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Letter

Four-Hundred Million Years of Conserved Synteny of Human Xp and Xq Genes on Three *Tetraodon* Chromosomes

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The freshwater pufferfish *Tetraodon nigroviridis* (TNI) has become highly attractive as a compact reference vertebrate genome for gene finding and validation. We have mapped genes, which are more or less evenly spaced on the human chromosomes 9 and X, on *Tetraodon* chromosomes using fluorescence in situ hybridization (FISH), to establish syntenic relationships between *Tetraodon* and other key vertebrate genomes. PufferFISH revealed that the human X is an orthologous mosaic of three *Tetraodon* chromosomes. More than 350 million years ago, an ancestral vertebrate autosome shared orthologous Xp and Xq genes with *Tetraodon* chromosomes 1 and 7. The shuffled order of Xp and Xq orthologs on their syntenic *Tetraodon* chromosomes can be explained by the prevalence of evolutionary inversions. The *Tetraodon* 2 orthologous genes are clustered in human XpII and represent a recent addition to the eutherian X sex chromosome. The human chromosome 9 and the avian Z sex chromosome show a much lower degree of synteny conservation in the pufferfish than the human X chromosome. We propose that a special selection process during vertebrate evolution has shaped a highly conserved array(s) of X-linked genes long before the X was used as a mammalian sex chromosome and many X chromosomal genes were recruited for reproduction and/or the development of cognitive abilities.

[Sequence data reported in this paper have been deposited in GenBank and assigned the following accession no: AJ308098.]

The highly compact genomes of the Japanese pufferfish, *Fugu rubripes* (400 Mb), and the freshwater pufferfish, *Tetraodon nigroviridis* (380 Mb), are particularly useful for large-scale comparative sequencing to characterize all human genes and to interpret the complex architecture of the human and other vertebrate genomes (Roest Crollius et al. 2000a; Venkatesh et al. 2000). In comparison with *Fugu*, the small and hardy *Tetraodon* has the added advantage that it can be maintained easily in the laboratory. However, as neither *Fugu* nor *Tetraodon* can be manipulated or bred in captivity, genetic analyses are not possible in these important animal models. Comparative sequencing approaches have shown that gene structure and short-range gene order (within megabase domains) are largely conserved between pufferfish and humans (Miles et al. 1998; Brunner et al. 1999), but the construction of chromosome homology maps has been difficult. A solution to this problem comes from fluorescence in situ hybridization of BACs containing the pufferfish orthologs of known human genes to *Tetraodon* chromosomes. In this study, we have established the syntenic relationships of human chromosome 9, which represents an ancestral mammalian autosome (Chowdhary et al. 1998), and of the human X chromosome with the pufferfish genome.

The X adopted its function as a sex chromosome ~240–320 million years ago after divergence of mammals and birds (Lahn and Page 1999). Conservation of the mammalian X in its entirety is thought to be a consequence of X inactivation to ensure dosage compensation for most X-linked genes between males and females (Ohno 1967; Lyon 1972). Only a few genes have been translocated between the X and autosomes during eutherian evolution. For example, *CLCN4* maps very close to the pseudoautosomal region of the X in humans and the wild Mediterranean mouse, but to chromosome 7 in the laboratory mouse (Rugarli et al. 1995). Pseudoautosomal genes have active partners on the Y and, therefore, are exempt from Ohno's law. It is plausible that *CNCL4*, and other genes, which at first glance appear to contravene Ohno's law, was originally pseudoautosomal in an eutherian ancestor and a translocation onto an autosome has occurred in the laboratory mouse (Marshall Graves 1996). Such X to autosome rearrangements are very rare in eutherian mammals, but gene order on the X has been rearranged extensively in many species (Iannuzzi et al. 2000; Kuroiwa et al. 2001).

In contrast, human chromosome 9 segments have been translocated onto nonhomologous chromosomes in a variety of species following the divergence of a common mammalian ancestor, for example, human 9 genes are distributed on four different mouse chromosomes (http://www.ncbi.nlm.nih.gov/Homology). Many genes from human 9pter-q31 have or-

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thologs on the chicken Z sex chromosomes, indicating the common ancestry of human 9 and chicken Z (Nanda et al. 1999, 2000; Schmid et al. 2000), which diverged ~350 million years ago (Kumar and Hedges 1998). Like the mammalian X, the entire Z appears to be conserved as a single syntenic block in birds (Shetty et al. 1999; Schmid et al. 2000). However, replication of the two Z chromosomes in male birds is not asynchronous (Schmid et al. 1989), and it is not clear whether the majority of Z-linked genes are subject to dosage compensation (Kuroda et al. 2001; McQueen et al. 2001).

