Supplementary figure legends EN-07-0425v3:

Figure S1: *PITX proteins regulate human FSHB transcription.*
HepG2 cells in 24-well plates were transfected with the indicated human *FSHB* promoter-reporters with expression vectors for either murine PITX1 or PITX2C. Control wells were transfected with empty expression vector (pcDNA3.0). Data were analyzed and presented as in Fig. 1D and show stimulation of human *FSHB* promoters of different lengths by PITX1 and PITX2C. For both PITX proteins, the greatest fold effects were observed with the shortest reporter tested, –126/+7 h*FSHB*-luc, whereas the intermediate length reporter, –486/+7 h*FSHB*-luc, showed the weakest responses.

Figure S2: *PITX proteins do not bind the putative PITX2C binding region between -233/-201 of the rat Fshb promoter*.
Gel shift assays were performed with a radio-labeled probe corresponding to –233/–201 of the rat *Fshb* promoter. The probe was incubated with CHO cell nuclear extracts containing over-expressed PITX1 (lanes 4-8), PITX2C (lanes 9-13), or pcDNA3.0 (1-3). Five hundred-fold molar excess of unlabelled -233/-201 rat *Fshb* (lane 2, 5, and 10) or –232/–200 murine *Fshb* (lanes 3, 6, and 11) were used as competitors. The analysis was performed with the same conditions described for Figs. 2A and 3 (upper panel) or with previously described methods (lower panel; Suszko *et al.,* 2003, *Mol. Endo*.). Both protocols produced comparable results, revealing the same three complexes (a, b, and c) whether using nuclear lysates from pcDNA3.0 or PITX transfected cells. Complexes were not super-shifted by PITX1 or PITX2 antibodies (lanes 8 and 13). Note the gel in the lower panel was run for a shorter time period than in the upper panel. It should also be noted that the rat and murine probes were similar in sequence with the exception of two base-pairs: one mismatch and one fewer bp in the rat sequence. Upon subsequent investigation, we discovered that rat probe described by Suszko *et al.* (2003) was missing a base-pair that actually exists in the rat promoter, and which is conserved in mouse. We confirmed the one base-pair mismatch between the two species. Nonetheless, we used the same rat *Fshb* probe sequence as described previously (Suszko *et al.*, 2003) and further showed here that the unlabeled murine probe was as effective as this rat probe in competing for binding to the radio-labeled rat –233/–201 probe.

Figure S3: *Mutation of lysine 50 in the PITX homeodomain inhibits DNA binding and confers dominant-negative activity*.
A and B) Western blots of nuclear extracts from transfected CHO cells confirmed equivalent protein expression levels of wild-type and K50 mutants of PITX1 (K139A) and PITX2C (K141A and K141E). **C)** Gel shift assay with radio-labeled -61/-40 murine *Fshb* probe and the nuclear extracts in A and B confirmed that the K50 mutants have greatly impaired DNA binding ability. **D)** To confirm that K50 mutants inhibit wild-type PITX2 function, we co-transfected HepG2 cells with –199/+1 m*Fshb*-luc along with wild-type PITX2C and K141A alone and together. PITX2C dose-dependently stimulated reporter activity, whereas K141A when transfected alone had no effect. When wild-type PITX2C was over-expressed at sub-saturating levels (100 ng/well), K141A dose-dependently inhibited its *trans*-activation function (compare three right-most bars with second bar). The PITX1-K139A mutant also inhibited wild-type PITX1 function in this system (data not shown).

Figure S4: *All the isoforms of PITX2 (PITX2A, PITX2B, and PITX2C) have similar effects on activin A-stimulated murine Fshb promoter activity.*
LβT2 cells were transfected with a murine *Fshb* promoter-reporter along with pcDNA3.0 or the indicated PITX2 isoforms and treated with 1 nM activin A for 24 h. Data are presented as described in Fig. 6A. PITX2A and 2C isoforms differed from each other but not from pCDNA3 or PITX2B (Tukey). The ligand x isoform interaction was not significant.

