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**Gene expression associated with the onset of hearing detected by differential display in rat  
organ of Corti**

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## ***Abstract***

The exquisite performance of the mammalian hearing organ results from a finely orchestrated array of cell types whose highly specialized functions are determined by their gene expression profile. In rodents, this profile is established mainly during the first two weeks of postnatal maturation. Here, we used the differential display technique on the rat organ of Corti to uncover transcripts upregulated in expression between postnatal stages P0 and P14. A total of 176 different genes were identified whose amount of mRNA increased during early postnatal development. The transcripts code for proteins serving a broad spectrum of cellular functions including intracellular signaling, control of growth/differentiation, regulation of protein synthesis/degradation/modification, metabolism and synaptic function. In addition, the set of upregulated transcripts contained several proteins of yet unknown function, hypothetical proteins, and so far unknown mRNA sequences. Thus, this study unravels the broad and specific transcription program that operates the maturation of the mammalian hearing organ. Further, as 49 of the genes found here map to at least one unspecified deafness locus, our study provides candidate genes for these and novel deafness loci.

## ***Keywords***

Deafness genes, hair cells, quantitative real-time PCR, deafness loci, hair cell differentiation

## ***Introduction***

The mammalian hearing organ, the cochlea, perceives and processes sound with extremely high sensitivity and frequency selectivity<sup>1</sup>. This remarkable performance essentially

results from the concerted action of a variety of highly specialized cell types that are organized in a well-ordered microarchitecture and serve quite a number of distinct functions, including ion transport and homeostasis, micromechanics and active amplification, sensory transduction, and electrical signal propagation<sup>1,2</sup>.

On the molecular level, this specialization, and hence perception and processing of sound, relies on the expression of a multitude of proteins, some of them specifically found in the inner ear, e.g. tectorin, cochlin and prestin<sup>3-6</sup>. This notion is supported by the considerable number of genes shown to be causative for inherited, non-syndromic forms of hearing impairment or deafness, both in humans and mouse models<sup>7,8</sup>. Furthermore, a series of proteins found both in the inner ear and in other tissues are known to be causative for inherited diseases which are associated with deafness. For example, Usher syndrome proteins like SANS are required for proper function of auditory hair bundles and photoreceptors in the retina<sup>9</sup>. For humans, the central database has so far collected a total of more than 140 different gene loci associated with non-syndromic deafness<sup>10,11</sup>. However, until now only around 50 have been assigned to proteins with defined function, and the majority still awaits identification and examination of the respective molecular pathomechanism<sup>10-12</sup>.

In rodents, pups are born deaf and the inner ear undergoes extensive remodeling during postnatal development<sup>13</sup> culminating in the onset of hearing around postnatal day 12 (P12) in rats<sup>14</sup>. Accordingly, proteins essential for perception and processing of sound are expected to be 'switched on' or to be upregulated on the transcriptional level during this period. Known examples that confirm to this developmental pattern and cause inherited hearing disorders in humans when mutated include, e.g., prestin (SLC26A5)<sup>15-17</sup>, KCNQ4<sup>18</sup> and Cav1.3<sup>7,8,19,20</sup>. Thus, postnatal maturation renders the rat organ of Corti an ideal model system for investigating genes required for hearing by analysis of transcripts being differentially expressed prior to and after the onset of hearing. The Differential Display on cDNA (cDNA-DD) of microdissected organs of Corti exhibited several benefits for our experimental design: first, it requires only small amounts

of tissue without the need of mRNA- or cDNA pre-amplification. Indeed, the high sensitivity of the cDNA-DD technique might increase the chance to detect expression differences for low abundance transcripts that are underrepresented in DNA array outcomes<sup>21</sup>. Second, the cDNA-DD allows detecting not only genes with database entries, but also new mRNA transcripts or splice variants can be identified. Third, several previous studies proved the reliability and robustness of the cDNA-DD<sup>22-24</sup>.

In this study, we used the cDNA-DD to identify genes that are upregulated in the rat organ of Corti between the very immature postnatal stage P0 and P14, which is two days after the onset of hearing.

