Original Contributions

Incorporating Mouse Genome
Mammalian Genome
From Genotype to Phenotype

Fxyd3 and *Lgi4* expression in the adult mouse: a case of endogenous antisense expression

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Abstract

We have investigated the expression of *Fxyd3* and *Lgi4* in the adult mouse by Northern blot analyses and in situ hybridization. Murine *Fxyd3* and *Lgi4* have been mapped to the same locus on mouse Chromosome (Chr) 7, where the last exon of *Fxyd3* completely overlaps with the 3′UTR in the last exon of *Lgi4*, which is transcribed in the opposite orientation. The *Fxyd3* gene (formerly called *Mat-8*) encodes an 8-kDa transmembrane protein that is upregulated in mammary tumors and can induce a chloride conductance upon RNA injection into *Xenopus* oocytes. *Fxyd3* is a member of the Fxyd family of which several members are tissue-specific regulators of ion channels. Murine *Lgi4* is a recently described member of the leucine-rich-repeat gene family Lgi. Northern blot analyses demonstrated a 0.6-kb *Fxyd3* transcript with abundant expression in the murine skin, colon, and mammary gland, but low level expression in the brain. In contrast, a 3.2-kb *Lgi4* transcript was abundant in brain, with lower level expression in colon. *Lgi4* transcription in the skin was detectable only by RT-PCR. A *Fxyd3*-specific sense cRNA probe hybridized to a transcript in Northern blots of brain and colon RNA that co-migrated with the *Lgi4* mRNA. In situ hybridization experiments revealed that both *Fxyd3* and *Lgi4* were expressed in the same tissue compartments in skin, uterus, intestine, mammary gland, and brain. These results demonstrate that *Fxyd3* and *Lgi4* transcripts potentially form double-stranded RNA molecules in many cell types in vivo, which may impact on their respective expression.

In recent years, the expression of endogenous antisense RNAs has been recognized as an important regulator of gene expression (reviewed in Lehner et al. 2002; Shendure and Church 2002). Several examples of endogenous antisense RNA effects have been characterized molecularly which attribute a role to antisense RNAs in X Chr inactivation, gene silencing, and interference with translation. Thus, the 40-kb-long Tsix RNA transcribed from the opposite strand of the *Xist* locus is associated with the future active X Chr and may regulate early steps in X Chr inactivation (Lee et al. 1999). Silencing of autosomal imprinted genes may also be caused by antisense transcripts. Recently it was shown that the non-coding Air RNA overlaps with the *Igf2r* gene transcribed from the opposite strand. The truncation of the AIR transcript by removing the region of sequence overlap causes loss of silencing of the *Igf2r*/*S1c22a2*/*S1c22a3* gene cluster on the paternal chromosome (Sleutels et al. 2002). These examples imply a potential interaction of these antisense RNA molecules and genomic DNA in genomic imprinting, although the exact molecular mechanism remains unknown.

Double-stranded RNA molecules formed by the expression of sense and antisense transcripts can also interfere with translation in vivo. During the development of the facial skeleton and the teeth, an Msx-1 antisense RNA is expressed in differentiated mesenchyme-derived cells, while the sense mRNA is found in the undifferentiated tissues. In vitro, the balance of *Msx1* expression and its endogenous antisense counterpart, *Msx-1 AS*, determines the expression of Msx1 protein in an odontoblastic cell line (Blin-Wakkach et al. 2001). Similarly, polyoma virus genes that are expressed early after infection of eukaryotic cells are down-regulated by nuclear antisense RNA transcribed from late genes. It has been proposed that these effects on early gene translation are caused by nucleotide modifications and nuclear retention of the early gene messenger RNAs, which would withdraw

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them from the cytosolic translation machinery (Kumar and Carmichael 1997).

A systematic in silico screen of ESTs expressed from the human and murine genomes has revealed that several hundred genes exist for which corresponding antisense ESTs can be found (Lehner et al. 2002; Shendure and Church 2002). Thus, regulation of gene expression by antisense transcripts may represent a more widespread natural mechanism than previously estimated. In order to corroborate this claim, however, it has to be demonstrated for each of these sequences that both sense and antisense transcripts of a given gene are expressed in the same cells at the same time.

In this report, we investigated the expression of the murine genes *Fxyd3* and *Lgi4*, which are transcribed from the same genomic locus on mouse Chr 7. The complete sequence of the murine genome has revealed that the *Lgi4* and *Fxyd3* genes are oppositely oriented and overlap in their 3′ terminal exon sequences.

