Genomewide Expression Analysis in Zebrafish *mind bomb* Alleles with Pancreas Defects of Different Severity Identifies Putative Notch Responsive Genes

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Background. Notch signaling is an evolutionarily conserved developmental pathway. Zebrafish *mind bomb* (*mib*) mutants carry mutations on *mib* gene, which encodes a RING E3 ligase required for Notch activation via Delta/Jagged ubiquitylation and internalization. Methodology/Principal Findings. We examined the *mib* mutants for defects in pancreas development using *in situ* hybridization and GFP expression analysis of pancreas-specific GFP lines, carried out the global gene expression profile analysis of three different *mib* mutant alleles and validated the microarray data using real-time PCR and fluorescent double *in situ* hybridization. Our study showed that the *mib* mutants have diminished exocrine pancreas and this defect was most severe in *mib*<sup>v528</sup> followed by *mib*<sup>m172</sup> and then *mib*<sup>909</sup>, which is consistent with the compromised Notch activity found in corresponding *mib* mutant alleles. Global expression profile analysis of *mib* mutants showed that there is a significant difference in gene expression profile of wt and three *mib* mutant alleles. There are 91 differentially expressed genes that are common to all three *mib* alleles. Through detailed analysis of microarray data, we have identified several previously characterized genes and some putative Notch-responsive genes involved in pancreas development. Moreover, results from real-time PCR and fluorescent double *in situ* hybridization were largely consistent with microarray data. Conclusions/Significance. This study provides, for the first time, a global gene expression profile in *mib* mutants generating useful genomic resources and providing an opportunity to identify the function of novel genes involved in Notch signaling and Notch-regulated developmental processes.

INTRODUCTION
The Notch pathway is an evolutionarily conserved signal transduction cascade that plays essential roles in a variety of developmental processes, such as pattern formation, cell fate determination and organ formation through local cell-cell interactions (reviewed in [1–2]). Apart from being important for normal development, Notch signaling is also related to several human congenital diseases, such as T-cell acute lymphoblastic leukemia/lymphoma [3–5], Alagille syndrome [6–8], a late onset neurological disease (CADDASIL) [9], and spondylocoetal dysostosis [10].

Notch receptor functions as a membrane-bound transcription factor that turns on specific genes in response to physiological cues that trigger ligand binding. Upon functional binding of DSL (Delta, Serrate/Jagged and Lag-2) transmembrane ligands, the membrane-bound Notch is proteolyzed by TNF-α-converting enzyme (TACE), metalloproteinases [11] and Presenilin, and the active form-Notch intracellular domain (NICD)–is released [12,13]. The NICD is then translocated to the nucleus [14–17] and binds to the conserved CSL (CBF1/RBPjκ, Su(H) and Lag-1) DNA-binding protein [18,19], which is converted from a transcriptional repressor to activator. This triggers the expression of downstream target genes such as Hes/Her (hairy/Enhancer of split related) family of bHLH transcription factors, which in turn modulate the expression of downstream genes and themselves [16,20–26].

Several E3 ligases, such as Mind bomb (*Mib*), Su(dx), Sel-10, Neuralized, and Deltex, have been shown to modulate the Notch signaling through ubiquitin-dependent protein degradation and/or endocytosis [27,28]. *Mib* is a ubiquitin ligase that is required cell non-autonomously for Notch signaling and lateral inhibition by controlling Delta protein internalization [27]. Several mutants of zebrafish Notch signaling components, such as after eight (ae8)/deltaD, deadly seven (des)/notch1a, beamter (bea)/deltaC, and mind bomb (*mib*) have been isolated in a large scale screen [24,27,29–32]. In all these mutants, the anterior somites are formed normally but the posterior somites are irregularly formed [29,30,33]. The zebrafish *mib2* ortholog was recently cloned [34]. The zebrafish *Mib* and *Mib2* have common and specific Delta substrates [35] and function redundantly [34]. Three different alleles of *mib*, viz., *mib*<sup>v528</sup>, *mib*<sup>m172</sup>.

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and mb 3591 with descending severity of Notch-dependent phenotypes have been characterized in our laboratory [34].

Genetic studies in mice [36] and zebrafish [37-40] have shown that Notch signaling is involved in pancreas development. Mice deficient in Notch signaling components, Dll1, RBP-Jk and Hes1, have an increase of endocrine pancreatic cells and depletion of progenitor cells [36,41]. Roles of some of the Notch-related transcription factors, such as Ngn3 [42,43], Isl1 [44], MaFa [45], Pax4 and Pax6 [46,47], in pancreas development have been demonstrated. However, the role of differential regulation of Notch signaling on pancreas development is not well understood.

Microarray is a useful method for genomewide expression profile analysis [40]. With this approach, several downstream genes of signaling pathways involved in controlling mouse pancreas development have been reported [49,50]. Following the leads from such microarray analysis, a novel transcription factor, Myt1 [50] has been shown to be involved in pancreas development. Similarly, Meltzer et al. also showed that a novel transcription factor, IA1, is Ngn3-regulated and required for normal differentiation of endocrine pancreatic cells [51]. High-density microarrays have been established for zebrafish and transcriptome profiles during embryogenesis have been documented [52]. In addition, new target genes involved in sonic hedgehog signaling pathway has been identified in zebrafish using microarrays [53]. However, a genomewide analysis of Notch signaling defective mutants has not been reported.

Hence, we have carried out this study with three major objectives, viz. (1) to examine the three mb mutant alleles for pancreas defects and understand the consequence of differential activation of Notch signaling on pancreas development using in situ hybridization and pancreas-specific GFP expression analysis, (2) to identify differentially regulated genes between the wt zebrafish and three different mb mutant alleles through microarray analysis and (3) to identify and validate the Notch-responsive genes involved in pancreas development. Through our present work, we have shown that the development of exocrine pancreas is diminished in mb mutants and this defect is most severe in mb 132 and mb 3591. Using microarray analysis, we have identified several characteristic genes, and novel genes/ESTs potentially involved in pancreas development. Validation of representative genes with real-time PCR and fluorescent double in situ hybridization supported the microarray data. Furthermore, this study has generated useful genomic resources identifying a number of uncharacterized ESTs/genes that may play a significant role in Notch-regulated developmental process.

RESULTS
Pancreas defects in mb mutant alleles
We carried out in situ hybridization for four pancreas-specific genes in mb mutants of 4-day post-fertilization (dpf) and their wild type (wt) siblings (Figure 1). The expression of exocrine pancreas-specific genes, elastaseA (Figure 1A and 1A–1D) and trypsin (Figure 1E and 1E–1H) were diminished in mb mutants. In comparison to their expression in wt embryos, these two genes were highly down-regulated in mb 3592 followed by those in mb 132 and then mb 3591, which are less down-regulated. This observation is in concurrence with the diminished Notch activity in these mb mutants [34]. We also analyzed the elastaseA-GFP expression in 4-dpf mb mutants and their wt siblings (Figure 2A–2D). The elastaseA-GFP expression was diminished in mb mutant alleles in the same order of decreasing Notch activity in these mutants, which corroborates our observation shown by in situ hybridization (Figure 1A and 1A–1D).