## ***Materials and Methods***

### **Differential Display**

#### *Tissue preparation*

Organs of Corti from six rats at day of birth and apical parts of the organs of Corti of seven rats at postnatal stage P14 were dissected under 5 to 10-fold magnification and immediately frozen in liquid nitrogen prior to mRNA isolation. Dissected tissue consists of the organ of Corti and minor parts of stria vascularis and spiral ganglion.

#### *mRNA isolation and cDNA synthesis*

mRNA was prepared with DYNABeads (Dyna, Oslo, Norway) according to the manufacturers instructions. mRNA concentrations in P0 and P14 samples were determined by UV absorption. cDNA was derived by reverse transcription of approximately 150 ng mRNA (each) with Superscript II (Invitrogen, Karlsruhe, Germany) in supplemented buffer and 10  $\mu$ M DTT, 1 mM dNTPs, 1 pmol oligo-dT primer, 0.3  $\mu$ l RNasin (Promega, Madison, WI) for 2 h at 42°C.

### *Differential Display*

The differential display on reverse transcribed mRNA of P0 and P14 organs of Corti was performed as described<sup>25</sup>. Briefly, arbitrary P-primers of the 'Delta Differential Display' kit (Clontech, Palo Alto, CA) were combined with each other or with two-base-anchored oligo dT-primers.

The differential display PCR was set up with two dilutions of reverse transcribed P0 and P14 mRNA (dilution *A*, using 2.4 ng/μl mRNA; and *B*, 0.6 ng/μl) and for negative controls with mRNA (without reverse transcriptase) or H<sub>2</sub>O as templates.

Bands displaying from P14 cDNA with no corresponding band at P0 or bands with higher intensity in both dilutions of P14 template cDNA compared to both P0 lanes were considered as 'differential'. The underlying gel pieces were cut out, DNA was eluted in 40 μl 1x TE at 100°C for 5 min and reamplified by a PCR using the original primer combination and 20-25 cycles of a standard PCR protocol.

### *Identification of cDNA bands*

Reamplified DNA fragments were analyzed by direct sequencing with fluorescent dye terminator cycle sequencing (Applied Biosystems, Weiterstadt, Germany). cDNAs for which no sequence information was obtained with this procedure were subcloned into pBluescript SK<sup>-</sup> (Stratagene) and three to five clones of each subcloned band were sequenced. In Supplementary Tables 2 and 3, only those genes were included where at least two inserts from independent clones were comprised of identical or overlapping sequences.

## **Quantitative Real-Time PCR**

### *RNA preparation and reverse transcription for qPCR analysis*

Quantification of transcripts from inner ear was performed as described<sup>26</sup>. mRNA of the respective developmental stage was prepared from the apical turns of two times six or seven organs of Corti. Total RNA was isolated with Trizol reagent (Invitrogen, Karlsruhe, Germany) as recommended by the manufacturer. RNA was transcribed into cDNA in 1x first strand buffer with 10  $\mu$ M DTT, 0.5 mM dNTPs, 0.3 x hexanucleotide Mix (Roche, Mannheim, Germany), 50 pmol oligo-dT-primer, 20 U RNasin (Promega) and 1  $\mu$ l Superscript II (Invitrogen) for 10 min at 25°C, 10 min 37°C and 70 min at 42°C. These cDNA samples were ethanol precipitated prior to qPCR.

#### *Design of intron-overspanning primers*

Primer pairs were designed by means of Primer Express 2.0 software (Applied Biosystems). Primers were either placed on neighboring exons, when introns spanned more than 1kb, or one of the primers annealed over the intron splice site, so that 5-8 nucleotides on the 3' site bound to the downstream exon. Amplification efficiency was evaluated in a separate qPCR experiment followed by melting curve analysis. Primer sequences are given in Supplementary Table 1.