Figure S5: *Pitx siRNAs knockdown expression of endogenous and over-expressed PITX proteins.*
(A) LβT2 cells were transfected with 10 nM control, *Pitx1*, or *Pitx2* siRNA pools. Western blot analyses confirmed specific knockdown of PITX1 protein. We further assessed the efficacy of the siRNA pools on murine PITX2C **(B)** and PITX1 **(C)** using a heterologous assay system. CHO cells in 10-cm plates were co-transfected with PITX2C or PITX1 expression constructs (3 μg/plate) along with control, *Pitx1*, or *Pitx2* siRNA pools (10 nM). Western blots of extracts showed that the *Pitx2* siRNA pool could suppress expression of PITX2C (panel B, lane 2), but had only a minor effect on PITX1 expression (panel C, lane 3). The *Pitx1* siRNA pool similarly suppressed PITX1 expression (panel C, lane 2) with a negligible effect on PITX2C expression (panel B, lane 3). The control (Ctrl) siRNA did not affect PITX2C or PITX1 expression (panel B and C, lane 1).

Figure S6: *Pitx siRNAs are functionally specific*.
To show the functional specificity of the siRNA pools, CV-1 cells were seeded in 12-well plates and transfected with the -199/+1 m*Fshb*-luc reporter along with expression vectors (300 ng/well, black bars) for PITX2C **(A)** or PITX1 **(B)** and 10 nM control, *Pitx1* or *Pitx2* siRNAs. Control wells (white bars) were transfected with the empty expression vector (pcDNA3.0). The *Pitx2* siRNA pool completely blocked the effect of PITX2C on m*Fshb* reporter activity (panel A) but did not alter the PITX1 response (panel B). Unexpectedly, the *Pitx1* siRNA increased PITX1-mediated promoter activity (panel B, middle black bar), whereas the *Pitx2* siRNA pool had no effect. The inset in panel B shows that the *Pitx1* siRNA pool efficiently knocked down PITX1 protein levels in CV-1 cells as it did in LβT2 and CHO cells (see Fig. S5). EV and PV denote the transfection of empty (pcDNA3.0) and PITX1 expression vectors, respectively. We therefore hypothesized that PITX1 may more effectively stimulate the *Fshb* promoter at lower concentrations. By knocking down expression of exogenous PITX1 we could therefore increase reporter activity. To test this idea, cells were transfected with the -199/+1 m*Fshb*-luc reporter and 3-300 ng/well PITX2C **(C)** or PITX1 **(D)**. Whereas PITX2C dose-dependently stimulated reporter activity, PITX1 had an inverted U-shaped effect. By decreasing the amount of expression vector 10-fold (from 300 to 30 ng/well) we actually increased promoter activity, consistent with our hypothesis. These data confirm the functional efficacy of the *Pitx1* siRNA pool and at the same time highlight a heretofore unappreciated difference in PITX1 and PITX2 regulation of *Fshb*. In all panels, bars with different letters differed from one another (Tukey).

Figure S7: *PITX2C does not confer activin A responsiveness to the Fshb promoter in heterologous cells.*
A) HepG2 cells were transfected with the activin responsive reporter, 3TP-luc, with combinations of Smad3 (Sm3) and Smad4 (Sm4) expression constructs. Cells were treated the following day with 1 nM activin A for 24 h. Activin A stimulated reporter activity and this effect was potentiated by Smad3 alone and in combination with Smad4. These data confirm the activin A responsiveness and the ability of Smad3 to stimulate transcription in these cells. **B)** HepG2 cells were transfected as in panel A, except here PITX2C and -355/+1 m*Fshb*-luc were used in place of Smad4 and 3TP-luc. PITX2C stimulated transcription as described in Fig. 1B. Neither activin A nor Smad3 stimulated *Fshb* transcription (unlike what we observe in LβT2 cells), and Smad3 antagonized the PITX2C response. Statistics revealed no significant Smad3 x Smad4 x ligand (panel A) or Smad3 x PITX2C x ligand (panel B) interactions, so pair-wise comparisons of individual means could not be performed.

Figure S8: *PITX1 can hetero-dimerize with PITX2 isoforms.*
CHO cells were co-transfected with Flag-PITX1 and PITX2A or PITX2Cβ alone or in combination. Based on previous results (Fig. 2B), myc-PITX1 was included as a positive control. Whole cell lysates were IP using anti-Flag M2 affinity gel. Bound proteins were eluted, separated by SDS-PAGE, and immunoblotted with the indicated antibodies. The results show that PITX1 can hetero-dimerize with the PITX2A and PITX2Cβ.