The murine *Fxyd3* gene, formerly known as *Mat-8*, codes for an 8-kDa transmembrane protein that induces a chloride conductance upon expression in *Xenopus* oocytes (Morrison et al. 1995). The Fxyd3 protein contains a protein motif PFXYD, which is the signature of the Fxyd gene family in mammals (Sweadner and Rael 2000). Differential expression of *Fxyd3* has been observed in studies of tumorigenesis in the mammary and prostatic glands, suggesting that *Fxyd3* expression is elevated in tumor progression (Morrison and Leder 1994; Schieman et al. 1998; Vaarala et al. 2000). *Fxyd3* is normally expressed in uterus, colon, mammary gland, thymus, and stomach (Morrison et al. 1995), but the expression pattern has not been investigated by in situ hybridization. Several members of the Fxyd gene family have been shown to associate with and regulate the Na, K-ATPase in a tissue-specific manner (Beguin et al. 2001, 2002; Cornelius et al. 2001; Crambert et al. 2002; Garty et al. 2002). Only one member of the Fxyd gene family, *Fxyd4*, previously called *CHIF*, has been investigated by knock-out strategy, suggesting that *Fxyd4* regulates K exchange in the kidney (Aizman et al. 2002).

Murine *Lgi4* has been identified as a member of the Lgi gene family encoding leucine-rich repeat proteins (Gu et al. 2002). The founder member of this family, human LGI1, has been implied in the development of lateral temporal epilepsy. By genome blasting, the human LGI4 gene maps to chromosome 19q13, a region that was associated with benign familial infantile convulsions (Guipponi et al. 1997). Since the LGI4 protein would not be predicted to have ion channel properties, the LGI4 gene represents an atypical candidate for a locus associated with epilepsy, because all other genes, in which mutations have been associated with inherited epilepsy, encode ion channel proteins.

In this report, we show that murine *Fxyd3* and *Lgi4* are expressed at different levels in various organs of the adult mouse, but in almost all tissues the transcripts are detected in the same tissue compartments. Since the co-expression of sense and antisense transcripts in the same cells is the prerequisite for antisense effects to be assumed, these findings support the idea that antisense effects may have a role in regulating *Fxyd3* and *Lgi4* expression in normal and pathological organ function.

Materials and methods

Tissue preparation and in situ hybridization. C57BL/6 mice were fixed by perfusion with Bouin's solution, and the organs were embedded in paraffin wax. In situ hybridizations were carried out on 5-μm paraffin sections essentially as described in the manual ''Guide to Non-radioactive In Situ Hybridization'' (www.roche-applied-science.com/prod_inf/manuals/InSitu/InSi_toc.htm, Roche Applied Science) by using 4–6 ng of DIG-labeled probe per section, and a hybridization temperature of 49°C in the presence of 50% formamide. Stringency washes were performed at 53°C. Hybridization of DIG-labeled probe was visualized with alkaline phosphatase-conjugated anti-DIG antibodies (Roche, Mannheim, Germany) and BCIP/NBT as described in the Roche manual.

Northern blot analysis. Five μg of total RNA prepared from tissues by using Qiagen RNeasy kits was subjected to gel electrophoresis in 1.2% agarose gels in the presence of 0.7% formaldehyde. The RNA was transferred to positively charged Nylon membrane (Roche) by vacuum blotting with a Model 785 Vaccum Blotter (BIORAD). Hybridization was carried out at 68°C in Dig Easy Hyb (Roche). Digoxigenin-labeled cRNA probes were synthesized with the DIG RNA labeling kit from Roche (Mannheim, Germany).

Hybridization probes. The Fxyd3 cDNA was synthesized by PCR from our own C57BL/6 murine skin cDNA library using Fxyd3-specific primers forward GTCCATACTCTGCTTTCTCC (first nucleotide corresponding to nucleotide 21 of the published Fxyd3 cDNA gi.:1085067) and reverse TGAACAAAGAGCCTGCTACC (first nucleotide is nucleotide 490 of the published Fxyd3 cDNA). The sequence of the cDNA was found to be 100% identical to nucleotides 20–490 of the published 522-bp Fxyd3

cDNA sequence. The PCR product was reamplified with the same primers adding the T7 and T3 RNA polymerase binding sites at the 5′-ends (primers sense Fxyd3-T3: AATTAACCCTCACTAAAGGGGTCCATACTCTGCTTTCTCC; antisense Fxyd3-T7: GTAATACGACTCACTATAGGGCTGAACAAAGAGCCTGCTACC) by using the following PCR protocol: 25 cycles, T_M = 52°C, 1 min extension time. Sense and antisense cRNA probes were derived from the PCR products by in vitro transcription in the presence of DIG-labeled NTPs with T7 and T3 RNA polymerase.