We further analyzed the expression of three endocrine pancreas-specific genes, somatostatin (β-cell-specific), insulin (β-cell-specific), and pbs1 (pancreas progenitor-specific). Levels of somatostatin (Figure 1I and 1I–1L) and insulin (Figure 1M and 1M–1P) were diminished in 4-dpf mb mutants. The insulin-GFP (Figure 2E and 2E–2H) and pbs1-GFP (Figure 2I and 2I–2L) expression analysis in mb mutants also showed that these genes are slightly up-regulated compared to their expression in wt embryos.

Genomewide expression profiling
We carried out the genomewide expression profiling of mb mutants at three different stages–24-, 48- and 72-hour post-fertilization (hpf). Through microarray analysis of 72-hpf embryos, we identified 1128 up-regulated and 936 down-regulated genes in mb 3592 mutants (Table S1, q = 0.0); 1464 up-regulated and 2210 down-regulated genes in mb 132 mutants (Table S2, q = 0.0); and 2081 up-regulated and 2538 down-regulated genes in mb 3591 mutants (Table S3, q = 0.0). Using a PERL script, we further identified the differentially expressed genes specific to each mutant allele and common to all three mb mutant alleles (Figure 3). In the list of up-regulated genes, the numbers of genes specific to mb 3592, mb 132 and mb 3591 mutant alleles were 93, 287 and 768, respectively; in the list of down-regulated genes, the numbers of genes specific to mb 3592, mb 132 and mb 3591 alleles were 33, 557 and 874, respectively (Figure 3A and 3B, Table S5, S6 and S7, q = 0.0 and score(d)>4.0). The majority of these differentially expressed genes were uncharacterized genes or ESTs (Table 1). There were 91 genes common to all three mb
mutant alleles, of which 31 were up-regulated and 60 were down-regulated (Figure 3A and 3B, Table 1). Of these 91 genes, only 27 genes were previously characterized and 64 were uncharacterized genes or ESTs (Table 1, Table S4, \( q = 0.0 \) and score(d) > 4.0). The cluster tree view showed the expression profile of these 91 genes: the up-regulated ones were shown in red and the down-regulated ones in green (Figure 4). We further categorized these 91 common genes according to their known function or predicted function based on their homology to mouse and human orthologs (Table S4). Of the 27 characterized genes, there are 6 up-regulated genes, including dab2, melta, meltb, fn1l, ttn and nppa, and 21 down-regulated genes, including pbs3b, gtm6aa (BI840762), olig2, tfhlp2, tfl13, gtm6aa (BI839927), atp1a1b, fabp7a, gtm6aa (BG306150), fshp5, gfp, opt1sw1, opt1sw2, opt1sw1, sxy1, pou30, mibta, tal1, dta, smp2 and her4.

Using the same PERL script, we analyzed the 48-hpf time point data for three \( \text{mib} \) mutant alleles, and data of 24-hpf, 48-hpf and 72-hpf time points for the \( \text{mib}^{952b} \) mutant allele. Numbers of differentially expressed genes common to all three mutant alleles or three time points, and specific to each mutant allele or time point are shown in the Venn diagram (Figure 3C–3F). At 48 hpf, there are 44 (30 up-regulated and 14 down-regulated) differentially expressed genes common to all three mutant alleles, and 129 (54 up-regulated and 75 down-regulated), 124 (44 up-regulated and 80 down-regulated) and 693 (635 up-regulated and 58 down-regulated) genes specific to \( \text{mib}^{952b}, \text{mib}^{952a} \) and \( \text{mib}^{1091} \) mutant alleles, respectively (Figure 3C and 3D, Table S8, S9, S10 and S11, \( q = 0.0 \)). For \( \text{mib}^{952b} \) mutants, there are 61 (32 up-regulated and 29 down-regulated) genes common to all three time points, and 1821 (1128 up-regulated and 693 down-regulated), 49 (14 up-regulated and 35 down-regulated) and 1851 (930 up-regulated and 921 down-regulated) genes specific at 24 hpf, 48 hpf and 72 hpf, respectively (Figure 3E and 3F; Table S12, S13, S14 and S15, \( q = 0.0 \)).

**Functional categories**

We used the microarray data of three \( \text{mib} \) mutant alleles (\( q = 0.0, \) score(d) > 4.0) and the 91 common genes at 72 hpf for functional analysis. We first searched for the gene ontology and the functional similarity of their human and mouse orthologs in the Zebrafish Chip Annotation Database. Then, we classified them into different functional categories based on their known functions in zebrafish or in mouse and human orthologs, such as ‘transcription factor/

**Table 1. Differentially expressed genes in 72-hpf \( \text{mib} \) mutants categorized based on their characterization status.**

| Gene set          | Characterized genes | Uncharacterized genes/ESTs |
|-------------------|---------------------|---------------------------|
|                   | Up-regulated        | Down-regulated | Up-regulated | Down-regulated |
| \( \text{mib}^{952b} \) | 51                  | 48            | 125         | 97           |
| \( \text{mib}^{952a} \) | 72                  | 380           | 452         | 632          |
| \( \text{mib}^{1091} \) | 165                 | 426           | 868         | 893          |

Genes sets for the analysis were selected based on the criteria: \( q = 0.0, \) score(d) > 4.0.

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nuclear’, ‘signaling’, ‘cell adhesion/matrix’, ‘cell cycle/apoptosis’, ‘transcription factors’, ‘hormone activity’ and ‘structural proteins’. Finally, we plotted them in a pie chart format either including the uncharacterized genes and ESTs (Figure 5A–5D) or excluding them (Figure 5E–5H). The majority (>70%) of the differentially expressed genes are uncharacterized genes or ESTs (Figure 5A–5D). Among the genes with known or related functions in the mib2302 data set, about 35% belong to the category of ‘transcription factor/nuclear’ related genes and the remaining genes to other categories, such as ‘transport proteins’ (25%), ‘signaling’ (19%), ‘structural proteins’ (8.6%), ‘cell adhesion/matrix’ (3.4%), ‘hormone activity’ (4.3%) and ‘cell cycle/apoptosis’ (3.2%) (Figure 5E).

Functional groups and pathway analysis by IPA

To further analyze our data set, we used IPA (see Materials and Methods) to identify functional groups and selected the top 15 ones as significantly enriched for their respective functions (Figure 6A). The significance of each function (calculated from negative log of p-value; -log 1.3 is equal to p=0.05) revealed that all the top 15 functions are highly and significantly enriched in all three mib alleles. Some of the significantly enriched functions are ‘cellular growth and proliferation’, ‘cellular development’, ‘gene expression’ and ‘embryonic development’. Genes related to ‘connective tissue disorders’, ‘skeletal muscular disorders’ and ‘cancer’ are significantly enriched in the mib2302 allele.

One of the tools in IPA enables us to identify the enrichment of genes in selected canonical pathways from the input data set. The top 15 canonical pathways that are enriched in the mib mutants include Notch and Wnt signaling pathways (Figure 6B). Genes related to Wnt signaling were significantly enriched in mib2302 and mib2091 mutants. Notch signaling genes were enriched to a lesser extent and their enrichment was not significant. Canonical pathways, such as ‘actin cytoskeleton signaling’, ‘integrin signaling’, ‘calcium signaling’ and ‘TGF-beta signaling’, were enriched in mib2302 and mib2091.

