after whole-genome duplication, and the conserved synteny is larger, including the loci of E2F3 and MBOAT1. E2F3, a cell cycle regulator, is not expressed in pancreas (19), and its fate after whole-genome duplication in zebrafish indicates that it is not a GRB target gene. The third risk region, an LD block of 49 kb encompassing a large part of the first intron of FTO (fat mass- and obesity-associated gene), also contains numerous HCNEs and lies within an extended region of conserved synteny with the IRX3/5/6 cluster, the closest gene of which is the transcription factor–encoding IRX3 (Fig. 1). Although our computational analysis and GRB visualization suggested that the target gene of the HCNEs within FTO is IRX3, no connection of IRX3 to pancreatic development or insulin secretion was apparent in the literature. To evaluate both candidates as GRB targets, we performed in situ hybridization for both fto and irx3a in zebrafish larvae, revealing barely detectable activity for fto (Fig. S1) and expression in endoderm, colocalized with insulin transcripts, for irx3a (Fig. S2).

To provide further functional evidence for our hypothesis that the aforementioned T2D-associated SNPs are in genomic regions devoted to long-range regulation of HHEX, SOX4, and IRX3 (the GRB targets), we examined the general landscape in the LD blocks containing reported SNPs and tested HCNEs within these LD blocks for the ability to drive expression consistent with that of the GRB target gene. Enhancer activity of human HCNEs from healthy, lean subjects was tested experimentally using a GFP reporter assay in zebrafish, whose validity in one case was confirmed by mouse transgenic reporter assays. A summary of tested elements is given in Table 2.

**Reporter Assays in Mouse and Zebrafish Support Long-Range Regulation of HHEX and SOX4.** Patients carrying the risk-associated C-allele of rs1111875 in the LD block overlapping with the HHEX GRB show lower acute insulin response (15). This highly associated SNP maps to a conserved region that, when tested in enhancer reporter assays in mouse or zebrafish, shows pancreatic islet expression (tested element 1; Fig. 2A–C). Although this does not prove that rs1111875 is the variant conferring disease risk, our finding that the normal version of the HCNE containing rs1111875 directs reporter expression to the islet is highly suggestive of an effect on β-cell function.

Fig. 1. Disease-associated SNPs (Top) and tested elements (elements 1–6) in the context of vertebrate GRBs around HHEX (A), SOX4 (B), and IRX3 (C). GRBs were defined by minimal synteny blocks to zebrafish (tan shading) or stickleback (light blue shading). Hapmap recombination hotspots (blue bars) mark the edges of LD blocks (blocks of interest shaded red). HCNE density plots for pairwise comparisons are colored yellow, orange, and red, based on the minimum percentage identity of the elements ±50 bp in size used to derive them: human vs. mouse (mm: ≥95%, ≥98%, 100% identity), chicken (gg: ≥90, ≥95, ≥98), Xenopus (xt: ≥70, ≥80, ≥90) and zebrafish (dz: ≥70, ≥80, ≥90). HCNE densities were calculated as number of bases in HCNEs in sliding windows of 300 kb, and curves for different species are drawn to the same scale. HCNEs (black bars) are shown at the lowest threshold for each pairwise comparison. All features, including the suggested regulatory target gene (red) and bystander genes (gray) are shown in human coordinates. Gene annotations were adapted from the UCSC Known Genes set.
which corresponds to a subdomain of sox4 expression (Fig. 2D) unrelated to that of the broadly expressed cdkal1 (Fig. S1). In addition, the human element directed expression to the pri-