The Lgi4 3′UTR fragment corresponding to nucleotides 1721 to 2618 of the published sequence (gi: 21362360) was synthesized by PCR on genomic DNA by using the primers Lf1 AGTGGCTGTGGGGTATTCTG and Lr1 AATTTCCCAGGACACCACTG (25 cycles, T_M = 56°C, 1 min extension time). The PCR product was TA-cloned into the pCRII vector (Invitrogen). The Digoxygenin-labeled cDNA probe of Lgi4 used in the Northern blot (Fig. 1b) was generated by PCR on the cloned Lgi4 3′UTR fragment by using primers Lf1 and Lr1 in the presence of Digoxygenin-labeled dNTPs. The cRNA probes for the in situ hybridization were derived by in vitro transcription from the pCRII cloned Lgi4 3′UTR fragment in the presence of DIG-labeled NTPs using T7 and Sp6 RNA polymerase. These probes were used for in situ hybridizations and the Northern blot hybridization in Fig. 1c.

RT-PCR. Exon-spanning RT-PCR analysis was performed on cDNA of day 10 pp dorsal skin of C57BL/6 mice (prepared with Clontechs SMART™ cDNA synthesis kit, according to the manufacturer's recommendations) with AccuTaq polymerase (Sigma-Aldrich). Lgi4-specific primers used were Lf2 forward GTGCTCTTTCACTGGGTTGG (the first nucleotide is 1249 of the published sequence), Lf3 forward TGCCACAAAACACTTTCAGG (the first nucleotide is 1317 of the published sequence), and reverse Lr2 CAAGAAGCTCCTGCCTTGAG (the first nucleotide is 2417 of the published sequence), utilizing the following PCR protocol: 30 cycles, T_M = 50°C, and 3-min extension time. Fxyd4-specific primers used were forward CTTCCTCTCTGCACCACTCC and reverse AAAATCAGCTTTGCCACACC with this protocol: 30 cycles, T_M = 52°C and 1-min extension time.

Results and Discussion

Tissue-specific expression levels of Fxyd3 and Lgi4 in the adult mouse. A sequence homology analysis revealed that the last exon of *Fxyd3* from nucleotides 332 to 518 of the published cDNA sequence is 100% homologous in reverse orientation to the sequence of the 3′UTR in the last exon of the murine *Lgi4* gene from nucleotides 3047 to 3233 (Fig. 1e). Thus, the genomic arrangement of the Fxyd3 and Lgi4 genes in the murine genome is similar to most of the known doubles-stranded RNA (dsRNA) loci, in which the two transcripts are encoded in tail-to-tail configuration (Shendure and Church 2002; Lehner et al. 2002). In order to investigate in which tissues *Lgi4* and *Fxyd3* are expressed, we performed Northern hybridizations using an *Lgi4*-specific cDNA probe derived from the 3′UTR, but outside the region of overlap with the *Fxyd3* gene, and an *Fxyd3*-specific probe that was a near full length antisense cRNA probe overlapping with *Lgi4* exon sequences (Fig. 1e).

The Northern blot hybridization of total RNA obtained from adult C57BL/6 organs using a Fxyd3-specific cRNA antisense probe (Fig. 1a) confirmed previous reports of a 0.55-kb Fxyd3 transcript (Morrison et al. 1995) that is abundantly expressed in colon, mammary gland, and uterus. This transcript was weakly detectable also in lung and thymus, but undetectable in brain, muscle, heart, testis, and ovary. Surprisingly, this experiment revealed that the highest *Fxyd3* expression level of all organs examined was in the skin (Fig. 1a).

Since *Fxyd3* is a member of a multigene family, we wanted to exclude that the *Fxyd3*-specific probes we used detected other Fxyd genes as well. Using the BLAST 2 Sequences program for nucleotide sequence comparison, we found that *Fxyd4* (also called *CHIF*) is the only member of the Fxyd gene family in mice that shows some homology with *Fxyd3* at the nucleotide level (76% homology between nucleotides 121 and 197 of *Fxyd3*). *Fxyd4* encodes a transcript of 552 bp, i.e., similar in size as *Fxyd3*, and is expressed in skin, kidney, epididymis, early embryos and oviduct (EST analyses given in *Fxyd4* Gene Card). RT-PCR analysis with gene-specific primers revealed that *Fxyd3* is expressed in the dorsal skin and colon, but not in kidney of C57BL/6 mice, while Fxyd4-specific primers generated RT-PCR fragments only in dorsal skin and kidney, but not colon (not shown). As we did not detect a signal in situ hybridizations on kidney with the *Fxyd3* cRNA probe (not shown), this probe should detect exclusively Fxyd3, but not Fxyd4 transcripts in hybridizations to blots and tissue sections with our procedures.