Notch and Wnt/β-catenin signaling were shown to be affected in mib mutants [27,54,55]. We mapped the genes that are involved in these two signaling pathways and differentially expressed in different mib alleles. The genes involved in Notch signaling, such as dll1, notch1, notch2, hey1 and herp, were down-regulated in one, two or three mib alleles (Figure S1A). In Wnt/β-catenin signaling, some genes, such as nlk, cdx3, sox2, sox3, sox4, sox9, sox11, tf7 and hba1, were down-regulated in one or three mib alleles; while sox2 and tab1 were up-regulated (Figure S1B). Few of the genes differed in the direction of expression change in different mutant alleles. For example, β-catenin was up-regulated in mib2302 while it was down-regulated in mib2302 and mib2091 (Figure S1B).

Putative Notch signaling related genes

As positive controls of our microarray data, we searched for the known Notch signaling related genes in our list of differentially expressed genes. As expected, heyL was up-regulated and dla, her4, herba, hes3, hey1, neurod, neurod4, notch1a, notch2 and notch3 were down-regulated (Table 2). To identify novel genes related to Notch signaling, we searched for the differentially expressed genes occurring at least two times in the gene sets common to all three mib mutant alleles at 72 hpf and 48 hpf, and the gene set common for the mib2302 mutant allele at 72 hpf, 48 hpf and 24 hpf. We identified 31 such genes, the majority of which are novel genes or ESTs (Table S16). Of these 31 genes, only eight genes are characterized and five of them are known to be directly related to Notch signaling: olg2 [56], her4 [57], hes5 [also known as her15.1, [58,59]], dla [60] and nort [61]. BI886648 is 98% identical to AB259590. Moreover, fn1l (also known as fn1b or fn3), in conjunction with its homolog, fn1a, has been shown to function cooperatively with Notch signaling via integrin3 in somitogenesis [62,63]. glap and gepfinal are glia- and neuron-specific structural...
proteins respectively and, therefore, indirectly linked to Notch-modulated gliogenesis and neurogenesis [64–66]. These observations suggest that a certain proportion of the 23 novel genes/ESTs are very likely to be involved in or related to Notch signaling.

Putative pancreas development related genes

We searched the gene expression data in the ZFIN database to identify the genes involved in pancreas development (Table 3, Table 4, and Table S17). There are 175 zebrafish genes in this group; however, only 98 of these genes are represented in our microarray chip. 25 of these 98 genes show significant difference (q<2.0) in gene expression in all three mib alleles: cx43, neurod, ins, fabp2, caco1, zgc:92530, pax6b, try, alfmu2, m2, xdp1, ksd11b2, zgc:92292, gadd45a, wu:fb59c09, ceb, tdb, gatp1, srd4, gata, rino2, atp1a3a, isl1, hoxc8a and notch1a (Table 3). The rest are differentially expressed either in one or two of these three alleles (Table S17). In addition, we identified 5 genes, namely gad1 [67], igfbp2 [68], zgc:112198 [69], wu:fc21h08 [70] and isl3 [71] based on the available functional analysis reports for their mouse orthologs, and 4 other genes, namely sfrs5, zgc:56374, AW281193 and BI846588 based on their gene ontology information available in the zebrafish gene annotation database (Table 4). These 9 genes are likely to play a role in zebrafish pancreas development.

Figure 5. Pictorial representation of significantly up-regulated or down-regulated genes (q = 0.0, score(d)>4.0) categorized based on known/related biological functions as assigned in the zebrafish gene ontology database (Unigene Build 85). Genes in each functional group were searched by using specific or related key words appropriate for that function in the lists of differentially regulated genes for each allele, common 91 genes in all three mib mutant alleles, and the genes specific to each mutant allele. Absolute number of genes in each category is shown next to the corresponding pie sector. (A–D) Pie charts show numbers of genes belonging to different functional categories. (E–H) Pie charts show numbers of genes (excluding the genes without any known function) in different categories. doi:10.1371/journal.pone.0001479.g005

Figure 6. Significantly up-regulated and down-regulated genes analyzed by IPA (see Materials and Methods) for (A) enrichment of functional groups and (B) enrichment of canonical pathways, shown in histograms. doi:10.1371/journal.pone.0001479.g006
Fluorescent double *in situ* hybridization validation of pancreas development related genes

To validate the microarray data for the genes predicted to be related to or involved in pancreas development, we carried out fluorescent double *in situ* hybridization at 3-dpf embryos for five down-regulated genes: *tpsin*, *isl1*, *cad*, *wu:fb59c09* and *notch1a*, and five up-regulated genes: *insulin*, *isl3*, *spon1b*, *glo1* and *tnap* (Table 3, Table 4 and Table S17). Exocrine *tpsin* and endocrine *insulin* are predictably down- and up-regulated, respectively (Figure 7). However, *notch1a*, *glo1* and *tnap* were not detected in the pancreas (see Discussion).

**Down-regulated genes** *isl1* encodes a zebrafish insulin gene enhancer binding protein and it has 81% identity to mouse LIM/homeodomain (Islet-1) and ISL2 transcription factor, *Hairy/enhancer-of-split related with YRPW motif* 1. It has high similarity to human ISL1 transcription factor, LIM/homeodomain (Islet-2). It has 81% identity to mouse LIM/homeodomain (Islet-3). *isl1* was expressed in organs such as nervous system, liver and pancreas at various developmental stages of zebrafish [58,72–77]. Its expression was strongly diminished in the nervous system (data not shown) of *mib* mutants; however, its expression was only slightly reduced in the endocrine pancreas compared to wt embryos (Figure 7A and 7B). Our microarray results also showed that *isl1* is down-regulated in *mib* mutants (Table 3).

*cad* encodes zebrafish carbamoyl-phosphate synthetase 2. Microarray analysis showed that *cad* is down-regulated (Table S17). *cad* was expressed in endodermal organs, such as pancreas, intestine and liver, which is similar to that observed in earlier reports [78,79]. In *mib* mutants, *cad* expression was down-regulated in all these tissues (Figure 7C and 7D; data not shown).

*wu:fb59c09* is a zebrafish EST with significant similarity to a hypothetical gene, LOC570477. The human ortholog of this gene is *Hairy-related* 8, which is involved in pancreas development. **Up-regulated genes** *spon1b* encodes an extracellular matrix protein, whose C-terminal contains five repeats identified previously in Thrombospondin and other proteins implicating in cell adhesion. Fluorescent double *in situ* hybridization results showed that this gene is expressed in endocrine pancreas, similar to earlier studies [77,79], and was up-regulated in *mib* mutants compared to that in their wt siblings (Figure 7G and 7H).

