parties or be used to prevent deception of third parties by a whistle-copying dolphin. Although vocal matching is common in birds (*22*), bottlenose dolphins are the only nonhuman mammals in which matching interactions with learned signal types have been found. The occurrence of such matching or labeling has been hypothesized to have been an important step in the evolution of human language (*23*, *24*). The results presented here show that reaching that step can be achieved in very different environments.

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PAX8-PPARγ1 Fusion in Oncogene Human Thyroid Carcinoma

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Chromosomal translocations that encode fusion oncoproteins have been observed consistently in leukemias/lymphomas and sarcomas but not in carcinomas, the most common human cancers. Here, we report that t(2;3)(q13;p25), a translocation identified in a subset of human thyroid follicular carcinomas, results in fusion of the DNA binding domains of the thyroid transcription factor PAX8 to domains A to F of the peroxisome proliferator–activated receptor (PPAR) γ1. PAX8-PPARγ1 mRNA and protein were detected in 5 of 8 thyroid follicular carcinomas but not in 20 follicular adenomas, 10 papillary carcinomas, or 10 multinodular hyperplasias. PAX8-PPARγ1 inhibited thiazolidinedione-induced transactivation by PPARγ1 in a dominant negative manner. The experiments demonstrate an oncogenic role for PPARγ and suggest that PAX8-PPARγ1 may be useful in the diagnosis and treatment of thyroid carcinoma.

Chromosomal translocations encoding fusion oncoproteins are common in leukemias/lymphomas and sarcomas (*1*) but have been identified in only a single adult human (thyroid papillary) carcinoma. Compared with fusion oncoproteins in noncarcinomas, those in thyroid papillary carcinoma occur at relatively low frequency and are derived from several distinct gene fusion events, the most common of which result from subtle chromosomal inversions (*2*). Most cytogenetic abnormalities characterized in carcinomas to date are deletions that remove growth-restraining tumor suppressor genes. These findings imply (i) that most human carcinomas develop through translocation-independent events, or (ii) that most carcinoma translocations are subcytogenetic alterations that are difficult to detect in complex carcinoma karyotypes (*3*). Distinction between these alternatives is important because carcinomas constitute up to 90% of human cancers.

We have determined the genetic consequences of t(2;3)(q13;p25), a chromosomal translocation identified in human thyroid follicular carcinomas. Three consecutive thyroid follicular carcinomas (*4*) karyotyped in our laboratory exhibited t(2;3)(q13;p25), which has been reported previously in thyroid follicular tumors, including one with lung metastases (*5*). We first mapped the 3p25 and 2q13 translocation breakpoints using interphase fluorescence in situ hybridization (FISH) (*6*). The 3p25 breakpoint region was narrowed to ~600 kb and was bordered by yeast artificial chromosomes (YACs) 753f7 (telomeric) and 903e6 (centromeric) (Fig. 1A). Hybridization with flanking YACs 753f7 and 932f3 confirmed 3p25 rearrangements in tumor but not normal cells (Fig. 1B). The 2q13 breakpoint was localized within overlapping YACs 989f12 and 896a8 (Fig. 2A) to a region containing *PAX8*, which encodes a paired domain transcription factor essential for thyroid development (*7*). A *PAX8*-containing bacterial artificial chromosome (BAC), 110L24, crossed the 2q13 breakpoint and cohybridized with 3p25 YAC 753f7 (Fig. 2B), consistent with involvement of *PAX8* and a 3p25 partner in the translocation.

To identify the 3p25 partner, we performed rapid amplification of cDNA ends (RACE) using 5′ *PAX8* primers (*8*). Sequence analysis of RACE products from t(2;3)-positive follicular carcinomas (*8*) revealed in-frame fusion of *PAX8* to the peroxisome proliferator–activated receptor γ (*PPARγ*) gene (Fig. 3A). *PPARγ* has been mapped to 3p25 (*9*), and a PPARγ-containing BAC, 321f13, crossed the 3p25 breakpoint and cohybridized with 2q13 YAC 989f12