In contrast to mammals and birds, the pufferfish, like most fish, does not possess heteromorphic sex chromosomes (Grützner et al. 1999), and the genetic mechanism(s) of sex determination is still unclear. It is known, however, that environmental and endocrine factors can strongly influence sex differentiation in fish (Baroiller et al. 1999). Because teleost fish diverged >400 million years ago (Kumar and Hedges 1998), they serve as a useful outgroup to test whether conservation of the mammalian X and avian Z is due to an intrinsic chromosomal property or their sex chromosomal status.

RESULTS AND DISCUSSION

To date, a random *Tetraodon* DNA sample of 800 Mb (equivalent to approximately two genomes) has been sequenced (http://www.genoscope.cns.fr/tetraodon). The pufferfish genome has a highly compact architecture and there is much more pufferfish than zebrafish sequence available for ortholog searches. These are important advantages of comparative mapping of teleost and tetrapod genes, which can help to infer the ancestral state of mammalian chromosomes. Using `Exofish` (Roest Crollius et al. 2000a), we identified 40 *Tetraodon* BACs that share orthologous gene sequences with human chromosomes 9 and X (Table 1). No *Tetraodon* orthologs

Table 1. List of Clones Containing *Tetraodon* Orthologs of Human Genes

Gene	Human chromosome band	Human chromosome Mb distance	*Tetraodon* chromosome	TNI BAC end sequence EMBL accession no.	Clone name
AK3	9p24	n.a.*	1	AL352756	C0AB028O11
GLDC	9p24	3.3	9	AL340373	C0AA037I03
TYRP1	9p23	9.3	11	AL339713	C0AB047I11
DMRT1	9p23	14.5	8	AL329663	C0AA052C14
CNTFR	9p21	33.5	8	AL333197	C0AA051P16
ANXA1	9q21	65	10	AL317970	C0AA051J11
GCNT1	9q21	68	8	AL329563	C0AA039H15
HNRPK	9q21	76.2	12	AL349127	C0AA017N19
HSD17B3	9q22	87.9	7	AL321714	C0AB002N22
TMOD	9q22.3	89.2	13	AL337508	C0AB012I03
EPB72	9q33	115	14	AL350810	C0AB008H21
PTGS1	9q33	116.5	8	AL351240	C0AA007A13
PBX3	9q33	120	15	AL340899	C0AA030I07
AK1	9q34	121.6	16	AL341655	C0AA050J03
ENG	9q34	121.6	17	AL346287	C0AA043F14
ASS	9q34	124.8	10	AL344332	C0AB011P19
ABL1	9q34	124	10	AL350025	C0AA031E21
ISPK1	Xp22	n.a.	1	AL321584	C0AA033J23
PIGA	Xp22	13.0	1	AL306766	C0AB026J12
PHKA2	Xp22	17.5	7	AL336707	C0AA025A16
PDHA1	Xp22.1	17.9	7	AL342845	C0AB001P05
SMS	Xp22.1	19.6	7	AL351833	C0AB025D12
SAT	Xp22.1	21.3	7	AL338831	C0AA019A15
POLA	Xp22.3	21.8	6	AL333304	C0AA017E04
OTC	Xp11.4	34.5	2	AL339149	C0AA010L18
EXLM1	Xp11	n.a.	2	AL344211	C0AB046K20
MAOB	Xp11.3	40.2	2	AL305902	C0AB020J13
PCTK1	Xp11	44.5	7	AL325789	C0AA046M22
P2RY4	Xq13	64.6	1	AL318925	C0AA020M21
K1F4A	Xq13	64.7	7	AL306871	C0AB032I13
HOPA	Xq13	65.6	1	AL334883	C0AB014D21
GJB1	Xq13	65.7	1	n.a.	C0AA019L09
TAF2A	Xq13.1	65.9	7	AL340618	C0AA022A22
PGK 1	Xq21.1	72.2	7	AL330772	C0AA032P18
LOC170240	Xq22	97.5	1	AL334745	C0TB001K13
*BTK***	Xq22	96.5	1	AJ308098	ICRFp551C0473Q6
GK	Xq22	96.9	2	AL311483	C0AB006D09
MID2	Xq22	103.4	1	AL323465	C0AB023P08
UBE2A	Xq24	113.2	1	AL344408	C0AA048M20
GRIA3	Xq25	119	7	AL347790	C0AA015K14
IDS	Xq28	145	1	n.a.	C0AA040L12