**DISCUSSION**

*mib* mutants have compromised Notch activity due to mutations in the *mb* gene and serve as a unique resource to study the role of Notch signaling on various developmental processes [27,29]. Moreover, there are several *mib* alleles with different
### Down-regulated genes

| Genbank ID | Gene symbol | Unigene Name | mib<sup>ta52b</sup> | mib<sup>m132</sup> | mib<sup>tfi91</sup> | Average fold change |
|------------|-------------|--------------|----------------------|-------------------|-------------------|-------------------|
| AF035481   | cx43        | Gap junction protein, alpha 1 | 0.7771              | 0.5925            | 0.8182            | 0.7293            |
| AF036148   | neurod      | Neurogenic differentiation | 0.4846              | 0.5223            | 0.7155            | 0.5741            |
| AF180921   | fabp2       | Fatty acid binding protein 2, intestinal | 0.6514              | 0.7622            | 0.5752            | 0.6630            |
| A1601297   | pax6b       | Paired box gene 6b | 0.7366              | 0.7788            | 0.8862            | 0.8005            |
| A297822    | try         | Trypsin       | 0.8176              | 0.8766            | 0.8712            | 0.8551            |
| AW115690   | olfm2       | Olfactomedin 2 | 0.6421              | 0.5667            | 0.8625            | 0.6904            |
| BG882996   | hsdl1b2     | Hydroxysteroid 11-beta dehydrogenase 2 | 0.8263              | 0.9139            | 0.7976            | 0.8400            |
| BI996418   | gatm        | Glycine amidinotransferase | 0.6641              | 0.8305            | 0.6792            | 0.7246            |
| B981058    | rifs2       | Regulating synaptic membrane exocytosis 2 | 0.5887              | 0.7021            | 0.9263            | 0.7390            |
| BM183338   | atp1a3a     | ATPase, Na<sup>+</sup>/K<sup>-</sup> transporting, alpha 3a polypeptide | 0.7161              | 0.7431            | 0.8743            | 0.7778            |
| D21135     | isl1        | Islet1        | 0.4843              | 0.8373            | 0.7885            | 0.7033            |
| YI4544     | hoxc8a      | Homeo box C8a | 0.8644              | 0.5562            | 0.7260            | 0.7155            |
| A497360    | zgc:92530   | Zgc:92530     | 1.1962              | 0.8602            | 0.8983            | 0.9849            |
| AW181487   | mt2         | Metallothionein 2 | 1.3597              | 0.7799            | 0.8513            | 0.9970            |
| B1709417   | wufb59c09   | Wufb59c09     | 1.2429              | 0.6583            | 0.8936            | 0.9316            |
| B1882972   | gstp1       | Glutathione S-transferase pi | 0.8352              | 0.7378            | 1.1683            | 0.9138            |
| B1885968   | ss4         | Signal sequence receptor, delta | 1.2889              | 0.4576            | 0.7009            | 0.8158            |
| X69088     | notch1a     | Notch homolog 1a | 0.8032              | 0.5803            | 0.7670            | 0.7168            |

### Up-regulated genes

| Genbank ID | Gene symbol | Unigene Name | mib<sup>ta52b</sup> | mib<sup>m132</sup> | mib<sup>tfi91</sup> | Average fold change |
|------------|-------------|--------------|----------------------|-------------------|-------------------|-------------------|
| AF305689   | casca1c     | Calcium channel, voltage-dependent, L type, alpha 1C subunit | 1.1653              | 1.1483            | 1.0988            | 1.1375            |
| BI672019   | gadd45a     | Growth arrest and DNA-damage-inducible, alpha | 1.1878              | 1.1591            | 1.0964            | 1.1478            |
| BI879550   | cbs         | Cystathionine-beta-synthase | 1.6677              | 1.3112            | 1.2058            | 1.3949            |
| AF036326   | ins         | Preproinsulin | 1.3530              | 0.8345            | 0.8377            | 1.0084            |
| AI029577   | xbp1        | X-box binding protein 1 | 1.3598              | 0.6465            | 1.1352            | 1.0471            |
| BI476180   | zgc:92292   | Zgc:92292    | 0.8291              | 1.1458            | 1.1390            | 1.0380            |
| B1882450   | tdh         | L-threonine dehydrogenase | 1.8600              | 1.0871            | 0.8719            | 1.2730            |

The in situ hybridization results for the endocrine pancreas-specific genes, such as insulin and somatostatin, showed slight increase in their expression in mib mutant alleles; however, a dose-dependent response of Notch signaling was not obvious (Figure 1). The GFP expression analyses in insulin-GFP and pdx1-GFP lines also showed a slight increase in endocrine pancreas in mib mutant alleles (Figure 2). Similar to our results, Zecchin et al. also showed that in mib mutants (mib<sup>ta52b</sup>) there is an increased expression of insulin and somatostatin [40].

### Microarray analysis in mib alleles

In this study, we have used the microarray chips with 16,416 probes, representing 15,800 unique zebrafish genes. Earlier studies have successfully used this version of microarray chips for elucidating genes involved in zebrafish embryogenesis [52], liver tumor progression [81] and Hedgehog signaling [53]. Comparison of our microarray results for three mib mutant alleles at 72 hpf showed that the number of differentially expressed genes (q = 0.0, score(d)>4.0) in mib<sup>tfi91</sup> allele (2352) is greater than that in mib<sup>m132</sup> (1536) and mib<sup>ta52b</sup> (321) alleles (Figure 3A and 3B). Interestingly, this is inversely related to the severity of their phenotypes, such as fused somite borders, diminished tail pigmentation, neuronal genetic severity available [34]. In this study, we have examined the mib mutants for defects in pancreas development using in situ hybridization and pancreas-specific GFP expression analyses, and compared the global expression profile of three mib mutant alleles and their wt siblings using oligo microarray chips.

**mib alleles are unique for studying Notch signaling**

The mib mutants have diminished exocrine pancreas development as evidenced by decrease in elastaseA and trypsin expression. The mib<sup>ta52b</sup> mutant allele showed maximal decrease in exocrine pancreas followed by mib<sup>m132</sup> and mib<sup>tfi91</sup> alleles. This indicates that there is a dose-dependent response of Notch signaling on pancreas development, since these alleles have different degree of compromised Notch activity [34]. Our GFP expression analysis in elastaseA-GFP lines also showed a similar effect of graded Notch signaling on pancreas development. Likewise, based on in situ hybridization results for exocrine pancreas-specific genes, such as mm2a, ptf1a and trypsin in mib<sup>ta52b</sup> mutants and DAPT-treated mib<sup>ta52b</sup> mutants, Zecchin et al. concluded that a blockage of Notch signaling decreases the number of exocrine pancreatic cells [40]. Earlier study by Ensi et al. also showed the role of Notch signaling in exocrine pancreas development of zebrafish [37].
hyperplasia and diminished her4 expression; most severe in mibm132 allele followed by those in mibm132 and mibm01 alleles [34]. So far, there is no known molecular mechanism to explain this finding. Future studies need to be focused in this direction.

We observed fewer differentially expressed genes at 48 hpf compared to that at 72 hpf for all three mib mutant alleles. This is in concurrence with the phenotypes of these three mib alleles, which are less obvious at 48 hpf compared to that at 72 hpf [34]. Owing to the large number of differentially expressed genes at these two time points, we narrowed our focus on the 72-hpf data set alone. In all three mib mutant alleles at 72 hpf, the majority (>70%) of the differentially expressed genes are uncharacterized genes or ESTs. This is because the zebrafish genome annotation has not been completed and the majority of genes remain as uncharacterized ESTs. Though a number of differentially expressed genes have been identified as full-length clones with ZGC IDs, they still remain unannotated. Gene Ontology (GO) analysis of differentially expressed genes of mibm132 allele showed that less than 29% of the genes are characterized, the majority (35%) of which belong to the category of 'transcription factors/ nuclear' followed by the category of 'signaling molecules' (19%). This could be due to the fact that the mib mutants are defective in Notch signaling, which is one of the fundamental signaling pathways required for proper development of an organism.

