

# Revelations of the RYK receptor

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## Summary

Significant progress has been made over the last decade in elucidating the mechanisms employed by receptor protein tyrosine kinases (RTKs) in transducing extracellular signals critical for the regulation of diverse cellular activities. Nevertheless, revealing the biological significance of a subset of the RTKs that contain catalytically inactive protein tyrosine kinase domains has proven more elusive. ErbB3 has served as the prototype for models of catalytically inactive RTK function, performing the role of signal diversification in heterodimeric receptor complexes with other ErbB subfamily members. The receptor related to tyrosine kinases (RYK) is unique amongst the catalytically inactive RTKs. Based on structural or functional properties of the extracellular domain, RYK cannot be classified into an existing RTK subfamily. Recent genetic analyses of mouse *Ryk* and its *Drosophila* orthologue *derailed* have defined a role for this novel subfamily of receptors in the control of craniofacial development and neuronal pathway selection, respectively. Recent biochemical data lead us to propose a model that involves RYK in signal crosstalk and scaffold assembly with Eph receptors. This model is consistent with the established roles of Eph receptors and ephrins in craniofacial and nervous system morphogenesis. *BioEssays* 23:34–45, 2001.

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## Introduction

Eukaryotes express a wide variety of transmembrane proteins at the cell surface responsible for the transduction of regulatory information into the cell (receptors). Information transferred across the plasma membrane by receptors is integrated to elicit appropriate cellular responses to developmental and physiological cues present in the extracellular environment. Loss or gain of receptor function can therefore uncouple cell behavior from these extrinsic inputs, often with serious pathological consequences.<sup>(1)</sup>

Type I, single-pass transmembrane proteins, which project a large glycosylated extracellular domain and possess a cytoplasmic portion containing a protein tyrosine kinase (PTK) domain, constitute the receptor protein tyrosine kinase (RTK) family.<sup>(2)</sup> Twelve conserved peptide sequence motifs, or subdomains, are the signature of the PTK catalytic domain and these involve some 13 invariant residues which fulfill vital structural or catalytic roles at the enzyme active site.<sup>(3,4)</sup> Members of the RTK family play cardinal roles in the control of a broad range of cellular activities, including metabolism (e.g. the insulin receptor), mitogenesis (e.g. the PDGF receptors), differentiation (e.g. the CSF-1 receptor), morphogenesis (e.g. *Drosophila* Torso), cell survival (e.g. the IGF-1 receptor), adhesion (e.g. *Drosophila* Trk), axon pathfinding (e.g. the Eph receptors), motility (e.g. the MET receptor) and oncogenesis (e.g. the ErbBs).

Major advances in our understanding of the mechanisms of RTK activation by growth-factor-type ligands have developed over the last decade.<sup>(5–7)</sup> The primary function of growth factors is the clustering of receptor chains into homodimeric or heterodimeric complexes.<sup>(8)</sup> This is achieved by many stoichiometric variations on a common theme,<sup>(9,10)</sup> involving a ligand with multivalent receptor binding sites; either oligomeric growth factor (e.g. dimeric PDGF-A), or monomeric growth factor multimerized through association with accessory factors (e.g. FGF with heparan sulfate proteoglycans), or clustered cell surface-anchored ligands (e.g. transmembrane ephrin-B ligands clustered by submembranous PDZ domains) or monomeric growth factor containing tandemly repeated receptor-binding motifs (e.g. collagen), drive the cross-linking of two RTK monomers through their extracellular domains. The resulting allosteric juxtaposition of PTK domains in the cytoplasm is responsible for activation of receptor phosphotransferase activity. This highly complex event is incompletely understood, and involves release of the catalytic domain from autoinhibition<sup>(11)</sup> and conformational changes in the receptor intracellular domain.<sup>(12)</sup>

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Abbreviations: AF-6, *ALL-1* fused gene from chromosome 6; *ap*, *apterous*; CSF-1R, colony stimulating factor-1 receptor; dpc, days post coitum; *drl*, *derailed*; *eg*, *eagle*; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; IGF-1, insulin-like growth factor-1; *lio*, *linotte*; LRR, leucine-rich repeat; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; NTR, neurotrophin receptor; PDGFR, platelet-derived growth factor receptor; PDZ, PSD95/DlgA/ZO-1; PTB, phosphotyrosine-binding; PTK, protein tyrosine kinase; PTPase, protein phosphotyrosyl phosphatase; RTK, receptor protein tyrosine kinase; RYK, receptor related to tyrosine kinases; SAM, sterile alpha motif; SH2, Src homology 2; SHP-1, Src homology 2 domain-containing PTPase-1; SHPS-1, SHP substrate-1; VEGFR-1, vascular endothelial growth factor receptor-1; WIF-1, Wnt inhibitory factor-1.