#### *Real-Time qPCR*

Quantitative PCR was performed with SYBr Green (2x Master Mix, Applied Biosystems) in an ABI PRISM 7000 or 7500 sequence detection system (Applied Biosystems). For the quantification of 18S rRNA samples were diluted 1:100 or 1:1000. All qPCR experiments were performed in triplicate; standard deviations (SD) of  $C_t$  values were in average 0.12 cycles, which reflects an SD in cDNA copy numbers of 5.3%. The SDs were smaller for high abundance genes, e.g. 18S rRNA quantification resulted in an SD of 0.02 cycles or 1.5% SD for the number of mRNA molecules, but higher for low abundance genes, e.g. Scamp3 ( $C_t$  values between 35 and 38 which is close to the detection limit).

To calculate mean  $C_t$  values, non-logarithmic numbers ( $2^{(42-C_t)}$ ) were calculated, those numbers were averaged and back-calculated to get the (logarithmic)  $C_t$  value. Transcript abundance in each sample was determined using the comparative  $C_T$  method, as described<sup>26,27</sup>.

## **Results**

### **Profiling upregulated transcripts by means of cDNA-DD**

In this study, we used a cDNA-DD with reverse transcribed mRNA of P0 and P14 organs of Corti to identify genes upregulated during postnatal development of the rat hearing organ (Fig. 1a). 28 combinations of 'arbitrary' and 'oligo-DT' primers were used for PCR-amplification of cDNA fragments from both transcriptomes. Separation of the radioactively labeled PCR products on high-resolution acrylamide gels showed distinct patterns between the two postnatal stages (Fig. 1b) and allowed for identification of cDNA fragments with higher abundance in the P14 amplifications. Altogether, 302 cDNA fragments were found upregulated in P14 organs of Corti, of which 289 were successfully recovered from the gel and reamplified. Of these, 166 allowed for direct sequencing, while 115 cDNA-fragments were identified only after subcloning. 14 of the subcloned fragments contained more than one DNA sequence (according to the criteria described in Materials and Methods). In total, sequences of 297 cDNA fragments were assigned to differential bands.

### **Identification and categorization of transcripts**

All sequences were screened for redundancy by comparison against each other (using *FASTA*<sup>28</sup>) and against the public 'non-redundant' or 'EST' databases on [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov) using *BLAST*<sup>29</sup>. In total, 176 individual genes were detected by this cDNA-DD, of which 114 were identified by cDNA fragments found in one cDNA-DD band each, while 62 genes were covered by DNA fragments extracted from two to eight bands (Table 1).



From the 176 genes, 116 fragments could be attributed to genes with well-established function (Fig. 2a, Supplementary Table 2). Another 19 sequences originated from genes encoding proteins with unknown function, while 13 cDNA fragments were assigned to predicted or hypothetical proteins. Further, 20 sequences matched to expressed sequence tags (ESTs) or uncharacterized mRNA sequences (Fig 2a), with two significantly exceeding the database entries which were therefore submitted for publication (dbEST 50172343, 50172344). Eight sequences were deposited in public databases (accession numbers 50172335 to 50172342) as they did not match any database entry for transcribed sequences but corresponded to genomic DNA entries.

The proteins with known function were grouped with respect to their role in cell physiology (Fig. 2b). As summarized in Supplementary Table 2, a substantial fraction of the developmentally upregulated genes are involved in (I) intracellular signaling pathways (including kinases/phosphatases and interacting molecules, GTP-binding proteins, receptors and enzymes modifying second messengers) as well as in (II) extracellular signaling dedicated mainly to regulation of growth and differentiation and in (III) transmembrane signaling and transport represented by subunits of ion channels and solute carriers. Further functional categories defined by the identified transcripts are (IV) synaptic/vesicular proteins, proteins involved in (V) regulation of protein biosynthesis and degradation (including transcriptional elements, chaperones and components of the protein degradation machineries), and (VI) proteins involved in maintenance of the cellular (sub-)structure and morphology. In addition, quite a number of the identified gene products exhibit enzymatic activities (VII) and participate in metabolism of various subcellular compartments (see Supplementary Table 2).