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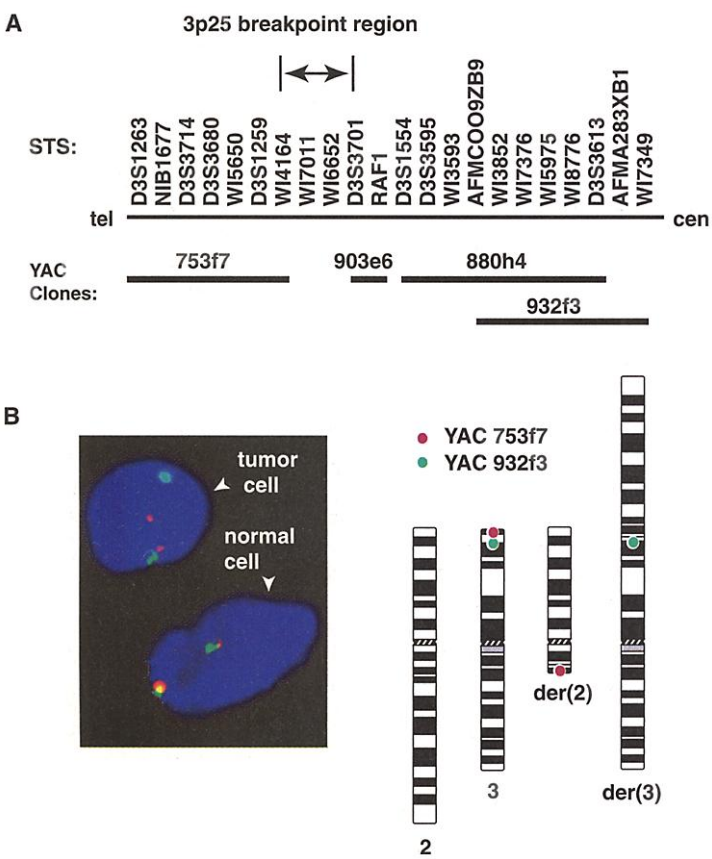
(*10*). Sequencing of partial and full-length *PAX8-PPARγ* transcripts from follicular carcinomas revealed fusion of *PAX8* exons 1 to 7, 1 to 8, 1 to 9, or 1 to 7 plus 9 to *PPARγ* exons 1 to 6 (Fig. 3A). The different *PAX8-PPARγ* forms were coexpressed (*10*) and appear to result from alternate splicing of *PAX8* (*11*).

The predicted PAX8-PPARγ fusion proteins (molecular mass 87 to 97 kD) are composed of the paired and partial homeobox DNA binding domains of PAX8 (*11*) fused to the DNA binding, ligand binding, RXR dimerization, and transactivation domains (A to F) of PPARγ1 (*9*) (Fig. 3A). The transactivation domains of PAX8 and the 28 NH_2-terminal amino acids of PPARγ (present in PPARγ2) are absent from the fusion proteins. Using a monoclonal antibody (mAb) to wild-type PPARγ, we immunoprecipitated a 98-kD putative PAX8-PPARγ1 protein from lysates of metabolically radiolabeled (*12*) t(2;3)-positive follicular carcinoma but not t(2;3)-negative follicular adenoma cultures (Fig. 3B). Wild-type PPARγ was not detected (Fig. 3B).

PAX8-PPARγ1 expression in thyroid follicular carcinomas was investigated by Northern blots and by reverse-transcription polymerase chain reaction (RT-PCR) (*8*). Using a *PPARγ* cDNA probe, we detected wild-type *PPARγ* (1.8 kb) in control Moser adenocarcinoma cells, normal thyroid tissue, and t(2;3)-negative thyroid follicular carcinomas (Fig. 3C, lanes 1 to 3). In contrast, wild-type and fusion *PPARγ* mRNA species (3 kb) were detected in t(2;3)-positive follicular carcinomas (Fig. 3C, lane 4). Hybridization with a *PAX8* cDNA probe confirmed that the 3-kb transcript was *PAX8-PPARγ1*, which migrated near wild-type *PAX8* (3.1 kb) (*10*). Nested RT-PCR with primers in exons 6 and 7 of *PAX8* and exon 1 of *PPARγ* confirmed the presence of *PAX8-PPARγ1* in five of eight follicular carcinomas, but not in 20 follicular adenomas, 10 papillary carcinomas, or 10 multinodular hyperplasias (*10*). One RT-PCR–negative follicular carcinoma contained t(2;3)(q13;p25) by cytogenetic analysis, suggesting that this translocation was associated with a different *PAX8-PPARγ1* breakpoint. The second RT-PCR–negative follicular carcinoma was immunoreactive for PPARγ (see below) and exhibited 3p25 but not 2q13 rearrangements by FISH (*10*), suggesting that this fusion consisted of *PPARγ* and a non-*PAX8* partner. The third RT-PCR–negative follicular carcinoma exhibited no evidence of *PAX8-PPARγ1*.