The expression of *Lgi4* in adult mouse tissues had previously been investigated only by RT-PCR (Gu et al. 2002). A Northern blot analysis of adult organs using a Lgi4 3′UTR-specific cDNA probe (probe Lgi4 3′UTR; see Fig. 1e) showed hybridization

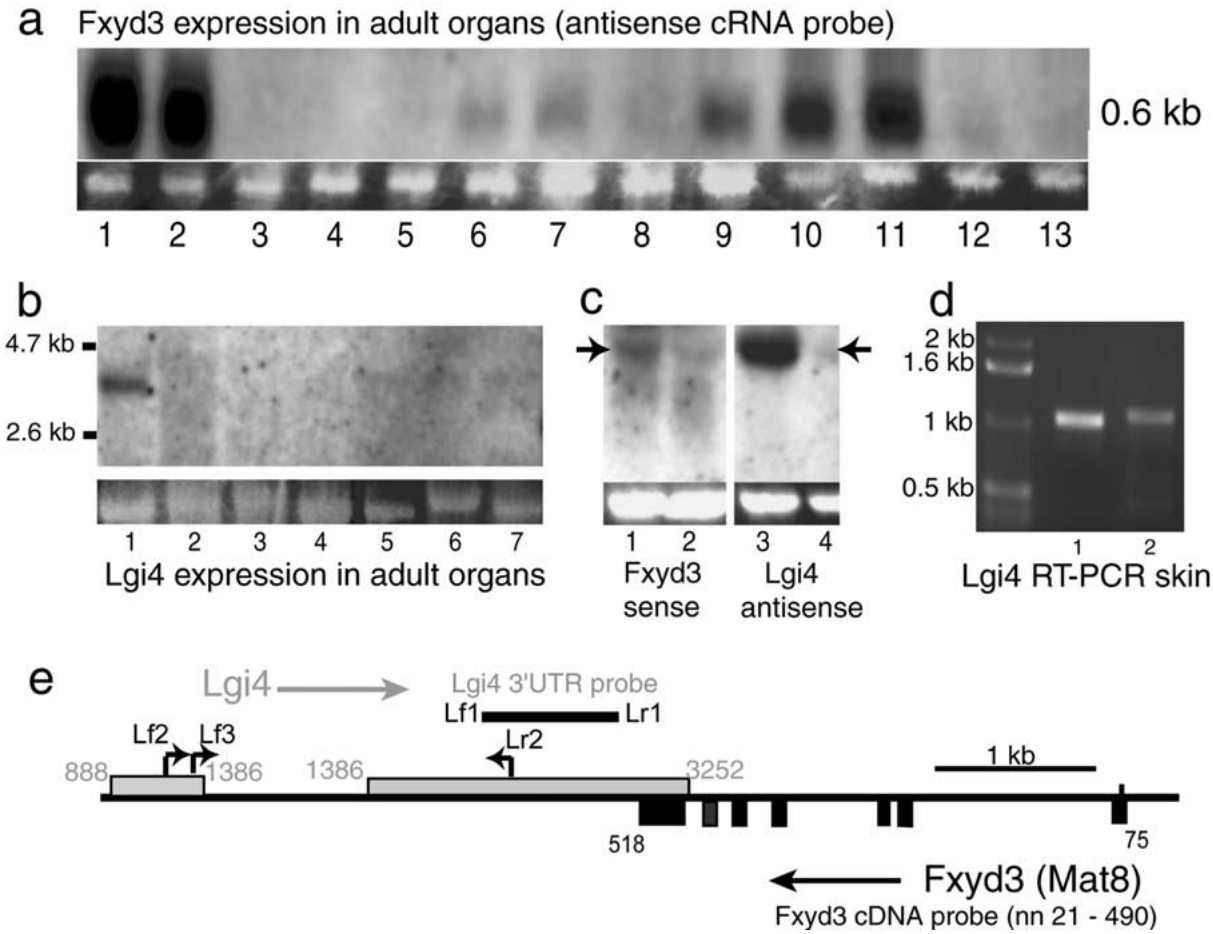

Fig. 1. Expression analysis of *Fxyd3* and *Lgi4* by Northern blot analysis. **a**. Northern blot hybridization of total RNA prepared from adult C57BL/6 organs by using a Fxyd3 cRNA antisense probe. Lanes 1, dorsal skin day 22pp; 2, dorsal skin day 90pp; 3, hindleg muscle; 4, forebrain; 5, heart; 6, lung; 7, thymus; 8, spleen; 9, colon; 10, mammary gland; 11, uterus; 12, ovary; 13, testis. **b**. Northern blot hybridization of total RNA prepared from adult C57BL/6 organs by using DIG-PCR labeled Lgi4 3′UTR DNA probe (see d). Lanes 1, forebrain; 2, dorsal skin; 3, colon; 4, thymus; 5, lung; 6, ileum; 7, mammary gland. The bars indicate bands of the DIG-labeled RNA molecular weight marker I (Roche). **c**. Northern blot hybridization of forebrain (1) and colon (2) total RNA using the Fxyd3 sense cRNA probe (left) and Lgi4 antisense cRNA probe (right), that were also used for the in situ hybridization experiments (Fig. 3). The blotting was done from the same gel; the filter was cut and hybridized separately with the probes and exposed to autoradiography film with the membranes realigned. **d**. RT-PCR on C57BL/6 dorsal skin (day10pp) cDNA using Lgi4-specific primers Lf1, Lf2, and Lr1 that span the last intron of Lgi4 (indicated in 1e). The RT-PCR reaction yields the expected 1.1-kb fragment indicating expression of Lgi4 mRNA in skin. **e**. Draft of the *Fxyd3* genomic locus (Fxyd3 exons in black) overlapping with the 3′ end of the Lgi4 gene (*Lgi4* exons in grey). The genomic location and orientation of the exon sequences was deduced by aligning the published cDNA sequences of Fxyd3 (gi:1085067), Lgi4 (gi;21362360) with the sequence of the genomic contig RP23-28D21 (gi:12658115).

to a transcript of the predicted length of 3.2 kb and demonstrated that *Lgi4* expression is high in the brain and low in small intestine, lung, and mammary gland (Fig. 1b). Northern blot hybridization with a labeled cDNA probe was not sensitive enough, however, to detect Lgi4 mRNA in skin, colon, and thymus. Yet, hybridization with a Lgi4-specific antisense cRNA probe, which binds to target mRNAs with higher affinity than cDNA probes, did detect the Lgi4 transcript in colon (Fig. 1c). *Lgi4* expression in skin was detectable only by RT-PCR (Fig. 1d). Thus, *Lgi4* is expressed in many organs, but at highly variable abundance.