*Not available
**cDNA clone

of the pseudoautosomal region Xp22.3 could be identified. The pseudoautosomal genes were added independently to the sex chromosomes of eutherian mammals and then became subject to progressive degradation (addition-attrition hypothesis) (Marshall Graves 1995; Lahn and Page 1999). To identify segments of conserved chromosomal synteny, *Tetraodon* BACs orthologous to chromosome segments of the human 9 and X were hybridized in situ to *Tetraodon* metaphase spreads. All BACs produced discrete hybridization signals on a single *Tetraodon* chromosome pair, allowing unequivocal chromosomal mapping.

History of the Mammalian X Chromosome

In this study, 10 X chromosomal genes were found to have orthologs on TNI 1, 4 on TNI 2 and 9 on TNI 7 (Fig. 1). Only 1 of 24 tested X orthologs, *POLA*, did not map to these 3 syntenic blocks. Hybridization of a TNI 1-specific microdissection DNA library revealed that TNI 1 corresponds to two smaller metacentric *Fugu* chromosomes. This explains the higher chromosome number (2n = 44) in *Fugu*, compared with *Tetraodon* (2n = 42) (data not shown). However, this split of TNI 1 does not affect the conservation of human X synteny. As 9 of 10 X orthologous genes on TNI 1 map to the short arm and the pericentromeric region (Fig. 2), we conclude that only these parts share homology with the human X. *UBE2A* was moved to the distal long arm of TNI 1 after the fusion of two ancestral pufferfish chromosomes (Grützner et al. 1999). This hypothesis is consistent with evidence that all TNI 1 orthologs tested map to the same small metacentric *Fugu* chromosome (data not shown). By comparing the relative position of orthologous genes on TNI 1 and human X (Fig. 2), it is evident that although large blocks of chromosomal synteny are conserved between pufferfish and humans, the gene order within the conserved blocks has changed. This indicates that intrachromosomal rearrangements occur much more frequently than interchromosomal rearrangements. This is also the case in the zebrafish (Barbazuk et al. 2000; Postlethwait et al. 2000; Woods et al. 2000) and chicken genomes (Burt et al. 1999; Schmid et al. 2000).

Comparative gene mapping data in different vertebrate species suggest that a sequence of fusion events has occurred (Fig. 1). Two X orthologous genes from TNI 1, *BTK* and *UBE2A*, and two X orthologous genes from TNI 7, *PHKA2* and *PGK1*, are syntenic on chicken chromosome 4 (Schmid et al. 2000). *PGK1* has also been mapped on the marsupial X (Watson et al. 1990). Therefore, we conclude that the chromosomal ancestors of TNI 1 and TNI 7 were fused before the avian-mammalian split. This ancestral vertebrate X_A, which evolved as an autosome >350 million years ago, already contained most genes (79% of the tested orthologs) from the present-day mammalian X, including many genes from the human X short arm. One X ortholog from TNI 7, *PDHA1*, is autosomal in marsupials (Fitzgerald et al. 1993). The most likely explanation for this is that the TNI 1 and TNI 7 syntenic groups fused to form the chromosome represented by the marsupial X and small segments of the ancestral X_A were translocated to a marsupial autosome(s), whereas most of the chromosome formed the X. It is plausible that genes that are syntenic in pufferfish and humans (*PDHA1* belongs to a group of 9 X orthologs on TNI 7) were linked in the last common ancestor of teleosts and tetrapods. In contrast, the autosomal localization of *OTC* and *MAOB* in monotremes and marsupials (Spencer et al. 1991) indicates that the TNI 2 block was added to the mammalian X after divergence of the eutherian, metatherian, and prototherian lineages ~170 million years ago. Consistent with this model and with one notable exception (*GK* was inverted onto the long arm), all TNI 2 orthologs map close to the putative fusion point in human Xp11 of the mammalian X-autosomal rearrangement (Wilcox et al. 1996). In addition, *OTC* maps to chicken chromosome 1 (Schmid et al. 2000) and is not syntenic with the chicken orthologs of TNI 1 and TNI 7 genes.