The reciprocal (*PPARγ1-PAX8*) fusion event was detected by nested RT-PCR in some follicular carcinomas but not on Northern blots or by immunoprecipitation (*10*). Cytogenetic analyses showed that the derivative chromosome harboring the reciprocal translocation was deleted in some follicular carcinomas (*10*). This finding suggests a reduced, if not negligible, oncogenic role for *PPARγ1-PAX8* relative to *PAX8-PPARγ1*.

Immunohistochemistry (*13*) performed with wild-type PPARγ mAb revealed strong, diffuse nuclear expression of PAX8-PPARγ1 in paraffin-embedded thyroid tumor sections in seven of the eight thyroid follicular carcinomas (Fig. 3D), whereas the 20 follicular adenomas, 10 papillary carcinomas, and 10 multinodular hyperplasias exhibited only faint, focal nuclear PPARγ expression (*10*). PAX8-PPARγ1 nuclear immunoreactivity in follicular carcinomas



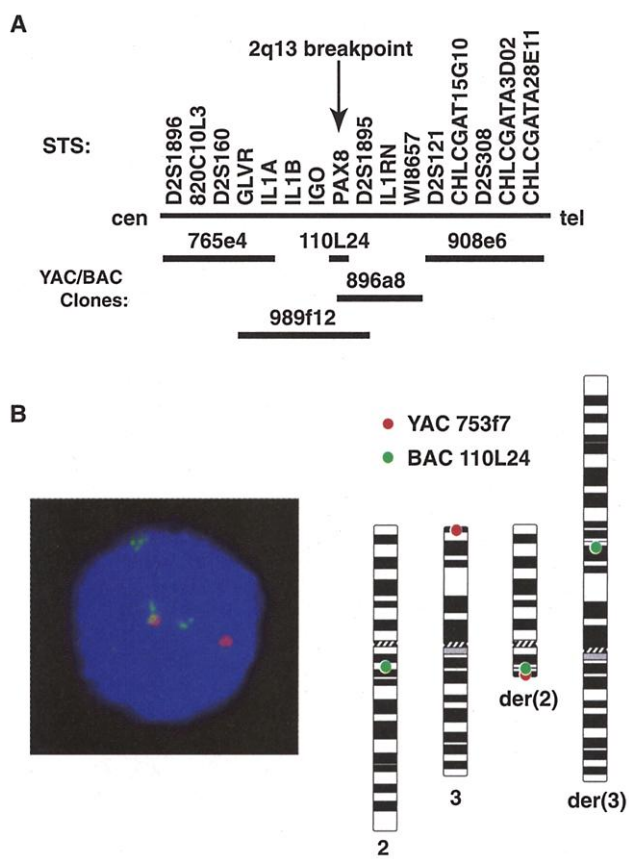


Fig. 1 (left). (**A**) 3p25 breakpoint in t(2;3)(q13;p25). Interphase FISH on touch preparations of thyroid follicular carcinoma cells (*6*) localized the 3p25 breakpoint to a ~600-kb region bordered by YACs 753f7 (telomeric) and 903e6 (centromeric). The 3p25 map is based on data from the Whitehead/Massachusetts Institute of Technology Center for Genome Research and chromosome 3 mapping efforts (*22*). (**B**) Detection of 3p25 rearrangements in thyroid follicular carcinomas. Splitting of YACs 753f7 (red) and 932f3 (green), which flank the 3p25 breakpoint, detected rearrangements in tumor but not normal cells. Yellow is generated by overlapping red and green signals. der, derivative chromosomes formed from t(2;3). **Fig. 2 (right).** (**A**) 2q13 breakpoint in t(2;3)(q13;p25). The 2q13 breakpoint was localized by interphase FISH within YACs 989f12 and 896a8 and BAC 110L24, which contained the *PAX8* gene. The 2q13 map is based on data from the Whitehead/Massachusetts Institute of Technology Center for Genome Research and chromosome 2 mapping efforts (*23*). (**B**) BAC 110L24 crosses the 2q13 breakpoint. BAC 110L24 (green) crossed the 2q13 breakpoint and cohybridized with 3p25 YAC 753f7 (red).

was inhibited by preincubation of the antibody with a blocking PPARγ synthetic peptide (Fig. 3D).