Together, the diversity of gene products detected by our cDNA-DD approach demonstrated that maturation of the hearing organ requires a broad transcriptional program covering a variety of cell physiological functions.

### **Association of the differential genes with hearing disorders**

Some proteins found here to be upregulated during inner ear maturation had been assigned to hearing disorders. Three of the genes, namely *Sbf2*, *Pmp22* and myelin protein zero lead to variants in Charcot-Marie-Tooth Syndromes which are associated with hearing impairments<sup>30,31</sup>. In a mouse model, deficiency in neurotrophic tyrosine kinase receptor type 2 (*Ntrk2*) was shown to be embryonic lethal but also led to a defect in hair cell innervation<sup>32,33</sup>. In addition, for a series of genes identified here, a close protein family member had been shown to cause deafness in humans. For example, protocadherin 15, sharing 42% sequence similarity with protocadherin  $\alpha$  (Supplementary Table 2, section II), is now well established to cause Usher 1F syndrome<sup>34,35</sup> as well as DFNB23<sup>36</sup>.

We next compared the genetic locus of the human homologous genes with deafness loci that had not been associated with a specific gene. If the position of the human gene lay within the finite linked deafness loci defined by linkage analysis, we considered this gene to be a potential candidate for a deafness gene. We found 49 genes that matched these criteria and were at the same time not assigned to a genetic human disorder in OMIM database (Supplementary Table 3). For example, two genes with yet unknown function, namely reticulon 3 and transmembrane protein 16A, both match to the deafness locus DFNB63, whereas in the genomic region comprising the deafness loci DNFNA24, DFNA27 and DFNB55, insulin-like growth factor binding protein 7 (*Igfbp7*) is encoded.

### **Upregulation of transcripts in the postnatal period**

The transcriptional upregulation implied in the cDNA-DD approach was verified in experiments that quantified the postnatal expression by quantitative PCR (qPCR). For this validation experiment we selected genes matching to several deafness loci, different genes encoded within the same deafness locus, and genes which might play an important role for organ of Corti function. The qPCR experiments revealed a substantial up-regulation between P0 and P14 for 21 out of the 27 tested genes (Fig. 3a). Upregulation was more than five-fold for seven

genes, including *Igfbp7*, Na-K-ATPase  $\beta 3$ , and *Sepp1*, whose human genes lie within several deafness loci. With this approach, we could not find a significant up-regulation for *Rab9*, protocadherin  $\alpha$ , transmembrane protein 16A, *prefoldin*, *Prkab2* and *pleiotrophin*.

For five genes we tested the course of up-regulation during five postnatal developmental stages of the organ of Corti (Fig 3b). *SLC20A1* was found to exhibit a linear increase in mRNA level between P0 and P21, while *SNAP91* and *Mpp6* display transient upregulation with peak levels around P10. mRNA expression of *Chp* ( $\text{Ca}^{2+}$ bp-P22) and *Gpm6b* elevates between postnatal days 1 and 9 and remains about constant thereafter (Fig. 3b). Thus, the up-regulation of the individual genes seems to happen at different time points during postnatal maturation of the inner ear.

### **Estimation of Completeness of Results**

Identifying a total amount of 176 distinct proteins poses the questions on how many genes may actually be upregulated in the maturing organ of Corti in total. We estimated this by statistical analysis using a custom developed algorithm<sup>25</sup>, which is based on the correlation that redundancy of randomly amplified genes decreases when the total number of templates increases.

We estimated that 16 % (90% confidence interval (CI) of [0.145, 0.178]) of the genes expressed in the organ of Corti are upregulated during postnatal maturation. Further, 441 differentially expressed genes are estimated to be theoretically detectable by the cDNA-DD using an infinite number of primer combinations (90% CI: [397, 485]). Accordingly, the cDNA-DD with a subset of 28 primer combinations that identified 176 distinct genes uncovered approximately 40% of the accessible differentially expressed genes. Finally, the total number of genes expressed in the organ of Corti is estimated to 2734 (90% CI [2485, 3037]). We consider this estimation as a lower limit for the actual protein encoding genes in the rat organ of Corti because of limitations of the cDNA-DD technique (discussed in ref<sup>25</sup>).