Since it has previously not been shown that the 3′UTR sequences of Lgi4 are indeed expressed in murine tissues and that both Fxyd3 and Lgi4 are co-expressed in the same tissue, we examined whether hybridization of both a Fxyd3 sense cRNA probe and a Lgi4 3′UTR-derived antisense cRNA probe would detect the 3.2-kb Lgi4 mRNA in Northern blot analyses of brain and colon. Indeed, both probes hybridized to comigrating transcripts of 3.2 kb and

also reflected the difference of abundance of *Lgi4* expression in brain versus colon (Fig. 1c). Therefore, the expression analyses have shown that the levels of *Fxyd3* and *Lgi4* expression were reciprocal in some mouse tissues, to name the extremes: the expression of *Fxyd3* was abundant in skin and low in brain, whereas *Lgi4* expression was high in brain and low in skin.

Fxyd3 and Lgi4 are expressed in the same tissue compartments. In order to examine in which tissue compartments *Fxyd3* and *Lgi4* were expressed, in situ hybridizations with Fxyd3-specific and Lgi4-specific antisense cRNA probes were performed. The probes used were from the same batches used for the filter hybridizations in Fig. 1a and 1c. Expression of both *Fxyd3* and *Lgi4* was found in the bronchial epithelium of the lung and free cells in the peribronchial tissue (Fig. 2a), the uterine epithelium and the uterine glands (Fig. 2b), the mammary gland (Fig. 2d), the epithelium of the small intestine (Fig. 2e), the Purkinje cells and less strong the granular cells of the cerebellum (Fig. 2f), the neurons of the dentate gyrus (Fig. 2g), large neurons in the cerebral cortex (Fig. 2h), scattered cells in the cortex of the thymus (not shown), as well as skin, colon, stomach and hippocampus (see Fig. 3). The *Fxyd3* in situ signal in the urinary bladder was very strong in the epithelium and in smooth muscle cells in the bladder wall, while only a weak signal with the Lgi4 probe was found in the epithelium. There was no in situ hybridization with either probe to cardiac muscle in the heart ventricle, skeletal muscle.and surrounding connective tissue, and liver (not shown).