Genes potentially involved in Notch signaling

Our microarray data is reliable, because some expected Notch signaling related genes did appear in the transcriptome, such as up-regulated hydL and down-regulated dlla. Comparison of three sets of genes (72 hpf and 48 hpf for three mib alleles and 24 hpf, 48 hpf and 72 hpf of the mibm132 allele) showed that there are 31 genes common to all three gene sets (Table S16). Five of these 31 genes, namely olig2 [56], her4 [57], her5 (also known as her5.1, [58,59]), dlla [60] and nort [61], are previously shown to be involved in Notch signaling. Particularly, based on similar global expression analyses of notch1a and notch3 morpholino morphants, Tsutsui and Itoh showed that nort is a putative noncoding RNA regulated by Notch signaling in zebrafish [61]. Therefore, it is likely that the rest of the genes in this group are a part of the Notch connected network and hence serve as a useful resource to further identify novel genes working downstream of Notch.

Genes potentially involved in pancreas development

As a major focus of our current study, we searched for the genes related to pancreas development. As of now, there is no bioinformatic tool available to classify the zebrafish genes according to their biological functions. Therefore, we carried out a detailed manual search for the genes related to pancreas development. Using the ZFIN in situ hybridization expression database, we found 98 zebrafish genes that have been previously shown to be expressed in pancreas (Table 3, Table 4 and Table S17). The microarray expression profile for these genes showed that their expression profile is significantly different (q<2.0) in at least one of the three mib alleles. Several genes, such as neural, isl3 and pax6b [82–84] were formerly shown to be responsive to Notch signaling in mice.

However, the fold change of expression for each gene is different in these three alleles. Only 25 out of these 98 zebrafish genes involved in pancreas development show significant difference (q<2.0) in expression profile in all three mib alleles. Out of these 25 genes, only 15 genes show consistent up-regulation (3 genes) or down-regulation (12 genes) in all three alleles. Apart from these 98 genes, we discovered 9 differentially expressed genes (Table S19), which are likely to play a role in zebrafish pancreas development based on their functional homology to mouse and human orthologs. It is definitely worth addressing in the future.

Is Mib Notch-specific?

So far, the experiments have unequivocally proved that Mib is an essential component of Notch signaling: it ubiquititates and then endocytoses Delta with the extracellular part of Notch and therefore allows the intracellular part of Notch to enter the nucleus in the receiving cell and activate downstream target genes [27]. There is also evidence to suggest that Mib may be linked to Wnt signaling. Riley, et al. showed that heat shock-driven wnt1 expression in mib mutants leads to a partial rescue of its hindbrain metameric
patterning phenotype [54]. Furthermore, knockdown of wnt3a and wnt6b in Df(3L)st5 mutants, where wnt1 and wnt10b are deleted, resulted in the loss of boundary cells in hindbrain, which is similar to that in mib<sup>−/−</sup> mutants [54]. However, this is in sharp contrast to wnt1 and tef3b morphants, where the boundary cells are increased [55]. Therefore, the mechanism remains to be determined.

From our IPA pathway analysis in all three mib alleles, we found enrichment of several differentially expressed genes belonging to various canonical pathways that have not been shown to directly link to Mib, including IGF-1, PDGF, EGF and IL-2 signaling pathways (Figure 6B). This raises the possibility that Mib may be somehow involved in these signaling pathways. Furthermore, mouse DAPK and zebrafish Jagged2a have been shown to be substrates of Mib E3 ligase [85,86]. With a yeast two-hybrid screen, we also found that Mib binds to proteins involved in endocytosis and the ubiquitin-proteasome pathway (Chengjin Zhang, Jason Kin Wai Koo, Qing Li, Haoying Xu and Y.-J. J., unpublished data). Similarly, Snx5 has been identified as a Mib-binding protein from a yeast two-hybrid screen, which is colocalized with Mib in early endosomes and required for hematopoiesis and vasculogenesis [87]. All these observations suggest that Mib may not be Notch-specific and can also work with other pathways, just as previously shown for another E3 ligase, Icch, which targets Notch receptor and links to TNF through JNK [88-90]. Alternatively, the gene expression change could simply reflect the tissue/organ defects that are inflicted by a failure in Notch signaling. Our pathway analysis on microarray data supplies a good resource for examining whether Mib functions beyond Notch signaling and/or for testing what genes are involved in the tissue/organ mis-patterning caused by compromised Notch activity.

**Validation of microarray data**

To validate the microarray expression profile, we carried out fluorescent double in situ hybridization for ten genes (5 down-regulated and 5 up-regulated) and real-time PCR for 25 genes. Out of these ten genes, insulin, tryptic, spon1b, cad, isl1, wu:fb59c09, notch1a, tsp1p and glo1 are previously shown to be involved in the development of and/or expressed in zebrafish pancreas; and isl3 is predicted to be related to pancreas development.

The microarray expression profile of tryptic and isl1 showed a down-regulation in mib<sup>−/−</sup> allele and this was validated by our fluorescent double in situ hybridization and real-time PCR (Figure 7 and Table 5), wu:fb59c09 was slightly up-regulated (1.24) in microarrays of mib<sup>−/−</sup> allele but it was down-regulated in mib<sup>−/−</sup> and mib<sup>−/−</sup> alleles (Table 3). However, our fluorescent double in situ hybridization and real-time PCR validations showed that it is down-regulated in the mib<sup>−/−</sup> allele (Figure 7 and Table 5). Notch expression was decreased in the mib<sup>−/−</sup> allele by double in situ, which is consistent with the microarray data (Figure 7 and Table S17). Earlier studies have shown that all these four genes are expressed in zebrafish pancreas at various developmental stages [37,75,78,79]. However, the role of Notch signaling on their expression profile in pancreas is not known except isl1. isl1 was shown to be up-regulated in primary neurons of mib mutants at 16s to 20s stage [91], but the effect of Notch signaling on its expression at later stages has not been studied. Furthermore, no observation has been made on its expression in pancreas. In contrast, our in situ hybridization results in 3-dpf embryos showed that the expression of isl1 in mib<sup>−/−</sup> allele is down-regulated in nervous system but its expression in pancreas is only slightly reduced compared to that in wt embryos. In support of this observation, our microarray and real-time PCR results also showed that isl1 is down-regulated in mib<sup>−/−</sup> mutants (Table 3 and 5).

Two up-regulated genes, insulin and spon1b, were validated. The microarray data showed that these two genes are up-regulated in the mib<sup>−/−</sup> allele and this is supported by our fluorescent double in situ hybridization. isl3 was slightly increased by fluorescent double in situ hybridization. However, it was consistently decreased in mib<sup>−/−</sup> allele by microarray analysis and real-time PCR (Figure 7, Table 4 and 5).

In contrast to the available information from ZFIN, which shows the gene expression pattern mainly up to 2 dpf, we did not detect notch1a, glo1 and tsp1p expression in the pancreas using fluorescent double in situ hybridization at 3 dpf, though we did detect notch1a expressed in, for example, hindbrain. It could be due to the stage difference or technical reasons. However, our real-time PCR results validated microarray expression for notch1a and glo1 (Table 5).