## ***Discussion***

In the present study, we identified a set of transcripts that are upregulated during postnatal maturation of the organ of Corti. The massive change in gene expression probably underlies the extensive physiological and morphological changes that lead to maturation steps essential for the onset of hearing.

### **Reliability of the Method**

To uncover differentially expressed transcripts in the mammalian inner ear, we chose the cDNA-DD method mainly because of its high sensitivity. Indeed, we successfully detected genes with low copy numbers, such as *Itgb11* or *Scamp3* (according to qPCR) and a series of 11 transcription factors. Further, this approach is known to produce a low fraction of false positives, which was reassessed here by qPCR. While 21 out of 27 genes were confirmed to be substantially upregulated (Fig. 3), transcript levels of six genes were unchanged in these experiments. This could be due to differences between organ of Corti microdissections for the cDNA-DD and for qPCR. For example, if a gene is upregulated in hair cells but down-regulated in spiral ganglion neurons, different amounts of spiral ganglion tissue attached to the organ of Corti in the different preparations could lead to such discrepancies.

Applying the cDNA-DD approach, we uncovered differences in mRNA expression, assuming that transcript abundance correlates with protein amounts for most of the genes. This presumption was found to be supported by earlier findings on the developmental upregulation of gene products involved in cochlear function. For example, immunostaining for KCNJ16 (Supplementary Table 2, section III) showed a rapid increase of protein levels in the rat cochlea from P10 to P16, correlating with observed changes in endocochlear potential<sup>37</sup>. Another well-studied example is the polyamine metabolism (Supplementary Table 2, section If): Highest

ornithine decarboxylase activity was observed at postnatal days 9 and 10, and it declined to about 1-2% in adulthood<sup>38,39</sup>. Moreover, deficiency in spermine synthase, an enzyme acting in this pathway, was shown to cause deafness in mice<sup>40</sup>. These examples strongly support the idea that differential transcripts found in our study might indeed reflect a postnatal upregulation of the respective proteins in the rodent inner ear.

In summary, the cDNA-DD approach turned out to reliably uncover upregulated transcripts in the inner ear. However, some proteins like KCNQ4, prestin or TMC1, have not been detected by this approach, despite our expectation to do so. For KCNQ4, whose mRNA has a GC content of 62%, we assume that it would not be amplified under our experimental conditions, but only upon addition of DMSO. Other proteins might have been missed as this cDNA-DD uncovered only 40% of the detectable genes.

### **Significance for identification of deafness genes and cochlear biology**

Normal hearing requires proper expression and function of an extensive set of specialized proteins, a notion reflected in the high number (~ 140) of gene loci correlated with various forms of non-syndromic hearing loss. Currently, 90 of these loci, mostly spanning 100 genes or more<sup>10,12,41</sup>, still await identification of the gene underlying this particular form of deafness. It is reasonable to suggest that in rodents many of those genes should be upregulated between birth and the onset of hearing. Here, we found 49 upregulated genes that have not yet been described as disease-associated but lie within a human deafness locus with so far unidentified underlying gene. Therefore, they are attractive candidates for geneticists that aim to identify genes defective in the respective forms of deafness. Further, interaction partners of the proteins unraveled here – although not directly identified in this study – might also be reasonable candidate genes for deafness loci.

Apart from the added value of our study for identification of candidate genes in novel deafness loci, this work gives insight into the molecular machinery that enables the organ of Corti to

transduce mechanical into electrical energy. The series of proteins serving most different cellular functions (Supplementary Table 2) might inspire research on different aspects of cochlear biology, such as establishment of the cytoskeleton, inter- and intracellular signaling and regulation of gene activation by transcription factors.

In the present study we show that maturation of the mammalian cochlea is associated with the transcriptional upregulation of a considerable number of proteins implicated in a wide array of cellular functions in the inner ear. Notably, many of the 176 distinct genes identified here match to so far uncharacterized deafness loci. Thus, this study provides a rational set of target candidates for the deafness genes within deafness loci that often span more than 100 genes<sup>41</sup>.