The amplification of cDNA fragments encoding PTK domains using degenerate PCR primers targeted to conserved catalytic motifs<sup>(13)</sup> resulted in a rapid increase in the identification of novel RTKs. As these screens relied only on the presence of PTK-related subdomains, rather than the ability to autophosphorylate, several RTKs that encode variant catalytic motifs were isolated. Reduced stringency hybridisation to genomic DNA has also been used to isolate related receptors in which not all of the PTK sequence motifs are conserved.<sup>(14)</sup> Subsequent biochemical analysis of these putative RTKs, comprising ErbB3, members of the CCK-4/Klg subfamily, EphB6, Ror1 and RYK, has defined a subset of RTKs devoid or severely attenuated in their ability to autophosphorylate on tyrosine residues and/or exhibit in vitro PTK activity towards exogenous substrates.<sup>(15)</sup>

Here we review the structure and function of the RYK receptor subfamily, summarizing knowledge derived from both *Drosophila* and mammalian systems. Recent data from our laboratory support a model for RYK subfamily function in developmental signalling mediated by a subset of Eph receptors.

### The RYK receptor tyrosine kinase subfamily

#### Structure

A full-length open reading frame encoding the mouse receptor related to tyrosine kinases (RYK) was first identified in 1992<sup>(16,17)</sup> following a screen for new RTKs by degenerate RT-PCR.<sup>(18,19)</sup> Subsequently, many independent isolations of cDNA fragments encoding mammalian RYK have been

**Table 1.** The RYK receptor tyrosine kinase subfamily

Species	RYK subfamily member <sup>a</sup>	Subdomain consensus sequence <sup>b</sup>	Variant subdomain sequence <sup>b</sup>	Tissue distribution (mRNA and protein)
Mouse	RYK	GXGXXG (I) VAVK (II) DFG (VII)	QEGTFG TFVK DNA	Adult renal tubular epithelium, adult liver, adult adrenal cortex, 14 dpc embryonic heart, red pulp of adult spleen, large and small intestines, placenta (pre-term) adult ovary, testis, uterus, tongue, salivary gland, stomach, duodenum, bone marrow, thymus, lymph node, lung, skeletal muscle, brain, eye, basal layer of skin and hair follicle (15.5-16.5 dpc), mature decidua cells of the uterine stroma, Lin <sup>+</sup> bone marrow cells (not Lin <sup>-</sup> )
Rat	RYK	GXGXXG (I) VAVK (II) HRDLXXXN (VI) DFG (VII)	QEGTFG TFVK HKDLATRN DNA	Embryonic central nervous system, in particular the ventricular zone and cortical plate; brachial arches, dorsal root ganglia, tail, brain (at 11 dpc); limb buds, dorsal root ganglia, brain (at 13 dpc); adult brain; isolated glomeruli, mesangial cells
Chicken	RYK	DFG (VII)	DNA	Unknown; cDNA fragment cloned from hemopoietic bone marrow
Human	RYK	GXGXXG (I) VAVK (II) HRDLXXXN (VI) DFG (VII)	QEGTFG AFVK HKDLAARN DNA	Primary tumors (neuroblastomas, primitive neuroectodermal tumors, Wilms' tumors, melanomas, hepatoblastomas, malignant ovarian tumors), heart, fetal lung, fetal brain, placenta, liver, skeletal muscle, fetal kidney, pancreas, breast, vas deferens, uterus, fallopian tube, ovary, lung, spleen, lymph node, tonsil, thyroid, blood vessels
Zebrafish <sup>c</sup>	Ryk	GXGXXG (I) VAVK (II) HRDLXXXN (VI) DFG (VII)	HEGTFG VFK HKDLAARN DNA	Not characterised
<i>Drosophila</i>	Derailed/Linotte	GXGXXG (I) VVK (II) HRDLXXXN (VI) DFG (VII)	QEGNFG VLVK HKDLAARN DSA	Subset of embryonic somatic muscles (21-23), embryonic epidermal cells, embryonic inter- and motoneurons crossing midline in anterior commissure, adult brain central complex and mushroom bodies
	Doughnut	GXGXXG (I) VAVK (II) HRDLXXXN (VI) DFG (VII)	QEGTFG VLVK HKDIATRN DSS	Embryonic epidermal cells adjacent to invaginating ventral furrow, cephalic furrow, foregut, hindgut, optic lobe and tracheal pits
<i>C. elegans</i>	CeRyk	GXGXXG (I) VAVK (II) E (III) HRDLXXXN (VI) DFG (VII)	MEGTFG VYTK D HNDIAARN DSA	Not characterised