Because of the genomic exon overlap of *Fxyd3* and *Lgi4*, we also examined whether a sense probe of Fxyd3 and an antisense probe of Lgi4 hybridize to the same cell populations in in situ hybrization experiments. Fxyd3 antisense, Fxyd3 sense, and Lgi4 antisense cRNA probes hybridized to the epidermis and hair follicle epithelium in the adult dorsal skin (Fig. 3a–c), to the epithelium of the gastric glands in the stomach (Fig. 3 e–g), to the crypts of the colon (Fig. 3i–k), and the pyramidal cells of the cornu ammonis in the hippocampus (Fig. 3m–o). In all these tissues, the Lgi4 3′UTR sense probe showed no hybridization, indicating that there was no unspecific hybridization in these tissue sections (Fig. 3d,h,l,p). Thus, using in situ hybridizations, we could demonstrate that, with the exception of the urinary bladder, *Fxyd3* and *Lgi4* are expressed in the same cell populations in the organs of the adult mouse.

In recent years, several examples of endogenous dsRNAs have been shown to be involved in diverse mechanisms of gene regulation such as genomic imprinting, RNA interference (RNAi), alternative splicing, translational control, X-inactivation, and RNA editing. The formation of dsRNA duplexes between murine Fxyd3 and Lgi4 in neurons of the brain may thus provide a unforeseen link between the *Lgi4* gene implied in the development of epilepsy, but without ion channel properties of its own, and *Fxyd3*, that can induce a chloride conductance upon expression in *Xenopus* oocytes.

In a computational survey of mouse and human EST-sequences (Shendure and Church 2002), 217 transcripts that may potentially form dsRNA were identified, most of them being novel. Thus, roughly 300 hypothetical dsRNA-transcripts are predicted, but for most of them no function has been proposed. However, before functional annotations to potential dsRNAs can be attempted, it should be demonstrated that the genes involved are expressed in the same cells. In this report, we have established by in situ hybridizations that the *Lgi4* and *Fxyd3* genes are indeed expressed in the same cell populations in a large variety of organs of the adult mouse.

Previously characterized examples of dsRNA transcripts can be categorized according to their respective location in the genome: first, antisense RNAs transcribed from loci distinct from their targets, e.g., *lin-4* in *C. elegans*, usually form imperfect duplexes with the complementary regions of their targets and are mostly short (Lee et al. 1993). On the other hand, those transcripts that originate from the opposite strand of the same genomic locus can form long and perfect duplexes with their target. The sequence of the murine genome has revealed that the murine *Fxyd3* and *Lgi4* genes belong to the latter group. The RNA of *Lgi4* displays a long 3′-UTR, not found in the other members of the LGI family (Gu et al. 2002). This 3′-UTR of murine *Lgi4* completely overlaps the last exon of the *Fxyd3* gene, which thus might form long perfect duplexes provided that both RNAs are transcribed in the same cells.

In most cases of dsRNA loci, the expressed antisense transcript is non-coding, as for example in *MsxI* (Blin-Wakkach et al. 2001) or represents only a subset of the exons, as for example in the *FGF-2* gene (Li and Murphy 2000). In both cases, the synthesis of the proteins encoded by these genes is suppressed by the transcription of the antisense sequences. In the case of the *Xist* and its antisense counterpart *Tsix*, neither transcript codes for a protein, but *Tsix* expression neutralizes the activity of *Xist* in X-Chr inactivation (Lee et al. 1999). Thus, the above-mentioned genes are examples of antisense pairs of genes, in which the non-coding partner may have been conserved in evolution for the main reason to regulate the expression or the activity of the other.