Comparative gene mapping in marsupials and monotremes (Watson et al. 1990; Spencer et al. 1991; Wilcox et al. 1996) has suggested that the human X long arm corresponds to the ancestral mammalian X, whereas most of the short arm was added in the eutherian lineage. In the pufferfish, many human Xp and Xq genes are syntenic on TNI 1 and TNI 7. In addition, zebrafish linkage groups (LGs) 9 and 23 are also endowed with both human Xp (*CXORF5* and *ASMTL* in LG 9; *EBP* in LG 23) and Xq orthologs (*API5L1* in LG9; *L1CAM*, *IDH3G*, and *SSR4* in LG 23). One possible interpretation of these findings is that most of the human X short arm was part of the ancestral X_A and does not represent a recent addition to the eutherian X. However, due to the lack of mapping data in species that are intermediate between fish and mammals, we cannot rule out that genes located on the short arm were separated from those on the long arm in mammalian ancestors during evolution.

In comparison with the pufferfish, the conservation of X chromosomal synteny appears to be relatively low in zebrafish (at least in current zebrafish maps). The human X chromosome genes are distributed on eight zebrafish LGs, each containing several orthologs and an additional three LGs with at least one X gene (Barbazuk et al. 2000; Postlethwait et al. 2000; Woods et al. 2000). However, because there is little overlap between the gene sets that have been analyzed in zebrafish and pufferfish, it is not possible to establish syntenic relationships between these two teleost models. In addition, the prevalence of duplicated chromosome segments in the zebrafish genome may confuse the results of zebrafish–human synteny mapping.

Disruption of Human Chromosome 9 Synteny

Comparative mapping of 17 human chromosome 9 orthologous BACs showed a considerably lower degree of conserved chromosomal synteny in pufferfish, compared with human X genes (Fig. 1). Four human 9 orthologous genes, including the evolutionarily conserved sex-determining gene *DMRT1* (Nanda et al. 1999; Raymond et al. 1999), are syntenic on TNI 8. Three other human 9 orthologs are linked on TNI 10. Numerous cohybridization experiments revealed that the remaining 10 (59%) human 9 genes tested are all distributed on different *Tetraodon* chromosomes. For example, *AK3* (from human 9p24) and *HSD17B3* (9q22) were colocalized with X orthologous BACs on TNI 1 and TNI 7, respectively. In the course of this study, we have identified marker clones for 17 of the 21 *Tetraodon* chromosomes (Table 1), which serve as in situ hybridization probes for anchoring linkage groups and sequenced contigs in the pufferfish map.

Because the zebrafish LG 5 contains 18 putative human 9 orthologs including *DMRT1* (Barbazuk et al. 2000; Postlethwait et al. 2000; Woods et al. 2000), it has been speculated that a common ancestor of human 9 and chicken