To test the biologic function of PAX8-PPARγ1, we measured its ability to transactivate PPARγ response elements (PPREs) in U2OS cells (*14*). PAX8-PPARγ1 was ineffective compared with PPARγ1 in stimulating troglitazone-induced transcription at a multimerized, perfect DR1 site (DR1), at a multimerized PPRE derived from the acyl CoA oxidase gene, or at a native PPRE from the aP2 enhancer (Fig. 4A). Coexpression of PAX8-PPARγ1 and PPARγ1 (1:1) led to complete inhibition of rosiglitazone-induced transactivation by PPARγ1 on the aP2 enhancer (Fig. 4B); hence, PAX8-PPARγ1 functions as a dominant negative suppressor of wild-type PPARγ activities.

Little is known about the pathogenesis of human thyroid follicular tumors. Our experiments show that formation of t(2;3)(q13;p25)/*PAX8-PPARγ1* is a frequent event in human thyroid follicular carcinoma. *PAX8* is a transcription factor essential for genesis of the thyroid follicular epithelial cell lineage (*7*). Transcription factors involved in lineage differentiation are frequent targets of chromosomal rearrangements in leukemias and sarcomas (*15*). PAX8-PPARγ1 functional domains are nearly identical to respective PAX and nuclear receptor functional domains in the rhabdomyosarcoma PAX3-FKHR (*16*) and acute promyelogeneous leukemia PML-RARα (retinoic acid receptor α) (*17*) oncoproteins. These similarities argue that homologous molecular cytogenetic mechanisms underlie at least some carcinoma and noncarcinoma types.

Our functional experiments indicate that PAX8-PPARγ1 does not stimulate thiazolidinedione-induced transcription and that PAX8-PPARγ1 can inhibit PPARγ1 transcriptional activation. The observation that PAX-PPARγ1 is expressed at higher levels than PPARγ in t(2;3)-positive follicular carcinomas is consistent with such a dominant negative mechanism. Recent studies showing that PPARγ ligands can inhibit growth and promote differentiation of cancer cell lines (*18*) and that heterozygous PPARγ point mutations in colon carcinomas impede ligand binding (*19*) have raised the possibility that abrogation of normal PPARγ function is important in cancer. The discovery of PAX8-PPARγ1, which likely plays an early, critical role in thyroid follicular oncogenesis, supports this hypothesis. It is also likely that PAX8-PPARγ1 deregulates PAX8 pathways in thyroid cells and that fusion of PAX8 and PPARγ engenders novel activities that promote thyroid carcinoma formation.

Our findings suggest that the type of fusion oncoprotein formed in thyroid follicular cells may determine the type of thyroid cancer produced. Whereas papillary thyroid carcinomas