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***Supplementary information*** is available at European Journal of Human Genetics' website.

### ***Conflict of interest.***

The authors declare no conflict of interest.

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### ***Table and Figure legends***

#### **Table 1**

***Frequency with which genes were identified within this cDNA-DD.***

#### **Fig. 1**

cDNA-DD on the developing rat Organ of Corti.

(a) Flowchart of the cDNA-DD. (b) Radioactively-labeled cDNA fragments were amplified and separated on a high-resolution acrylamide gel. A PCR using one primer combination results in 4 neighboring lanes, where two different cDNA concentrations (higher on left, lower

on right) from postnatal day 0 (P0) and P14 were used as templates. Small letters indicate bands with higher abundance in P14 compared to P0; examples for bands identified by cycle-sequencing are (a) *Kcnj16*, (b) mineralocorticoid receptor, (c) glutaredoxin, (d) annexin A2, (e)  $\text{Ca}^{2+}$ bp P22 (Chp), (f,r) Transcribed sequences Rn.4180, (g)  $\text{Na}^+/\text{K}^+$ -ATPase  $\beta$ 1, (h,m) Snap91, (i) SLC20A1, (j) sphingolipid- $\Delta$ 4-desaturase, (k)  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha$ 3, (l) CUG-BP1, (n) *Cpeb1*, (o) annexin A5, (p) cathepsin B, (q) transcribed sequences Rn.48946, similar to human hypothetical protein KIAA0256.

### **Fig. 2**

Summary of the cDNA-DD results.

(a) Alignments of the sequences with entries in the public databases reveal the fraction of proteins of known function vs. less characterized genes. (b) Proportions of the cellular functions of the 116 proteins of known function identified in this cDNA-DD.

### **Fig. 3**

Up-regulation was quantified with quantitative PCR.

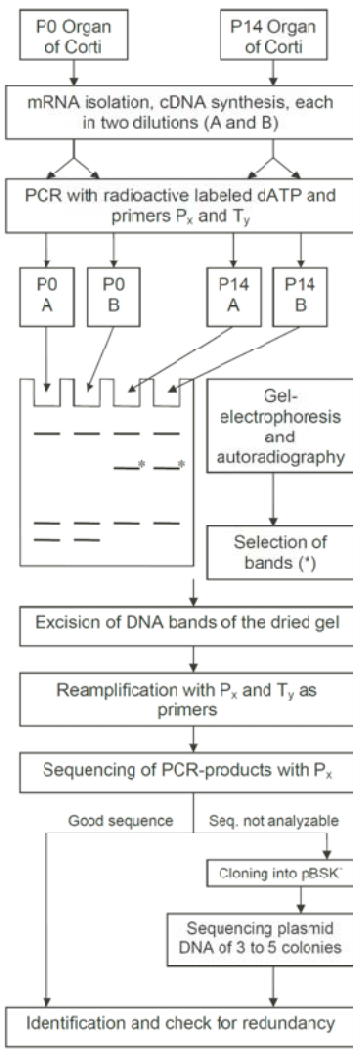
(a) mRNA from two times six organs of Corti of P0 and P14 rats was isolated and reverse transcribed. qPCR with SYBr green was pipetted in triplicates for each of the four cDNA samples and each pair of intron-overspanning primers. cDNAs were normalized on 18S rRNA (dilution of template DNA 1:1000). The bar graphs show the mean amount of cDNA at P14 compared to P0. Standard errors of the mean (SEM) are calculated from the relative cDNA amounts in the four cDNA preparations (compared one by one). (b) Time course of mRNA upregulation in the apical turn of the rat organ of Corti determined for a sample set of genes (mean  $\pm$  SEM).