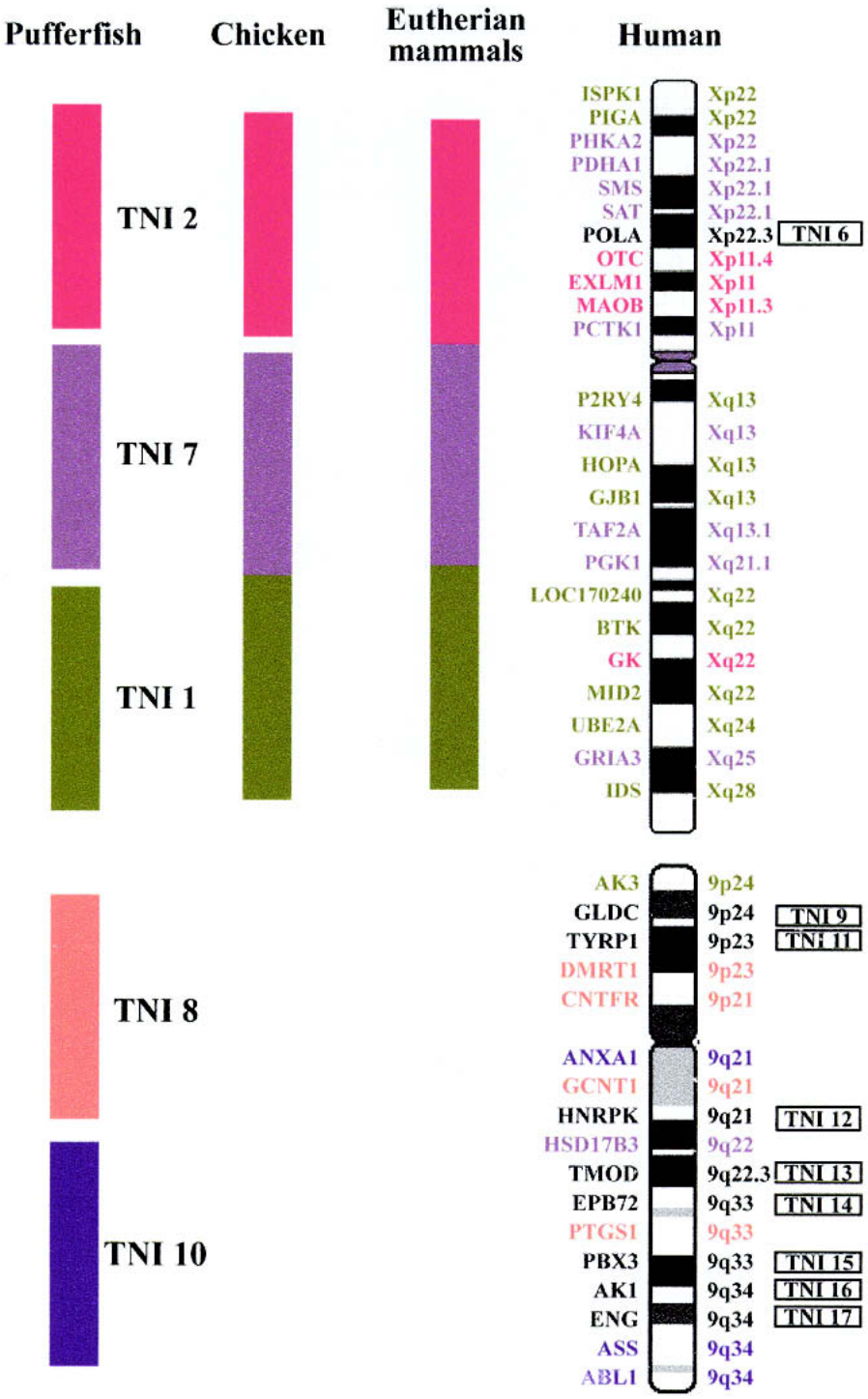

Figure 1 Syntenic relationships of human chromosomes X and 9 with the *Tetraodon* genome and an evolutionary model for the mammalian X. The *top* shows the common phylogenetic origin of pufferfish chromosomes TNI 1, TNI 2, and TNI 7 and the human X. At *left*, the three *Tetraodon* blocks of conserved synteny are depicted as green (TNI 1), red (TNI 2), and purple (TNI 7) bars. At *right* is shown the mosaic synteny of human X and the chromosomal localizations of the comparatively mapped genes. The gene order is based on genomic sequence data (http://www.genome.ucsc.edu/cgi-bin/hgGateway December 2001). The *POLA* gene, indicated in black, maps to a different *Tetraodon* chromosome (TNI 6) and, thus, does not belong to the delineated synteny groups. The two central schematic drawings represent hypothetical ancestral X chromosomes. Because human X orthologous genes from TNI 1 and TNI 7 are linked in chicken, we conclude that an ancestral X autosome contained these two sets of Xp and Xq genes even before the avian-mammalian split. Because the human X orthologs belonging to the TNI 2 block are autosomal in marsupials and monotremes, they represent a recent addition to the eutherian sex chromosomes. For simplicity, the three X syntenic *Tetraodon* chromosomes are depicted as blocks on the hypothetical X ancestors. However, this does not reflect the real gene order, which may differ significantly from both the order in the three *Tetraodon* chromosomes and in the human X. At *bottom* is shown a much lower degree of synteny conservation for human chromosome 9 in the pufferfish than for the X. Comparative mapping of human 9 orthologous genes reveals two syntenic blocks on TNI 8 (orange) and TNI 10 (blue). One gene each reside on TNI 1 (green) and TNI 7 (purple), respectively. The genes indicated in black all map to different TNI chromosomes.