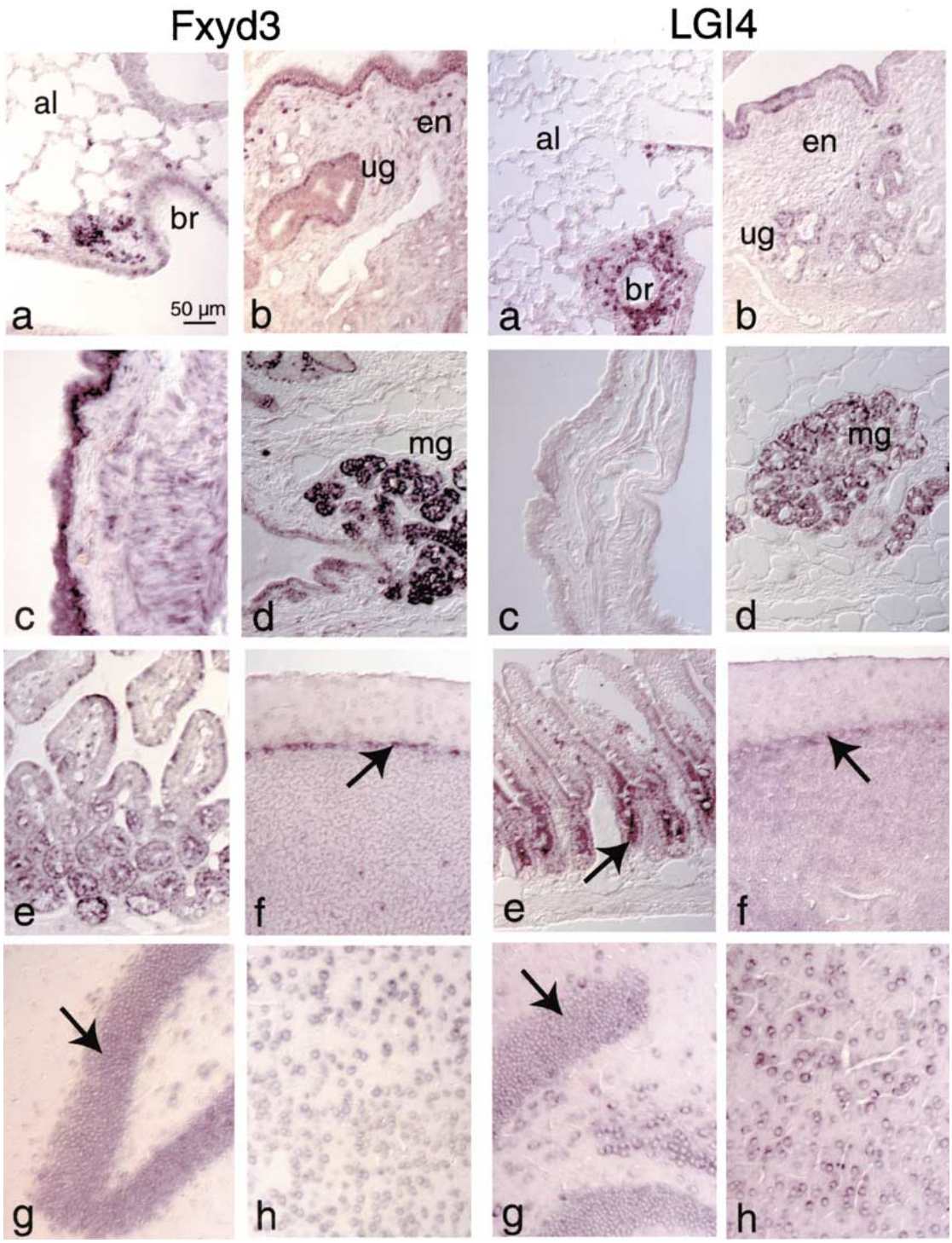

Fig. 2. In situ hybridization with Fxyd3-specific (left) and Lgi4 3′UTR-specific antisense cRNA probes on C57BL/6 adult organs. **a**, lung; **b**, uterus; **c**, urinary bladder; **d**, mammary gland; **e**, ileum; **f**, cerebellum; **g**, hippocampus; **h**, cerebral cortex. al, alveoles; br, bronchus; mg, mammary gland; en, endometrium; ug, uterine glands. Arrows in e point at the crypts of the intestinal mucosa, in f at Purkinje cells in the cerebellum, in g at neurons in the dentate gyrus. Probes are indicated on the side of the images. Size reference bar in a equals 50 µm. Same magnification in all images.

The antisense unit represented by the genes *Fxyd3* and *Lgi4* is one with both genes coding for proteins, which is true for just over half the overlapping, oppositely oriented transcriptional units in the mammalian genome (Shendure and Church 2002; Lehner et al. 2002). Both *Fxyd3* and *Lgi4* are members of