**Table 1. Gene frequency**

frequency	genes	bands
1	114	114
2	38	76
3	12	36
4	2	8
5	3	15
6	3	18
7	2	14
8	2	16
9	0	0
Sum	176	297

Figure 1

a



b

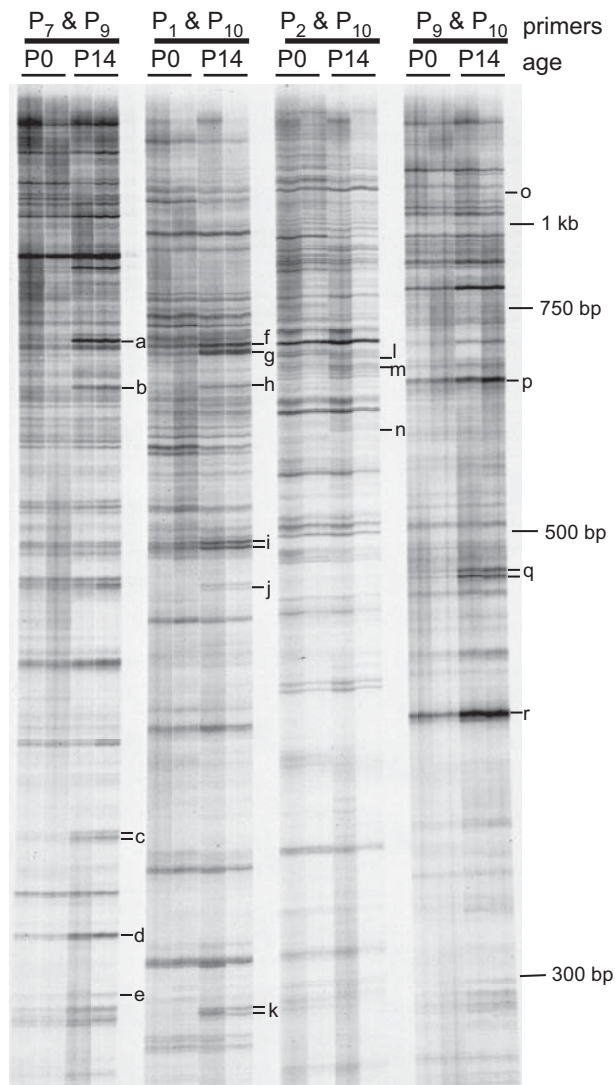
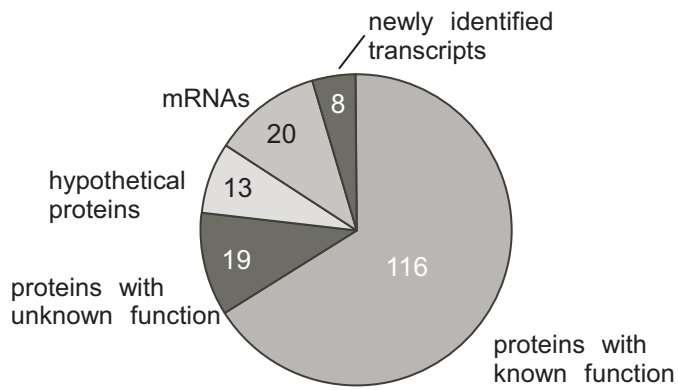


Figure 2

a



b

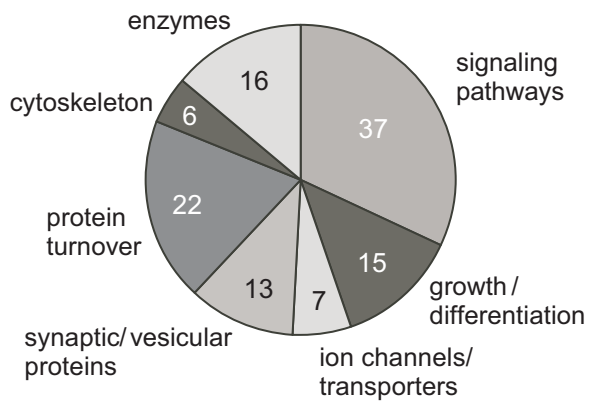


Figure 3