The ratio comparison between microarray and real-time PCR for genes, such as igf1, pep, p2, isl3, spon1b and BL246388, showed that there is slight up-regulation in microarray, but down-regulation in real-time PCR. However, it is evident that the overall expression alteration of all 25 genes is statistically
comparable, although the actual fold change values in real-time PCR and microarray are not always commensurable. Such variation in values is likely due to the difference in sensitivity [80]. Nevertheless, our in situ hybridization results are highly consistent with our microarray profile.

In conclusion, the microarray analyses carried out in this study provide a useful resource of global gene expression profile of mib mutants defective in Notch signaling. Functional analysis of differentially expressed genes will shed light on their role in Notch signaling and various developmental processes.

**MATERIALS AND METHODS**

**Zebrafish wild type embryos and mind bomb mutants**

We used AB strain wild type (wt) and three different mib alleles of different genetic severity, viz., mib<sup>ta52b</sup>, mib<sup>m132</sup> and mib<sup>tfi91</sup> [34]. mib<sup>ta52b</sup> carries a missense mutation (M1013R) in the C-terminal-most RING finger domain; mib<sup>m132</sup> carries a nonsense mutation (C785stop) leading to a truncated protein and mib<sup>tfi91</sup> contains a nonsense mutation (Y60stop) [27]. mib<sup>ta52b</sup> and mib<sup>m132</sup> are strong and weak antimorphic alleles, respectively, whereas mib<sup>tfi91</sup> is a null allele [34]. All animal procedures were approved by the Biological Resource Centre, A*STAR.

**Fish maintenance and sample collection**

Fish were maintained in the IMCB zebrafish facility according to standard procedures. Crosses were set up in the evening and the barrier was lifted in the next morning. After half an hour, the fertilized embryos were collected and maintained at 28.5°C in egg water supplemented with methylene blue. For microarray analyses, the wt embryos were collected at 24 hpf, 48 hpf and 72 hpf, snap-frozen in liquid nitrogen and stored at -80°C. Mutants were separated from their wt siblings and frozen stored in the same way. At least two independent biological replicates were taken for each sample. All the samples were collected from the same cohort of fish to maintain a uniform genetic background.

### Table 5. Real-time PCR validation of microarray data.

**A. Down-regulated genes**

| Genbank ID | Unigene Name | Gene symbol | Microarray fold change ratio | Real-time PCR ratio |
|------------|--------------|-------------|-----------------------------|---------------------|
| AJ297822   | Trypsin      | try         | 0.81                        | 0.18                |
| D21135     | Islet1       | isl1        | 0.48                        | 0.17                |
| X97332     | Hairy-related 4 | her4    | 0.35                        | 0.04                |
| U57975     | Notch homolog 3 | notc3   | 0.38                        | 0.12                |
| A522447    | DeltaA       | dla         | 0.45                        | 0.22                |
| X69088     | Notch homolog 1a | notc1a | 0.80                        | 0.22                |
| AF109373   | Ospin 1 (cone pigments), short-wave-sensitive 1 | opn1sw1 | 0.14                        | 0.004               |
| AF017266   | Glutamate decarboxylase 1 | gad1       | 0.65                        | 0.25                |
| BI891976   | Zgc56374     | zgc56374    | 0.86                        | 1.15                |
| AY583322   | elastase A   |             | 0.89                        | 0.07                |
| AW019843   | Somatostatin 1 | sst1       | 0.62                        | 0.13                |
| AW26071    | Zgc112198    | zgc112198   | 0.59                        | 0.10                |
| AW281193   | similar to rat insulinoma gene |         | 0.93                        | 0.49                |
| BI887742   | Splicing factor, arginine-serine-rich 5 | sfrs5     | 0.97                        | 0.87                |
| AF198033   | Insulin-like growth factor binding protein 2 | igf2b2   | 0.99                        | 0.13                |
| BI709417   | Wufkb59c99 (Danio rerio similar to Peroxiredoxin 4) | pxn4     | 0.93                        | 0.88                |

**B. Up-regulated genes**

| Genbank ID | Unigene Name | Gene symbol | Microarray fold change ratio | Real-time PCR ratio |
|------------|--------------|-------------|-----------------------------|---------------------|
| AB062116   | Heat shock cognate 70-kd protein | hsp70 | 5.14                        | 11.55               |
| AF036326   | Preproinsulin | ins         | 1.35                        | 1.22                |
| BM18964    | Glyoxalase 1 | glo1        | 1.40                        | 1.08                |
| AF036325   | Insulin promoter factor 1, homeodomain transcription factor | ipf1 | 1.18                        | 0.20                |
| BI890045   | PERP, TP53 apoptosis effector | perp | 1.66                        | 0.70                |
| BI846588   | Transcribed locus, weakly similar to insulin-like growth factor-dependent IGF binding protein-4 protease |       | 2.02                        | 0.48                |
| BI533195   | Wufk210h08 (Danio rerio insulin-like growth factor 2 precursor) | igf2 | 1.35                        | 0.86                |
| D38454     | Islet3       | isl3        | 1.12                        | 0.19                |
| AB006087   | Spondin 1b   | spn1b       | 1.28                        | 0.36                |

The real-time PCR ratio in mib<sup>ta52b</sup> allele at 72 hpf is compared with the microarray data expressed as fold change values. The Genbank ID, Unigene name and Gene symbol were obtained using the Zebrafish Chip Annotation Database, (Unigene Build 85), http://giscompute.gis.a-star.edu.sg/~govind/zebrafish/version2/.

* Average microarray ratio of three mib mutants.

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which inhibits pigmentation, after 12 hpf and fixed/colllected at appropriate stages.

**In situ hybridization and fluorescent double in situ hybridization**

DNA clones for making in situ hybridization probes were obtained from the Expressed Sequence Tag (EST) clone collection at the Genome Institute of Singapore (GIS) and the Institute of Molecular and Cell Biology (IMCB). Whole mount in situ hybridization using digoxigenin (DIG) (Roche) labeled RNA probes was carried out as previously reported [92]. Goat anti-DIG antibody conjugated to alkaline phosphate (AP) was used for probe detection and NBT-BCIP was used as the substrate for color development. In situ hybridized embryos were observed using light microscope and the photos were taken with Zeiss Imagor M1 microscope.

DIG- and fluorescein-labeled probes were generated via standard protocols. Embryos were proceeded for fluorescent double in situ hybridization with protocols previously reported [32,86]. However, the incubation temperature for probes was changed to 60°C to reduce the background. Photos were taken with Olympus Fluoview FV1000 microscope.

**GFP expression analysis**

To screen for defects in pancreas development, we have used three pancreas-specific GFP transgenic lines, viz., elastase4-GFP (elast-GFP) [93], insulin-GFP and pdx1-GFP lines [94]. These GFP lines were crossed with the heterozygous mib mutants and the offspring (F1) were grown until maturity. The F1 siblings carrying mib mutations were intercrossed to obtain embryos for GFP expression analysis. Photos were taken with Leica MZ FLIII microscope.