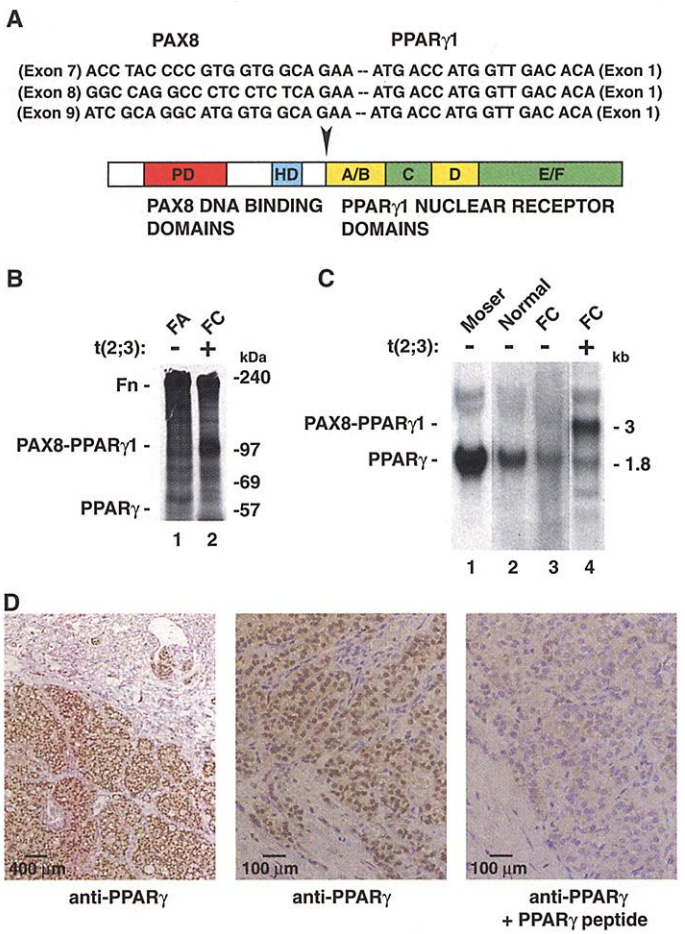


Fig. 3. (**A**) PAX8-PPARγ1 structure. The *PAX8-PPARγ1* mRNA breakpoints juxtapose exons 7, 8, or 9 of *PAX8* with exon 1 of *PPARγ*. The predicted PAX8-PPARγ1 fusion proteins contain PAX8 paired (PD) and partial homeobox (HD) DNA binding domains and all PPARγ1 nuclear receptor domains (A to F). (**B**) A PAX8-PPARγ1 fusion protein (98 kD) is immunoprecipitated (*12*) from thyroid follicular carcinoma (FC, lane 2) but not follicular adenoma (FA, lane 1) cells. (**C**) t(2;3)-positive follicular carcinomas express *PAX8-PPARγ1* mRNA. Moser colonic adenocarcinoma cells (lane 1), normal thyroid (lane 2), and t(2;3)-negative follicular carcinomas (lane 3) expressed wild-type *PPARγ* (1.8 kb), whereas t(2;3)-positive follicular carcinomas expressed both wild-type *PPARγ* and fusion *PAX8-PPARγ1* (3 kb) transcripts. (**D**) Diffuse, strong nuclear immunoreactivity for PAX8-PPARγ1 was observed in t(2;3)-positive follicular carcinomas stained with wild-type PPARγ mAb (*13*). The nuclear immunoreactivity was inhibited by preincubation of the antibody with a synthetic peptide against which it was raised.

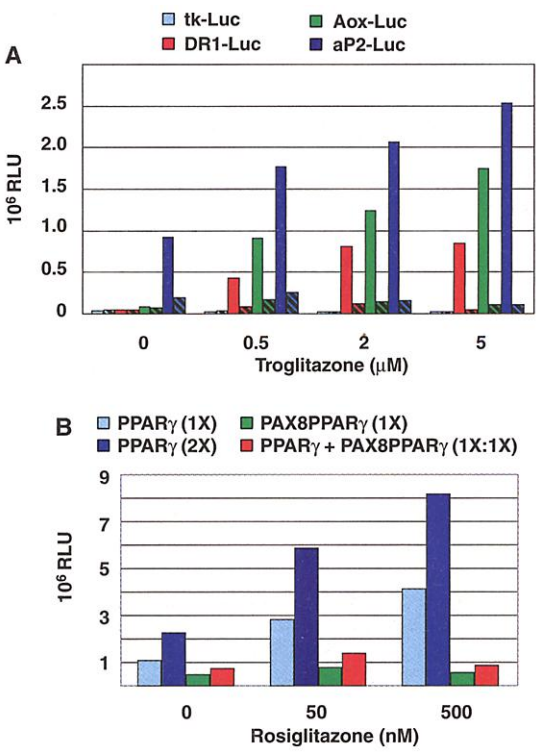


Fig. 4. (**A**) PAX8-PPARγ1 is ineffective at promoting troglitazone-induced transcriptional activation in U2OS cells. PAX8-PPARγ1 (hatched bars) was ineffective compared to PPARγ1 (solid bars) at promoting transcriptional activation of luciferase reporters containing multimerized perfect DR1 PPRE (red), a multimerized PPRE derived from the acyl CoA oxidase gene (Aox) (green), and a native PPRE from the aP2 enhancer (dark blue) in transient transfection experiments (*14*). A luciferase reporter lacking a PPRE (light blue) served as a control (RLU, relative light units). (**B**) PAX8-PPARγ1 acts through dominant negative inhibition of PPARγ1. Cotransfection of *PAX8-PPARγ1* and *PPARγ1* (1:1) resulted in complete inhibition of rosiglitazone-induced transcriptional activation by PPARγ1 at the aP2 PPRE.

express the receptor tyrosine kinase oncoproteins RET or NTRK1 (*2*), follicular carcinomas express the transcription factor oncoprotein PAX8-PPARγ1. This may account, at least in part, for the phenotypic and clinical differences between these two tumors.