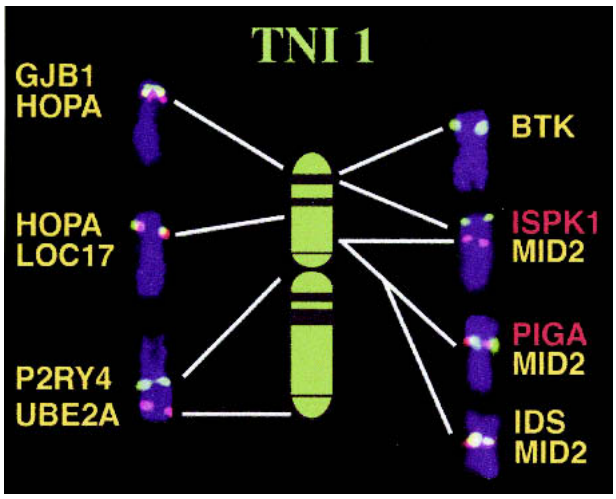

Figure 2 Localization of human Xp (indicated by red gene symbols) and Xq (green) orthologous genes on a TNI 1 ideogram. TNI 1 chromosomes with representative FISH signals of 10 genes were cut out from different metaphases.

Z may have already existed before the split of zebrafish and tetrapods, possibly functioning as a cryptic sex chromosome. Although our in situ hybridization results suggest disruption of human 9 and zebrafish LG 5 orthologous blocks in the pufferfish (i.e., *ANXA1* and *ASS* from LG 5 are syntenic on TNI 10, whereas *DMRT1* is on TNI 8), this does not exclude the possibility that *DMRT1* plays a crucial role in sex determination in fish. In addition, zebrafish LGs 21 and 25 show synteny of 6 and 15 human 9 orthologs, respectively.

Evolutionary Implications

The observed disruption of human chromosome 9 and chicken Z synteny in pufferfish suggests that, despite an excess of evolutionary inversions, interchromosomal changes must also have occurred in the teleost lineage. The extraordinary conservation of X synteny could be due to intrinsic chromosomal properties that confer selective pressure on large parts or the entire X to conserve its synteny. One such factor may be the enrichment for genes with a similar functional spectrum and/or expression pattern. The human X is known to contain an (approximately fourfold) excess of genes that are associated with general cognitive abilities (Marshall Graves and Delbridge 2001; Zechner et al. 2001). Many of these genes are expressed in both brain and testis and seem to be related to reproduction (Saifi and Chandra 1999). In evolutionary terms, these genes are usually highly conserved and engaged in basic cellular mechanisms, such as mRNA stabilization, cytoskeleton organization, and signaling cascades (First International Workshop on Comparative Genome Organization 1996; Lahn and Page 1999). We propose that this highly conserved X-linked array(s) of functionally important genes was already selected before the mammalian sex chromosomes evolved. This implies that many of these conserved genes must have acquired new or additional (brain and testis) functions that exert the so-called large X chromosome effect on general intelligence (Zechner et al. 2001) and fertility (Wu and Davis 1993; Turelli and Orr 1995) in humans.