**RNA extraction**

Total RNA from the frozen embryos was extracted with Trizol (Gibco BRL) and cleaned with the Qiagen RNeasy mini kit. RNA quality was determined by gel electrophoresis, and the concentration was measured with a UV spectrophotometer. To reduce the bias, we used a common reference RNA for each time point, which was prepared at one time by extracting RNA from stage-matched wild type embryos. The RNA extracts were stored at −80°C.

**Microarray construction, target preparation and hybridization**

The zebrafish microarrays were printed at GIS [32]. Oligonucleotide probes for this array were designed by Compugen (USA) and synthesized by Sigma Genesis (USA). For each gene, one 65-mer oligonucleotide probe was designed from the 3′ region sequence of the gene. Each probe was selected from a sequence segment that is common to a maximum number of splice variants predicted for each gene. The arrays contained 16,416 probes, representing 15,800 unique zebrafish genes (UniGene build 85). In addition, the arrays also contained 170 spots representing β-actin gene probes as controls. The probes were suspended at a concentration of 20 μM in 3X SSC and spotted onto poly-L-Lysine coated microscope slides using custom-built DNA microarrayer.

For fluorescent labeling of target cDNAs, 20 μg of total RNA (10 μg, when the RNA quantity was limited) from reference and experimental samples were reverse-transcribed in the presence of Cy3-dUTP and Cy5-dUTP (Amersham Biosciences), respectively. Labeled target cDNAs were combined, concentrated and resuspended in DIG EasyHyb (Roche). Hybridizations on microarray slides were performed at 42°C for 16 h using MAUI Mixer FL (BioMicro Systems) as explained earlier [52,95]. At least two independent biological replicates were taken for each sample. At least two to three independent replicate hybridizations (technical repeats) were performed for each biological repeat sample (Table S20).

**Scanning, filtering and data normalization**

The arrays were scanned by the GenePix 4000B microarray scanner (Axon Instruments) to generate 16-bit TIFF image files. GenePix Pro 4.0 image analysis software (Axon Instruments) was used to measure the fluorescent signal intensity of the array features and local background on TIFF images. Only the gene features with signal background ratio more than 1.5 were used for analysis. The 16-bit TIFF image files and the gpr files with Cy3 and Cy5 signal intensities were uploaded into the GIS-developed Microarray Database (madb). Median normalization of the sample and reference channel intensity values was performed using the intensity-based log ratio median method [96]. The extracted intensity data from the madb database were normalized by Lowess normalization method and analyzed by modified t-statistic Significance Analysis of Microarrays (SAM) [97]. The microarray data files have been submitted to the Gene Expression Omnibus (GEO) and the accession number is GSE8522.

**Gene annotation**

The gene annotations were carried out by using the Zebrafish Chip Annotation Database (MySql) (UniGene build 85) (http://giscompute.gis.a-star.edu.sg/~govind/zebrafish/version2) developed and maintained by GIS. This database contains putative annotations for the probes in the zebrafish oligonucleotide array. We queried this database with the Genbank ID to obtain the following information: (1) Compugen description, (2) Zebrafish UniGene ID (build 85), (3) Zebrafish UniGene description (build 85), (4) Entrez Gene description, (5) Entrez Gene ID and Gene symbol, (6) GO term, (7) Locus Link, (8) UniGene protein sequences or the longest available gene sequences were obtained (technical repeats) were performed for each biological repeat sample. Since we have different number of replicates for different alleles, we used different thresholds (q = 0.0, score(d)>4.0) [97]. The frame of value of score(d) is greater than 4.0, namely, |score(d)|>4.0 at 72 hpf; q = 0.0 at 48 hpf; q = 0.0 at 24 hpf) to select similar number of genes. However, the thresholds we used are all stringent (in every case the q-value is less than 2) and hence the false discovery rate (FDR, value expressed in %) in each case does not exceed 5. The FDR indicates the outcome with which the gene selected to be differentially expressed by the SAM analysis is likely to be occurring by chance. The score(d) indicates a statistic parameter, which is numerator(r) divided by denominator(s) and hence serves as a cut-off point along with the q value. The numerator(r) value indicates the actual gene expression change shown as log2 value.

**Microarray data analysis**

We applied Significance Analysis of Microarrays (SAM) [97] to identify statistically significant genes in each case. Since we have different number of replicates for different alleles, we used different thresholds (q = 0.0, score(d)>4.0) [97]. This represents that the absolute value of score(d) is greater than 4.0, namely, |score(d)|>4.0 at 72 hpf; q = 0.0 at 48 hpf; q = 0.0 at 24 hpf to select similar number of genes. However, the thresholds we used are all stringent (in every case the q-value is less than 2) and hence the false discovery rate (FDR, value expressed in %) in each case does not exceed 5. The FDR indicates the outcome with which the gene selected to be differentially expressed by the SAM analysis is likely to be occurring by chance. The score(d) indicates a statistic parameter, which is numerator(r) divided by denominator(s) and hence serves as a cut-off point along with the q value. The numerator(r) value indicates the actual gene expression change shown as log2 value.

For the analysis of 72-hpf time point data, we selected those genes with q = 0.0 and score(d)>4.0 from the SAM generated data for all three mib alleles (Table S1, S2 and S3). The description for all the gene sets was obtained from the Zebrafish Chip
Annotation Database [52]. Based on the most recently available information of the zebrafish gene annotation, these gene sets were classified as characterized and uncharacterized genes (Table 1). Furthermore, we manually searched for two sets of genes: one with functions related to Notch signaling and the other with functions related to pancreas development (Table 2-4). The expression profile values (log2) for the genes involved in the Notch pathway and pancreas development were obtained from the SAM analyzed data set. The in situ hybridization gene expression data for the genes related to pancreas development were obtained from the ZFIN database (http://zfin.org/cgi-bin/webdriver?MIval = aa-spateseltep). If the gene was previously shown to be involved in pancreas development, we classified them as the ‘genes involved in pancreas development’. If there is no functional relationship to zebrafish pancreas development but only functional homology to the human or mouse genes related to pancreas development, we classified them as the ‘genes predicted to be involved in pancreas development’.

A PERL script was used to identify the differentially expressed (q = 0.0, score(d)>4.0) genes (Genbank IDs) that are common among all three mib alleles (Table S4), between two different alleles and specific to each allele (Table S5, S6 and S7) at 72 hpf, and to remove duplicate genes (Genbank IDs), if any. Using this method, a group of 91 genes common to all three mib alleles were classified based on their function and characterization status (Table S4). These 91 genes were hierarchically clustered with TreeView_overs_1.60 software [98] and tree view image was generated using Adobe Illustrator (Fig. 4).

For the analysis of 48-hpf time point data, the gene sets with q = 0.0 were used. The same PERL script was also used here to find the common genes and the allele-specific genes (Table S8, S9, S10 and S11). The SAM data (q = 0.0) for the mibta52b on all three time points (24 hpf, 48 hpf and 72 hpf) were analyzed and queried using the PERL script to find out the gene sets that are common to all time points and specific to each time point (Table S12, S13, S14 and S15).