PAX8-PPARγ1 may aid in the differential diagnosis of follicular carcinomas (potentially malignant) from follicular adenomas (benign) in fine needle aspiration biopsies. This would help to reduce the number of thyroid surgeries performed, increase the percentage of malignancies resected, and reduce the costs of treating patients with thyroid nodules (*20*). Notably, nuclear receptor ligands for PML-RARα have proven highly effective in treatment of patients with acute promyelogeneous leukemia (*21*). It will therefore be important to determine whether ligands involving PPARγ pathways can benefit patients with thyroid carcinoma as an adjunct or alternative to standard surgery and radio-iodine therapy.

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12. Metabolic radiolabeling, immunoprecipitation, and SDS–polyacrylamide gel electrophoresis were performed as described [T. Kroll *et al.*, *J. Biol. Chem.* **269**, 9270 (1994)] using Easy tag EXPRESS protein labeling mix (NEN-Dupont). Immunoprecipitations were performed with PPARγ mAb E8 (SC-7273) and corresponding blocking peptide (SC-7273P; both from Santa Cruz). Fibronectin was precipitated by virtue of its nonimmunospecific association with protein A–Sepharose. Primary thyroid follicular adenoma and follicular carcinoma cultures were obtained by mechanical dissociation and collagenase treatment of tumor fragments in F10 medium containing 5% fetal calf serum. Attached cells were cultured in F10 medium containing 5% fetal calf serum and mitomycin C.
13. Immunohistochemistry was performed on paraffin-embedded human thyroid tissues using microwave antigen retrieval for 30 min at 199°F in 10 mM citrate buffer, pH 6. Sections were incubated with PPARγ mAb E8 (*12*). The LSAB avidin-biotin-complex and DAB (Dako) were used for immune complex detection.
14. Transactivation assays were performed in U2OS cells as described [P. Sarraf *et al.*, *Mol. Cell* **3**, 799 (1999)] using Fugene VI (Roche). Full-length *PAX-PPARγ1* cDNAs with kozak sequences were TA-cloned or ligated into the pCR3.1CMV expression vector (Invitrogen). Most experiments were performed with a *PAX-PPARγ1* form containing exons 1 to 7 plus 9 of *PAX8* and exons 1 to 6 of *PPARγ1*. The luciferase reporters were as described [R. Brun *et al.*, *Genes Dev.* **10**, 974 (1996)]. Duplicate or triplicate samples were used for each condition; the standard deviation of the mean for all conditions was less than 20%.
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Regulated Cleavage of a Contact-Mediated Axon Repellent

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Contact-mediated axon repulsion by ephrins raises an unresolved question: these cell surface ligands form a high-affinity multivalent complex with their receptors present on axons, yet rather than being bound, axons can be rapidly repelled. We show here that ephrin-A2 forms a stable complex with the metalloprotease Kuzbanian, involving interactions outside the cleavage region and the protease domain. Eph receptor binding triggered ephrin-A2 cleavage in a localized reaction specific to the cognate ligand. A cleavage-inhibiting mutation in ephrin-A2 delayed axon withdrawal. These studies reveal mechanisms for protease recognition and control of cell surface proteins, and, for ephrin-A2, they may provide a means for efficient axon detachment and termination of signaling.

Repulsion by direct cell contact is one of the basic mechanisms of axon guidance (*1*) and allows patterning of neural connections in a spatially precise manner (*2, 3*). However, this mechanism raises inherent questions. The binding of an axonal receptor to its cell surface ligand would be expected to favor adhesion, so how is this reconciled with repulsion? Also, how is contact-mediated repellent signaling terminated? These questions are further emphasized by the known properties of the ephrins, which are well-characterized cell surface axon repellents: The ephrins and their receptors are expressed at high density; the receptors do not appear to be down-regulated upon ligand binding; and the receptor-ligand interaction is mul-