<sup>a</sup>Mouse and human RYK have also been cloned under other names: JTK5, PTK1, Nyk-r, Ntbk-1, Vik, Stk-4 and 9B4.

<sup>b</sup>Single-letter amino acid code. Only those PTK subdomains which differ from the consensus<sup>(3, 4)</sup> are listed.

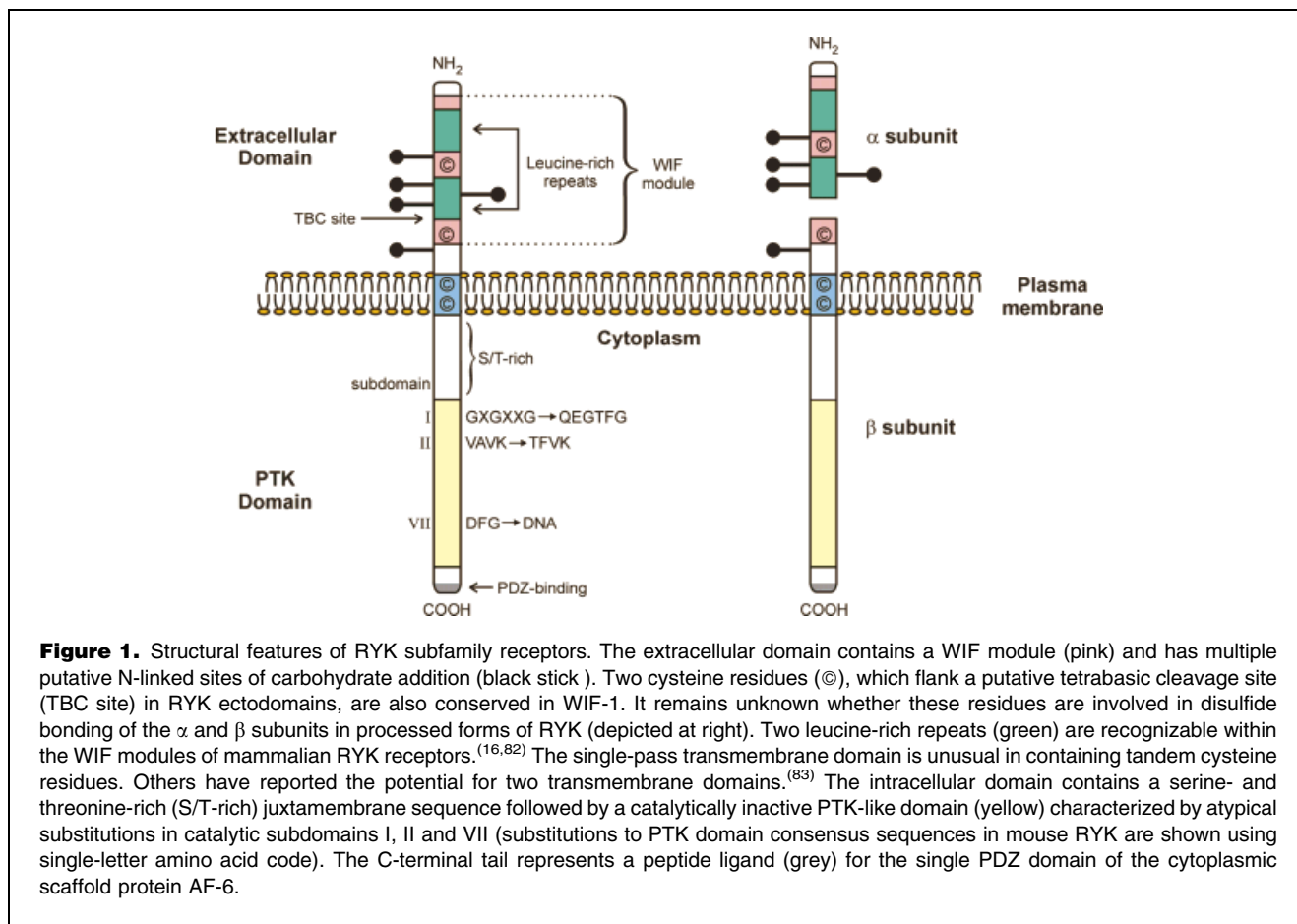
<sup>c</sup>CP Do et al. (personal communication)