eminent functionally important region regulating IRX3.variants rs8050136, rs9939609, rs1421085, and rs17817449 were found to be highly associated with T2D and obesity and lie in an LD block of 49 kb in the FTO gene (4, 6, 7). FTO contains numerous conserved non-coding elements in its introns and is located adjacent to a gene
desert next to IRX3, a transcriptional regulator expressed in the kidney, notochord, forebrain, hypothalamus, and endodermal derivatives (22). We tested the expression driven by the 2 most deeply conserved HCNEs from the obesity-associated LD block: in both cases, we obtained expression patterns consistent with that of the IRX3 gene (Fig. 2E and F). Element 3 drove GFP expression in pronephric duct: Irx3 is expressed in the developing kidney (22) and directs nephron segment identity (23). Element 4 drove reporter expression in the notochord, also a subdomain of the IRX3 expression pattern (22). These results show that HCNEs located within the FTO obesity-risk LD block are most likely acting on IRX3, whereas zebrafish fto is at these stages not significantly expressed in the embryo. Neither of these subdomains, however, suggested a link to obesity, and no disease-associated SNPs map to those HCNEs. Upon testing the elements that overlap with risk SNPs rs1421085 and rs9939609, we noted that the majority of transgenes of the former (5 of 8) and a single transgenic line of the latter directed reporter expression to the pancreas.

**Table 2. Summary of tested elements**

<table>
<thead>
<tr>
<th>Test HCNE-containing element</th>
<th>Human coordinates (hg18)</th>
<th>Relation to SNP position</th>
<th>Relation to closest gene</th>
<th>GRB target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>chr10:94452612–94453112</td>
<td>Contains rs1111875</td>
<td>10 kb downstream of HHEX</td>
<td>HHEX</td>
</tr>
<tr>
<td>2</td>
<td>chr6:20769594–20770392</td>
<td>Closest HCNE to rs7754840 in the corresponding LD block</td>
<td>In fourth intron of CDKAL1</td>
<td>SOX4</td>
</tr>
<tr>
<td>3</td>
<td>chr16:52364673–52365826</td>
<td>Contains rs1477196</td>
<td>In first intron of FTO</td>
<td>IRX3</td>
</tr>
<tr>
<td>4</td>
<td>chr16:52397232–52397632</td>
<td>Most conserved HCNE in the corresponding LD block</td>
<td>In first intron of FTO</td>
<td>IRX3</td>
</tr>
<tr>
<td>5</td>
<td>chr16:52358243–52358842</td>
<td>Contains rs1421085</td>
<td>In first intron of FTO</td>
<td>IRX3</td>
</tr>
<tr>
<td>6</td>
<td>chr16:52377745–52378287</td>
<td>Contains rs9939609</td>
<td>In first intron of FTO</td>
<td>IRX3</td>
</tr>
</tbody>
</table>

*The equivalent element in Xenopus is contained within the tested genomic interval scaffold_444:319497–322675 in genome assembly xenTro2.*

![Fig. 2. Reporter expression patterns directed by non-coding elements from T2D risk regions.](image)

(A and B) Live dorsal image (A) and cryosection (B) of a 72-hpf transgenic zebrafish showing GFP-reporter expression driven by the Xenopus element equivalent to the rs1111875-containing human HCNE downstream of HHEX (element 1). Red arrowheads point to the pancreatic anlagen. The horizontal line in (A) shows the level of section in B. The cryosection (B) was stained for DNA using DAPI (blue), for muscle actin using rhodamine-phalloidin (red) and for GFP using anti-GFP (green). (C) Sections through the pancreas of a 14.5-d mouse transgenic for rs1111875-containing human HCNE downstream of HHEX (element 1). LacZ staining is seen in the pancreatic anlagen. (D) Live dorsal image of a 48-hpf transgenic zebrafish showing GFP-reporter expression driven by the human HCNE closest to rs7754840 in CDKAL1 (element 2). Arrowheads point to expression in the hindbrain (red) and the primordium of the swim bladder (green). (E and F) Live lateral images of 48-hpf transgenic zebrafish showing GFP-reporter expression driven by the most deeply conserved HCNEs in the obesity-associated LD block of FTO. Element 3 (E) drives expression in the pronephric duct (red arrowhead), whereas element 4 (F) drives expression in the notochord (green arrowheads) and in hindbrain rhombomeres (red arrowhead). (G and H) Live dorsal images of 48-hpf transgenic zebrafish showing GFP-reporter expression driven by the rs1421085-containing HCNE and the rs9939609-containing HCNE in the FTO intron 1. Red arrowheads point to expression in the pancreatic area.
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hlxb9

and

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in zebrafish).