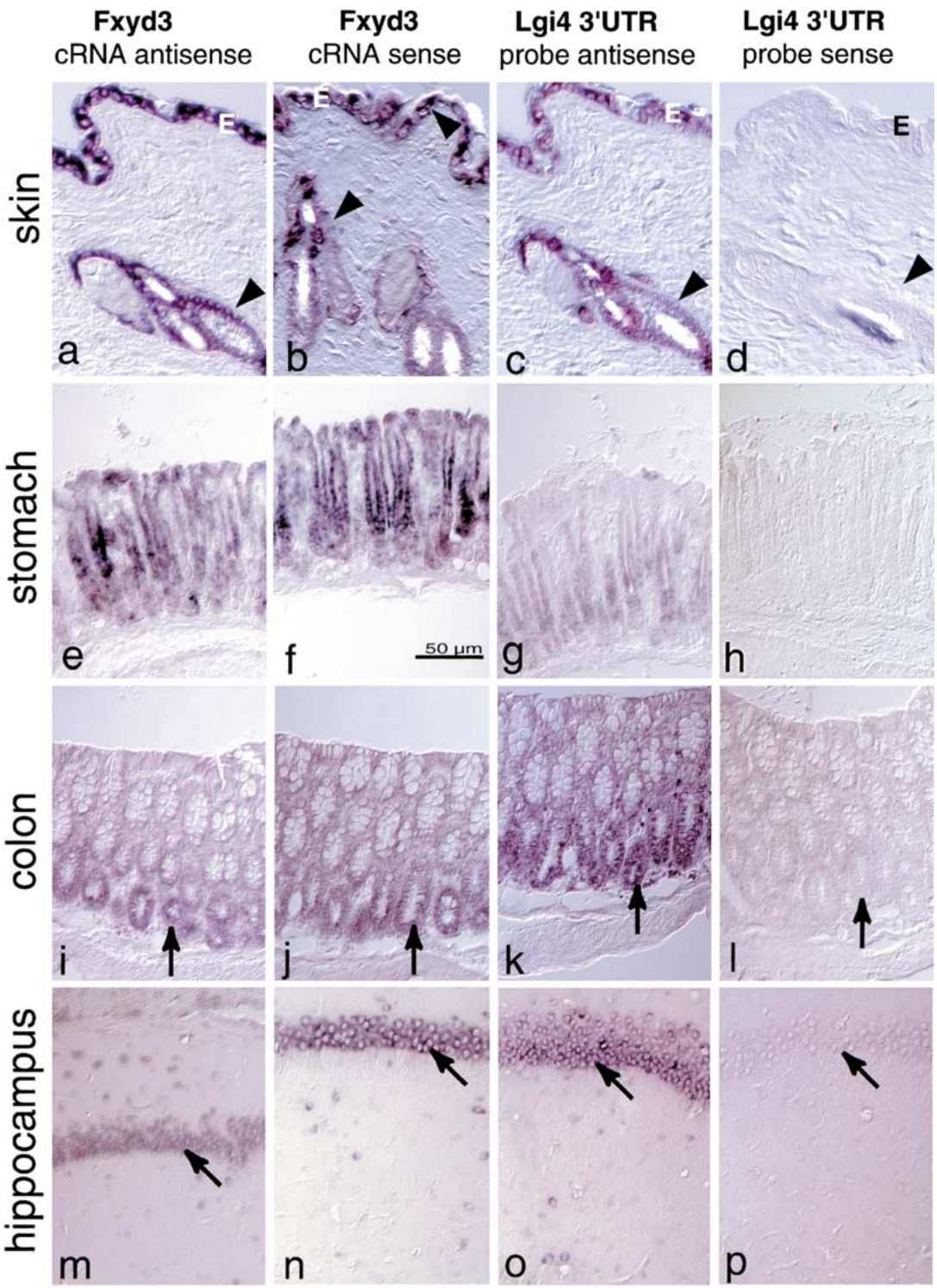

Fig. 3. In situ hybridizations with Fxyd3-specific antisense and sense cRNA probes, and Lgi4 3′UTR-specific sense and antisense cRNA probes. Tissues: a–d dorsal skin (E, epidermis) with telogen hair follicles (arrowheads); e–h, stomach, corpus ventriculi. i–l, colon (arrows indicate crypts); m–p, hippocampus, cornu ammonis (arrows indicate pyramidal neurons). Size reference bar in f. The probes applied are indicated on top of the columns of images.

multigene families, and it is at present not known from mutant mice whether their normal functions are indispensable for development or organ function.

In this report, we have shown that the murine *Fxyd3* and *Lgi4* genes produce partially overlapping transcripts of opposite orientation in the adult

mouse and may thus form double-stranded RNA duplexes in vivo. Future studies involving the exogenous control of the expression of *Fxyd3* in murine cells and tissues will address the question whether a putative reciprocal antisense regulation of *Fxyd3* and *Lgi4* expression is of biological relevance.

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