described from a wide variety of cDNA sources (Table 1). RYK orthologues from lower eukaryotes have also been identified (Table 1), notably the *Drosophila* paralogues *derailed*<sup>(20)</sup> and *doughnut*,<sup>(21,22)</sup> zebrafish *Ryk* (C. Do, personal communication) and *Caenorhabditis elegans Ryk*.<sup>(23,24)</sup> Zoo blotting suggests that a *Ryk* gene may be common to all metazoan organisms.<sup>(24)</sup>

Several structural features define members of the RYK subfamily (Fig. 1). The glycosylated extracellular domain of RYK subfamily members (<250 residues) is smaller than those of all other RTKs (typically >400 residues) and exhibits significant sequence homology with the N-terminal domain of the secreted Wnt inhibitory factor-1 (WIF-1). Identification of this “WIF module” in RYK,<sup>(25)</sup> which is necessary and sufficient for Wnt binding by WIF1,<sup>(26)</sup> suggests a role for RYK subfamily receptors in binding members of the Wnt ligand family. The potential for proteolytic cleavage of the extracellular domain and disulfide bonding of the two resulting fragments to generate an  $\alpha$  (extracellular)– $\beta$  (transmembrane) heterodimer is reflected in the conservation of a putative tetrabasic protease cleavage (TBC) site flanked by two

cysteine residues in all RYK subfamily members except *C. elegans Ryk*, in which the tetrabasic cleavage site is not fully conserved.<sup>(24)</sup> Such post-translational modification involving TBC sites is seen during maturation of the insulin and IGF1 receptors into  $\alpha_2\beta_2$  tetramers<sup>(27,28)</sup> and MET subfamily members into  $\alpha\beta$  heterodimers<sup>(29–32)</sup> in the constitutive secretory pathway. Although proteolytic processing of the RYK extracellular domain has been detected in MCF-7 and 41M cell lines<sup>(24,33)</sup> and in neonatal mouse kidney,<sup>(34)</sup> the functional consequences of cleavage at the TBC site and whether the two resulting subunits are disulfide bonded remains to be determined.

In all RYKs, subdomain I (consensus GXGXXG), which functions to chelate the ATP–Mg<sup>2+</sup> phosphodonor complex in active PTKs,<sup>(35)</sup> displays substitution of the first glycine residue (G) with a bulky glutamine (Q), histidine (H) or methionine (M) (Table 1). In the case of a human TrkA extracellular domain fused to the human RYK transmembrane and cytoplasmic domains (a TrkA-RYK chimera) expressed in NIH 3T3 cells, the ATP-binding site was not functional as assessed by its inability to bind a non-hydrolysable ATP



analogue.<sup>(36)</sup> Substitution of Q with G in subdomain I of the TrkA-RYK chimera could not restore catalytic activity to the chimeric receptor in the presence of NGF.

C terminal to the catalytic loop (subdomain VI) of RYK subfamily members, highly unusual substitutions in subdomain VII (DFG → DNA in mouse and man) are observed. Although the aspartate residue (D), which functions to chelate the Mg<sup>2+</sup> moiety of the phosphodonor complex, is conserved, “retrosubstitution” of N with F, A with G or NA with FG in the DNA motif of the TrkA-RYK chimera is sufficient and necessary to activate the ability of the chimera to phosphorylate itself or an exogenous synthetic substrate on tyrosine residues.<sup>(36)</sup> Substitution of other variant residues in human RYK for the prototypical residue (e.g. subdomain II and/or VI; see Table 1) could not by themselves restore PTK activity to the TrkA-RYK chimera. Homology modelling of the RYK PTK-like domain suggests that the net effect of RYK-specific subdomain substitutions is disruption of the catalytic cleft between the two lobes of the PTK domain.<sup>(36)</sup> It is this cleft in which the ATP–Mg<sup>2+</sup> complex and protein substrate are positioned during the phosphotransfer reaction.<sup>(2)</sup> Reactivation of RYK PTK function by restoration of the DFG motif may reflect reconstitution of the ATP–Mg<sup>2+</sup> binding site given that subdomains I and VII are believed to cooperate in this regard.