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encodes

irx3a

might serve a role related to that of IRX3

irx3a

D

expression.

hlxb9

elucidates the power of GRB analysis of the results of GWA

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ε

morphant embryos. The

morphant embryos (| and

Ragvin et al. Pancreatic buds of 48-hpf zebrafish development and/or regulation of insulin secretion, the case of IRX3

H

, suggesting that these HCNEs might regulate IRX3 expression in pancreas, and that its effect upon T2D might act through changes in glucose homeostasis. As we detected transcripts of the zebrafish IRX3 orthologue irx3a in the embryonic area with the earliest insulin expression (Fig. S2), we next tested whether irx3a affects cell identity in the pancreas, akin to the transcriptional regulator Nkx2.2, which has been shown to regulate the number of ghrelin- and insulin-producing cells in the pancreatic islet in both mouse and zebrafish (24, 25).

Zebrafish irx3a Affects the Ratios of Insulin-, Ghrelin-, and Glucagon-Producing Islet Cells. The risk allele mapping to the LD block in FTO is linked to the development of T2D in obese subjects and a deregulation of the relative numbers of β- (i.e., insulin-producing) and ε- (i.e., ghrelin-producing) cells would be a plausible explanation for the co-occurrence of obesity and T2D. We therefore reasoned that IRX3 might serve a role related to that of Nkx2.2 during pancreas development.

To test this hypothesis, we generated morpholinos against the translation start site and the first splice junction of irx3a in zebrafish. The knockdown efficiency was tested by RT-PCR for skipping of the second exon of irx3a and at optimal conditions reached >99% (Fig. S3). Subsequent in situ hybridization for ghrelin and insulin revealed a significant increase for the former and a large decrease for the latter (Fig. 3 and Table 3 and Table 4), establishing the involvement of zebrafish irx3a in regulating the ratio of β- to ε-cells. In addition, knockdown of irx3a also diminished the number of α-cells producing glucagon, but not of those producing somatostatin (Fig. 3 and Table 4).

Neither IRX3 nor any of its vertebrate orthologues had any prior association with pancreatic development, but mouse Irx3, together with Nkx2.2, acts as a potent repressor of Hlxb9 during the development of spinal cord interneurons (26). Hlxb9 encodes a transcriptional regulator whose loss of function also affects pancreas development (27, 28). We therefore tested how knockdown of irx3a combined with that of nka2.2a would affect expression of hlb9 in a hlb9:GFP transgenic zebrafish line (29). Knockdown of irx3a and nka2.2a resulted in reduction of the numbers of hlb9:GFP-expressing cells in the islet (Fig. S4A), whereas in the spinal cord, the expression domain of hlb9 predictably expanded dorsally in irx3a knockdowns and ventrally in nka2.2a knockdowns (Fig. S4B and Table S1). This indicates that, although irx3a, nka2.2a and hlb9 are involved in regulating the development of both spinal cord neurons and pancreatic islet cells, the interaction of irx3a and nka2.2a with hlb9 appears to be different in these tissues.

Both the conservation of the genic neighborhood of FTO/IRX3 across vertebrates and the finding that the strongest genome-wide signals for obesity map to an LD block containing HCNEs driving reporter expression in the pancreas strongly suggest that FTO is a bystander gene harboring HCNEs that drive IRX3 expression. In the case of lower IRX3 expression, the number of ghrelin-producing epsilon cells may increase at the expense of the pancreatic β- and α-cells, suggesting that a deregulation of pancreatic peptide hormones can underlie obesity and T2D with regard to the FTO association variants.

Discussion

Our analysis of 3 LD blocks containing T2D and obesity risk variants identified regulatory regions highly likely to belong to GRBs targeting developmental transcriptional regulators. This led us to link the regions containing the disease-associated SNPs to candidate genes that had a prior association with pancreatic development. None of our observations represents an unequivocal identification of a functional disease-causing SNP, but this was not our aim: instead, our results provide strong evidence that the enclosing LD blocks are within evolutionary conserved GRBs devoted to the regulation of particular developmental genes (HHEX, SOX4, and IRX3). Although, for both HHEX and SOX4, evidence was already available for their involvement in pancreas development and/or regulation of insulin secretion, the case of IRX3 elucidates the power of GRB analysis of the results of GWA