METHODS

Tetraodon Probes

The construction and sequencing of *Tetraodon* BAC libraries were described previously (Roest Crollius et al. 2000b). The selection of BAC clones for in situ hybridization followed a forward-reverse similarity search strategy on the basis of end sequences. This ensured that the *Tetraodon* BAC probes contained orthologs of the desired human genes within the limits of known sequences from the human and *Tetraodon* genomes. In the forward experiment, a database of 734 partial and complete protein sequences from genes of human chromosomes 9 (107 genes) and X (627 genes) was compiled by combining information from SWISSPROT (Bairoch and Apweiler 2000) and Refseq (Wheeler et al. 2002). The set of human proteins was compared by use of `Exofish` (Roest Crollius et al. 2000a) to 951,256 *Tetraodon* whole genome shotgun sequences, including 47,599 BAC and 903,657 plasmid end sequences. This dataset represents more than two equivalents of the *Tetraodon* genome in randomly distributed single reads, or ~87% genome coverage. A total of 96 *Tetraodon* BAC end sequences were identified as matching one of the 734 human genes with `Exofish` criteria in addition to numerous additional plasmid end sequences. Each of the 96 human protein sequences was then aligned to the BAC and plasmid end sequences using the Smith-Waterman algorithm (Smith and Waterman 1981). Alignments were inspected manually to identify where the human protein sequence aligns best to a BAC end sequence as opposed to a plasmid end sequence. By use of these criteria, seven BAC end sequences were rejected for X chromosome genes. In the reverse experiment, each of the 89 remaining *Tetraodon* sequences was compared by use of the Smith-Waterman algorithm to the human International Protein Index (Apweiler et al. 2001) to verify that no other human protein sequence aligns better to the *Tetraodon* BAC end than the gene of interest from human chromosome 9 or X. Of 89 *Tetraodon* sequences, 40 found the original gene (17 on human 9 and 23 on X) in this reverse experiment. These 40 pairs of *Tetraodon* BAC ends and human genes were considered orthologs on the basis of a global screen between the available 24,147 entries in the human International Protein Index and ~87% of the *Tetraodon* genome sequence.

Clone ICRFp551C0473Q6 was isolated by hybridization of a microdissected TNI 1 library to arrayed *Tetraodon* cDNA clones (RZPD library no. 551). Sequencing and sequence comparisons revealed that it is the *Tetraodon* ortholog of human *BTK* (GenBank accession no. AJ308098).

Chromosomal Mapping (PufferFISH)

Metaphase spreads were prepared from primary *Tetraodon* fibroblast cultures, as described elsewhere (Grützner et al. 1999). For FISH, the slides were treated with 100 µg/mL RNase A in 2× SSC (pH 7.0), at 37°C for 30 min and with 0.01% pepsin in 10 mM HCl at 37°C for 10 min. After refixing for 10 min in 1× PBS, 50 mM $MgCl_2$, 1% formaldehyde, the preparations were dehydrated in an ethanol series. Slides were denatured for 1 min at 90°C in 70% formamide, 2× SSC (pH 7.0), and again dehydrated.

BAC DNAs and *BTK* cDNA were labeled with either biotin-16-dUTP or digoxigenin-11-dUTP by nick translation. For hybridization of one slide, 400 ng of biotinylated and/or digoxigenated probe DNA was coprecipitated with 50–100 µg sheared *Tetraodon* genomic DNA (as competitor), and 10–20 µg sheared human placental DNA (as carrier), and redissolved in 50% formamide, 10% dextran sulfate, 2× SSC. The hybridization mixture was denatured for 10 min at 80°C. Preannealing of repetitive DNA sequences was carried out for 30 min at 37°C. Next, the hybridization mixture was applied to each slide and sealed under a coverslip. The slides were hybridized

for at least 3 d in a moist chamber at 37°C. The slides were then washed three times for 5 min in 50% formamide, 2× SSC at 42°C and once for 5 min in 0.1× SSC (pH 7.0), at 60°C and blocked with 4× SSC, 3% BSA, and 0.1% Tween 20 at 37°C for 30 min. Probes were detected with FITC-conjugated avidin and Cy3-conjugated anti-digoxin antibody. Chromosomes and cell nuclei were counterstained with 1 µg/mL DAPI in 2× SSC for 1 min and mounted in 90% glycerol, 0.1 M Tris-HCl (pH 8.0), and 2.3% DABCO.

Images were taken with a Zeiss epifluorescence microscope equipped with a thermoelectronically cooled CCD camera (Photometrics CH250), which was controlled by an Apple Macintosh computer. `Vysis` imaging software was used to capture gray scale images and to superimpose the source images into a color image.

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http://www.ncbi.nlm.nih.gov/Homology; human-mouse homology map. This site provides access to various comparative maps between human and mouse chromosomes.

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