#### *Expression of mammalian RYK*

The large range of RNA sources from which mammalian *Ryk* cDNA fragments have been amplified and cloned, together with the broad tissue distribution of *Ryk* mRNA and protein (Table 1), indicates that transcription of the *Ryk* gene occurs in many different tissues both during development and in adult animals. Although *Ryk* expression has been assessed in many different cell lines (of neuronal, haemopoietic, epithelial and embryonic origin), the majority of which are positive,<sup>(16,18,33,37–41)</sup> this information has provided no clues to the in vivo functions of the gene product. In vivo expression data has revealed that *Ryk* is expressed in almost every mammalian tissue examined, (see Ref. 23 for review) suggesting either a widespread, possibly housekeeping, function or a widely transcriptionally active promoter whose activity is important in only a subset of expressing tissues during development.

Importantly, the temporal and spatial profile of *Ryk* expression is not uniform within particular organs or systems (Table 1). For example, in haemopoietic cells,<sup>(39)</sup> *Ryk* is expressed in a lineage- and stage-specific manner. *RYK* mRNA is localized to the epithelial, endothelial and stromal compartments of human brain, lung, colon, kidney and breast<sup>(33,42)</sup> and there is cell-type-specific *Ryk* mRNA distribution in the developing rat central nervous system.<sup>(43)</sup> Differentiation-specific expression of mouse *Ryk* mRNA and protein occurs in many epithelial tissues, particularly decidualizing uterine stroma.<sup>(23)</sup> RYK protein expression is re-

stricted to the parenchymal compartments of adult mouse adrenal gland and spleen, to the tubular epithelium of the kidney and to the stroma and epithelium of intestinal villi.<sup>(24)</sup> Together, these data point towards a temporospatially regulated function for RYK in vivo.

Consistent with membership of the RTK family, RYK can behave as an oncoprotein. Overexpression of human RYK in NIH 3T3 cells confers anchorage-independent growth in vitro and tumourigenicity in vivo.<sup>(42)</sup> A role for RYK in tumour progression is suggested by upregulation of its mRNA and protein in epithelial, stromal and vascular compartments at early and subsequent stages of several types of ovarian cancer.<sup>(33,42)</sup>

#### *Signalling properties of RYK*

Although RYK is devoid of detectable PTK activity,<sup>(16,33,36)</sup> several observations indicate that RYK is involved in the transduction of extracellular signals across the plasma membrane. Firstly, the transmembrane topology of RYK, with its PTK-like intracellular domain and glycosylated extracellular domain, establishes RYK as a member of the RTK family.<sup>(16,17)</sup> Without exception, it has been repeatedly demonstrated that well-characterized members of this family, including the catalytically inactive receptor ErbB3, function in the transduction of important growth-regulatory information across the plasma membrane to intracellular signal relay systems. Furthermore, PTK activity has been demonstrated to be dispensible for the execution of certain in vivo functions by RTKs such as VEGFR-1,<sup>(44)</sup> VAB-1<sup>(45–47)</sup> and EphB2.<sup>(48)</sup> The detailed functional characterization of Drl in *Drosophila*, a putative orthologue of mammalian RYK, indicates an important role in the embryonic central nervous system where it most likely transduces a chemorepulsive signal in navigating growth cones.<sup>(49,50)</sup>

Katso et al.<sup>(36)</sup> have employed a chimeric receptor approach to investigate the signalling properties of the human RYK transmembrane and cytoplasmic domains. Their chimeric TrkA-RYK receptor can be dimerized by recombinant human NGF, however there is no detectable increase in phosphotyrosyl content of the aggregated chimeric receptor, consistent with a lack of intrinsic PTK activity. In spite of this, a mitogenic signalling capacity of the wild-type human RYK receptor is suggested by its ability, when stably overexpressed in NIH 3T3 cells, to confer morphological transformation in vitro and tumourigenicity in nude mice.<sup>(36)</sup> Remarkably, NGF stimulation of NIH 3T3 cells stably expressing the TrkA-RYK chimera, but not the parental TrkA-negative cell line, triggered a MAPK cascade as assessed by increased reactivity with anti-Erk activation-specific antibodies.<sup>(36)</sup> Events downstream of Erk activation, in particular the activation of p90 Rsk3 activity, a ribosomal S6 kinase, was found to be comparable in magnitude to that induced by EGF stimulation of cells expressing the EGFR. This observation may, however, be