Table 3. irx3a knockdown affects the numbers of pancreatic ε-, β-, and α-cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Embryos with increased ghrelin expression (%)</th>
<th>Relative ghrelin area staining (average, n = 18)</th>
<th>Embryos with decreased insulin expression</th>
<th>Relative insulin area staining (average, n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO2-irx3a</td>
<td>39</td>
<td>25/39 (64)</td>
<td>39/39</td>
<td>0.3</td>
</tr>
<tr>
<td>Controls</td>
<td>47</td>
<td>0/47</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Twenty-five of 39 morphants (64.1%; P < 0.0001) displayed a dramatic increase of ghrelin expression and a reduction in the amount of insulin transcripts compared to un.injected embryos. By ImageJ-assisted area calculations on 18 morphant embryos, we estimated a mean reduction to 32.7% of the β-cell mass and an increase of the total number of ghrelin-expressing cells of approximately threefold.
studies for the identification of hitherto unsuspected players in vertebrate development and human disorders. Although IRX3 had no prior association with pancreatic development, we found among possible clues in the literature that mouse Irx3, together with Nkx2.2, acts as a potent repressor of Hlxb9 during the development of spinal cord neurons (26). Hlxb9 encodes a transcriptional regulator whose loss of function also affects pancreas development (27, 28). In the mouse, Hlxb9 is necessary for early dorsal pancreas development as well as for β-cell development (27), whereas in zebrafish, hlxb9 deficiency affects only β-cells (21). As extension of the early expression window of Hlxb9 is also detrimental for murine pancreas development (30), one might speculate that Hlxb9 repression during this period is also dependent upon Irx3 and Nkx2.2, but this could not be ascertained in the zebrafish. Additionally, a direct effect of irx3a/irx2.2a in the pancreas on hlxb9 would not explain the effect of their knockdown upon α-cells, which, in the mouse, do not express Hlxb9 (27). Any interaction of nks2.2a and irx3a with hlxb9 in pancreatic development therefore remains speculative at this point and will be the subject of future studies.

It is of note in this regard that 3 genes encoding transcriptional regulators, namely Nkx2.2, Pax4, and Pax6, were already identified as regulators of β- versus ε-cell fate (24, 31), with Nkx2.2 and Pax6 acting in the same pathway. It will be interesting to see whether any of these genes are also involved in the development of obesity, and how IRX3 will fit into these pathways. We note that the principal site of ghrelin production in the mouse is the gastrointestinal tract, where there is no expression in zebrafish at the stages of our assay. However, loss of function of Nkx2.2 also affects the identity of peptide hormone producing cells in the mouse gut (32), suggesting that Irx3 may serve a related role in mammals. Interestingly, mouse Irx1 and Irx2 are also expressed in the pancreatic islets and in glucagon-producing cells, and act downstream of Ngn3 (33). Together with our finding that glucagon-producing cells are also affected in the zebrafish irx3a morphant, these results suggest the elucidation of the regulation of islet cell identity as a promising avenue for future research in human metabolic disorders.

Although additional evidence will be required for any of the SNPs associated to disease to be functional, we note that the expression patterns driven in transgenic zebrafish by human HCNEs can yield useful clues whether they could be functional in a given expression context, in addition to ascertaining their regulatory function through potential changes in transcription factor–binding sites (7). It is of interest that the tested human elements are not conserved in the zebrafish genome, but still can be interpreted by the zebrafish embryo, similar to what was previously reported by Fisher and colleagues (34). While the present work was under consideration, a knockout of the mouse Fto gene was described, reporting postnatal growth retardation and increased energy expenditure (35). Notably, the targeted deletion removed exons 2 and 3, and thus did not touch upon the interaction of HCNEs in Fto intron 1 with the Irx3 target gene. This knockout therefore did not test the function of the risk LD block, nor do these results contradict our findings. A second publication reported human FTO to be a gene causing recessive multiple malformations and lethality in early childhood, also accompanied by growth retardation (36). Interestingly, the authors noted the absence of obesity in the heterozygous parents.