Functional groups and pathway analysis

Differentially expressed genes of three mib mutant alleles were subjected to Ingenuity Pathways Analysis (IPA) to identify the enrichment of genes in specific functional groups and pathways (IPA, Version 4, Ingenuity® Systems, http://www.ingenuity.com). The IPA accepts human UniGene IDs as one of the identifiers for data upload and analysis. For this reason, the differentially expressed genes of mib mutants were mapped to their human homologs using the HomoloGene database and zebrafish UniGene mapping tool established at the GIS (http://giscompute.gis.a-star.edu.sg/~govind/unigene_db/). Human homologs of up- and down-regulated genes of the mutants were analyzed by using IPA tools and the enrichment of functional categories and canonical pathways with reference to the Ingenuity Pathways Knowledge Base (IPKB) were documented. Initially, differentially expressed genes of the three mutant alleles were individually analyzed. Subsequently, the enrichment patterns were compared among the mutants to identify the conservation of functional groups among the mutants.

Using the input data set (human homologs of zebrafish genes differentially expressed in the mib mutants), IPA identified a set of genes that are enriched for a particular function or pathway and the enrichment is represented as ratio. The ratio refers to the number of input genes associated with each function/pathway versus the total number of genes (available in IPKB) involved in that particular function/pathway. The ratios may be affected by the variations in the total number of input identifiers. In order to find the significance of enrichment in a particular function, IPA calculates the significance value based on the measure of involvement of the gene in the input data set to their respective molecular functions/signaling pathways. Using the right-tailed Fisher’s Exact Test, the p-value (significance) is calculated by comparing the number of user-specified genes of interest that participate in a given function or pathway, relative to the total number of occurrences of these genes in all functional/pathway annotations in the IPKB.

Real-time PCR

To validate the microarray results, we carried out the real-time PCR for 25 genes and beta-actin gene was used as a reference. The primers used for amplifying each gene were listed in Table S18. cDNA was generated using the same purified RNA preparations from 72-hpf embryos (one biological repeat of wt and two biological repeats of mibta52b) used in microarray, and two other biological repeats of reference RNA from 72 hpf and one biological repeat sample RNA from mibta52b mutants. RT-PCR was carried out using the LightCycler® FastStart DNA Master-PPLUS SYBR Green kit (Roche) and the Light Cycler machine as per the instructions of the manufacturer. The products of the RT-PCR were analyzed on the agarose gel electrophoresis for a single band of expected size. Relative cDNA amounts were calculated using the comparative CT method as explained in the real-time PCR manual of Applied Biosystems and normalized to the expression of beta-actin.

Analysis of correlation between microarray data and real-time PCR results

Subsequent to microarray and real-time PCR data analysis, an evaluation of linear correlation was performed for a set of 25 genes (Table 5), and the statistical significance of the correlation was determined using One-way ANOVA in SPS software. For the correlation analysis, the data input of the microarray was the fold change of expression and the data input of the real-time PCR was the ratio of relative expression for each gene. Both sets of ratios were obtained from the 72-hpf time point for the mibta52b allele.

SUPPORTING INFORMATION

Table S1 List of significantly expressed genes (q = 0.0) in 72-hpf taf52b mutants generated with SAM.
Found at: doi:10.1371/journal.pone.0001479.s001 (1.75 MB XLS)
Table S2 List of significantly expressed genes (q = 0.0) in 72-hpf m132 mutants generated with SAM.
Found at: doi:10.1371/journal.pone.0001479.s002 (3.12 MB XLS)
Table S3 List of significantly expressed genes (q = 0.0) in 72-hpf tfi91 mutants generated with SAM.
Found at: doi:10.1371/journal.pone.0001479.s003 (3.92 MB XLS)
Table S4 List of significantly expressed genes (q = 0.0, score(d)>4.0) common to all three mib mutant alleles at 72 hpf.
Found at: doi:10.1371/journal.pone.0001479.s004 (0.09 MB XLS)
Table S5 List of significantly expressed genes (q = 0.0, score(d)>4.0) specific to ta52b mutants at 72 hpf.
Found at: doi:10.1371/journal.pone.0001479.s005 (0.09 MB XLS)
Table S6 List of significantly expressed genes (q = 0.0, score(d)>4.0) specific to m132 mutants at 72 hpf.
Found at: doi:10.1371/journal.pone.0001479.s006 (0.52 MB XLS)
Table S7 List of significantly expressed genes (q = 0.0, score(d)>4.0) specific to tfi91 mutants at 72 hpf.
Found at: doi:10.1371/journal.pone.0001479.s007 (0.93 MB XLS)
Table S8  List of significantly expressed genes (q = 0.0) common to all three mib mutant alleles at 48 hpf. Found at: doi:10.1371/journal.pone.0001479.s008 (0.04 MB XLS)

Table S9  List of significantly expressed genes (q = 0.0) specific to ta52b mutants at 48 hpf. Found at: doi:10.1371/journal.pone.0001479.s009 (0.08 MB XLS)

Table S10  List of significantly expressed genes (q = 0.0) specific to m132 mutants at 48 hpf. Found at: doi:10.1371/journal.pone.0001479.s010 (0.09 MB XLS)

Table S11  List of significantly expressed genes (q = 0.0) specific to tfi91 mutants at 48 hpf. Found at: doi:10.1371/journal.pone.0001479.s011 (0.05 MB XLS)

Table S12  List of significantly expressed genes (q = 0.0) specific to all three time points for ta52b mutants. Found at: doi:10.1371/journal.pone.0001479.s012 (0.38 MB XLS)

Table S13  List of significantly expressed genes (q = 0.0) specific to 24 hpf time point in ta52b mutants. Found at: doi:10.1371/journal.pone.0001479.s013 (0.01 MB XLS)

Table S14  List of significantly expressed genes (q = 0.0) specific to 48 hpf time point in ta52b mutants. Found at: doi:10.1371/journal.pone.0001479.s014 (0.03 MB XLS)

Table S15  List of significantly expressed genes (q = 0.0) specific to 72 hpf time point in ta52b mutants. Found at: doi:10.1371/journal.pone.0001479.s015 (0.05 MB XLS)

Table S16  List of significantly expressed genes (q = 0.0) common in at least two data sets. Found at: doi:10.1371/journal.pone.0001479.s016 (0.02 MB XLS)

Table S17  Zebrafish pancreas development related genes. Found at: doi:10.1371/journal.pone.0001479.s017 (0.02 MB XLS)

Table S18  Sequence of primers used in real-time PCR validation on microarray data. Found at: doi:10.1371/journal.pone.0001479.s018 (0.07 MB XLS)

Table S19  Genes predicted to be related to zebrafish pancreas development. Found at: doi:10.1371/journal.pone.0001479.s019 (0.02 MB XLS)

Table S20  Summary of biological and technical repeats. Found at: doi:10.1371/journal.pone.0001479.s020 (0.01 MB XLS)

Figure S1  Changes of expression levels in the canonical pathways. (A) The Notch signaling pathway and (B) the Wnt/β-catenin signaling pathway. The nodes in these pathways are highlighted with expression data from the m132 data (red: up-regulated; green: down-regulated). Nodes with a histogram chart next to it represents gene expression in the i) ta52b, ii) m132 and iii) tfi91 data set from left to right. (TIF)

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Author Contributions

Conceived and designed the experiments: YJ AH. Performed the experiments: AH KT NQ SH XQ. Analyzed the data: KG YJ JG AH SM. Wrote the paper: YJ AH SM.

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