complicated by the expression of endogenous EphB3 in NIH 3T3 cells (see below). Importantly, there have been no reports of RYK subfamily members, in either wild-type, chimeric or mutant forms, being phosphorylated on tyrosine residues, as is invariably the case for RTK-mediated activation of MAPK cascades.<sup>(51)</sup> Substitution of the subdomain II lysine (K) residue, essential for the catalysis of phosphotransfer in active PTKs<sup>(52)</sup> for alanine (A) abolished the ability of NGF-stimulated TrkA-RYK to induce phosphorylation of Erk1/2 or activate p90 Rsk3. This result may reflect the observed conservation of variant and typical PTK subdomains within RYK subfamily members, which may have been preserved in such a context to provide for a qualitatively and/or quantitatively unique, but as yet uncharacterised, catalytic activity.

### Biological functions of RYK: clues from *Drosophila*

Initial description of the *derailed* (*drl*) phenotype originated from experiments that exploited an enhancer trap screen in which a modified P element incorporating the *tau-lacZ* fusion gene<sup>(53)</sup> was used to identify Tau- $\beta$ -Gal reporter activity in fasciculating subsets of embryonic neurons. The Tau portion, which binds microtubules, allowed for histochemical visualisation of  $\beta$ -Gal activity in axonal processes. Probes to the *drl* gene were subsequently isolated by plasmid rescue of the modified P element from a line in which  $\beta$ -Gal activity was also observed in a subset of embryonic muscles and epidermal cells. Isolation of a full-length cDNA demonstrated that *drl* encodes an RTK with significant homology to mammalian RYK proteins, particularly within the PTK-like domain.<sup>(20)</sup>