It is striking that the risk sequence variants fall within GRBs of regulatory genes associated with multiple phenotypes (i.e., pleiotropic genes), which is not the case for T2D-associated SNPs that affect the coding region of genes, such as that of SLC30A8 (1–4). In the former case, the affected genes we have suggested have complex spatiotemporal expression and multiple roles in development and differentiation, so different SNPs within large regions can affect different roles of the same gene, whereas the latter represents an islet-specific gene whose coding mutation is not expected to affect other tissues. Elucidation of the functional relationship between HCNEs and their target genes may hold a key to understanding the disease mechanism behind the T2D- and obesity-associated genomic variation. As the risk SNPs in FTO are common variants, it is tempting to speculate that they confer a selective advantage under specific environmental conditions. Such an advantage may be the “thrifty genotype” postulated by Neel (37), whereby natural selection during famine may enrich human populations for variants decreasing energy expenditure and increasing adiposity in times of plenty (38).

Materials and Methods

HCNE Selection for Experimental Testing. We identified human-zebrafish noneonic (i.e., noncoding and non-UTR) evolutionary conserved sequences using the HCNE identification procedure described later, complemented with the Vertebrate MULTIS Alignment and PhastCons Conservation and Chained Alignments tracks in the UCSC Genome Browser (39) for the human March 2006 assembly. Sequences were extracted from the UCSC Genome Browser for the human March 2006 assembly (hg18) or the Xenopus tropicalis August 2005 assembly (kenTro2).

Generation of Transgenic Zebrafish. Candidate HCNEs were amplified by PCR on human and Xenopus genomic DNA using the Advantage 2 PCR Enzyme System (Clontech). The final enhancer test vector contained an HCNE in front of the zebrafish gata2 promoter coupled to the EGFP gene and an polA signal, all flanked by To1 transcription sequences. Microinjection and screening were done as described (12). For description of bioinformatic methods, cloning of HCNEs, generation of transgenic mice, in situ hybridization, histology, and RT-PCR, see SI Materials and Methods.

Morpholinos. To knock down irx3a expression, we have used the following morpholinos: MO1-irx3a (AGCGTGGGGAGAGACACATTTGATG) and MO2-irx3a (GTGTCCTCCTAAAACACAGAAGACT), targeting the ATG codon and the second intron-exon boundary, respectively. Solutions were prepared and microinjected into the yolk of one-cell stage embryos according as previously described (40). The morpholino for nks2.2a (MOnk-5UTR, 5'-TGGAGCATTTGATGCAGTCAAGTTG) was the one reported by Pauls et al. (25). For the controls we used an unrelated/ unspecified morpholino (GTATAACAGGAGATTAGGATTAG).

ACKNOWLEDGMENTS. We gratefully acknowledge imaging help by Mary Laplante, and the Sars Centre zebrafish facility for animal maintenance. We thank Dr. Koichi Kawakami for the tol2 vector, Dr. Robb Krumbiak for the IacZ vector, Dr. Dirk Meyer for the hlxb9:GFP transgenic line, and Albert V. Smith for discussion. This work was funded in part by grants from the Sars Centre (G.O., P.N., B.L., and T.S.B.), grants from the University of Bergen (A.M., P.R.N., and T.S.B.), a grant from the Institut du Cerveau et de la Moelle épinière, Paris, France (T.S.B.), the FUGE Program at the Research Council of Norway (A.R., A.M., P.R.N. and B.L.), Helse Vest (A.M. and P.R.N.), Innovest (A.M. and P.R.N.), The Translational Fund (A.M. and P.R.N.), the YFF Program of the Research
Council of Norway and Bergen Forskningsstiftelse (B.L.), European Commission Grant LSHG-CT-2003-503649 as part of the ZF-Models integrated project in the 6th framework program (to T.S.B., F.A., and E.M.), grants BFU2007-00349/BMC, and CSD2007-00008 from Ministerio de Educación y Ciencia of Spain (co-funded by Feder) (to J.L.G.S. and F.C.), Junta de Andalucía Grants CVI00260 and CVI 2658 (to J.L.G.S. and F.C.), Grant BFU2005-00025 from the Spanish Ministry of Education and Science (to M.E.A. and M.M.), the CONSOLIDER-INGENIO Programme Grant 25120 (to J.L.G.S., F.C., M.E.A., and M.M.). M.J.T. was supported by Fundação para a Ciência e Tecnologia, Portugal.