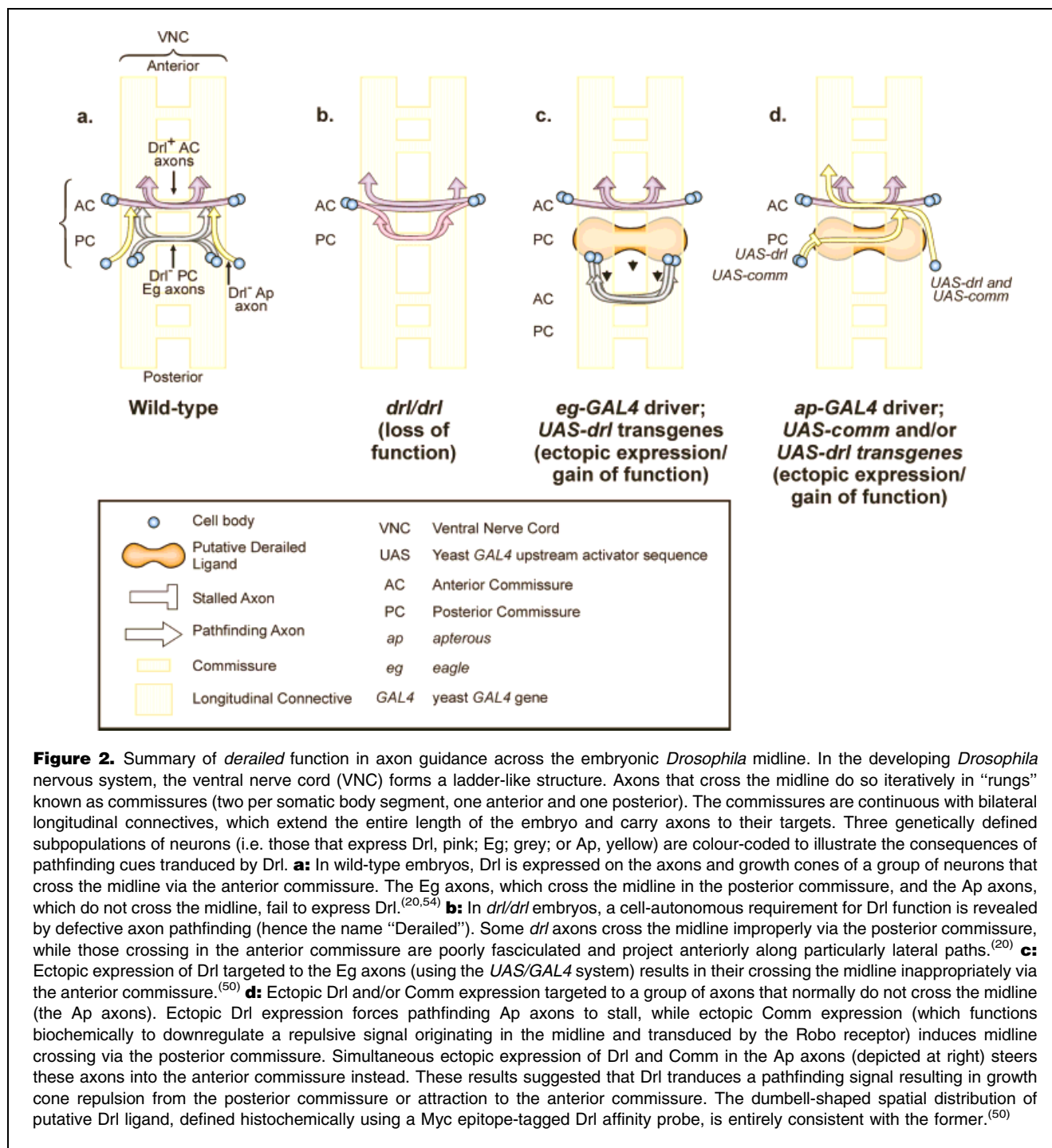
The function of *drl* in the embryonic *Drosophila* nervous system has been examined in detail by analysis of loss-of-function mutants as well as strains that ectopically express the Drl protein (Fig. 2). In wild-type *Drosophila* embryos, Drl is expressed on a heterogeneous group of neurons (interneurons and motor neurons) which share the common property of projecting axons across the midline in the anterior commissure and combine to give the staining pattern observed in the *drl* enhancer trap line (Fig. 2a).<sup>(20,50,54)</sup> In loss-of-function *drl* mutants, which lack easily detectable *drl* mRNA, axon pathfinding defects in  $\beta$ -Gal-expressing neurons are observed (Fig. 2b). Although the Drl-deficient neurons cross the midline, they are poorly fasciculated relative to the heterozygote and a subset cross abnormally via the posterior commissure. When they reach the contralateral connective, *drl* neurons fail to extend along the appropriate pathways and follow a variety of inappropriate paths, particularly those in very lateral positions. Expression of Drl in wild-type embryos is observed on the cell bodies, axons and growth cones of neurons crossing the midline, but only on the cell bodies and axonal portions within the anterior commissure subsequent to axon projection within the contralateral connectives. These observations, therefore, suggest that Drl is critical for selection of the correct longi-

tudinal pathway after crossing the midline, after which it is rapidly downregulated from that portion of the cell surface found within the longitudinal connective.

Drl marks a subset of neurons that project axons within the anterior, but not the posterior, commissure.<sup>(50,54)</sup> Ectopic expression of Drl in neurons that normally cross the midline in the posterior commissure forces them to take instead the anterior commissure (Fig 2c). Furthermore, ectopic expression of Drl and Comm targeted to a thoracic axon tract (the Ap axons, which never cross the midline) resulted in midline crossing via the anterior commissure (Fig. 2d). Comm is responsible for downregulating a repulsive signal from the midline, which is transduced by the Robo receptor. Ectopic expression of Comm alone in the Ap neurons diverts them to cross the midline in the posterior commissure (Fig. 2d), suggesting that Drl transduces a signal resulting in avoidance of the posterior commissure or attraction to the anterior commissure. Using a soluble receptor affinity probe (the Drl extracellular domain fused C-terminally to 5 tandem Myc epitope tags), the likely spatial distribution of the Drl ligand was defined histochemically as a dumbbell-shaped domain overlapping with the posterior commissure and adjacent longitudinal connectives (Fig 2c,d). This result suggested that Drl transduces a repulsive signal, localized around the posterior commissure, which guides Drl<sup>+</sup> growth cones crossing the midline into the anterior pathway.

*drl* mutants also exhibit defective embryonic muscle attachment site selection,<sup>(55)</sup> suggesting shared mechanisms for the recognition of appropriate axonal pathways and muscle insertion targets. Drl expression is observed in embryonic abdominal hemisegments A2–A7, in three of the 30 somatic muscles that establish a reiterated stereotypical array by growing towards and inserting into predetermined epidermal attachment sites. In *drl* mutants, somatic muscles 21–23 grow beyond their attachment sites and insert into the epidermis more ventrally than appropriate in 30% of somatic hemisegments. A cell-autonomous role for Drl in selection of the epidermal attachment site by the elongating muscles was demonstrated by targeted expression of Drl specifically to muscles of *drl* mutants, which fully rescued the muscle bypass phenotype. When expression of Dlg (a second *Drosophila* RYK homologue) was targeted to muscles of *drl* flies, only a partial rescue of the bypass phenotype was observed,<sup>(21)</sup> indicating incomplete complementation of function.

An independent *Drosophila* strain, *linotte* (*lio*), identified in a screen for three hour memory deficits after classical conditioning of an olfactory avoidance response,<sup>(56)</sup> represents an allelic *PlacW* element insertion into the *drl* gene.<sup>(57)</sup> Conflicting reports regarding the identity of the gene trapped in the *lio*<sup>1</sup> strain, and the associated phenotype resulting from loss of function,<sup>(57,58)</sup> are apparently due to the divergent effects of subtly different insertional mutagenesis events on the expres-



sion of adjacent transcriptional units. It is now clear that the *lio*<sup>1</sup> strain characterized by Dura et al.<sup>(57)</sup> contains a hypomorphic *lio/drl* allele. A *lio*<sup>2</sup> null allele, generated by imprecise excision of the *PlacW* element, is associated with a more severe memory deficit than the *lio*<sup>1</sup> homozygote.<sup>(57)</sup> A requisite role for the Lio/Drl protein in axon pathway selection during morpho-

genesis of the mushroom bodies, central complex and other central brain axons crucial for olfactory learning has been now conclusively established.<sup>(49,59)</sup>

In summary, the *Drosophila* RYK subfamily member Drl/Lio clearly fulfills a key embryonic role in the transduction of repulsive signals involved in axon guidance across the midline.

Drl/Lio also appears to execute a related function in the *Drosophila* brain that is crucial for the morphogenesis of neural structures involved in olfactory learning. The evolutionary conservation of axon pathfinding mechanisms and overall structure of RYK subfamily members between flies and vertebrates<sup>(20,24)</sup> suggests that RYK could be involved in similar processes in mouse and man.

### **Analysis of vertebrate RYK function: evidence for a role in Eph receptor signalling**

The analysis of RYK-deficient mice has recently demonstrated an absolute requirement for RYK in normal development and morphogenesis of craniofacial structures and the limbs.<sup>(34)</sup> Mice homozygous for a null allele of the *Ryk* gene die soon after birth and exhibit a complete cleft of the secondary palate plus a distinctive craniofacial appearance. In RYK-deficient mice, the face is typically flattened, the snout shortened and the shape and size of bones forming the cranial vault is subtly altered to give a characteristically rounded profile to the skull. In addition, long bones of both the forelimbs and hindlimbs are shortened by up to 25%.

Histochemical examination of reporter activity from the targeted *Ryk*<sup>*βgeo*</sup> allele<sup>(34)</sup> revealed initial activation of the *Ryk* promoter in the developing face at 12.5 days post coitum (dpc). Strong staining of the tips of growing palatal shelves and subepidermal mesenchyme of the tongue, together with weaker staining of the palatal shelf mesenchyme, is consistent with a requisite role for RYK in palate formation. Gene targeting studies have previously implicated elements of growth factor and cytokine signaling pathways in palate formation, including TGF $\alpha$ /EGFR,<sup>(60)</sup> Eph receptors B2 and B3,<sup>(61)</sup> TGF $\beta$ 2,<sup>(62)</sup> and TGF $\beta$ 3.<sup>(63,64)</sup> The distressing condition of cleft palate has a high incidence in humans, and, therefore, an understanding of its multifactorial genetic and environmental etiology is crucial to the long-term aim of rational prevention and improved treatment.<sup>(65)</sup> Histological and in vitro analysis of palatogenesis in RYK-deficient mice showed that the cleft palate was a result of physical obstruction of palatal shelf elevation at 13.5–14.5 dpc by an improperly positioned tongue. It therefore seems that, although a primary role for RYK in palate formation is unlikely, its function is essential for regulation of normal craniofacial morphogenesis.

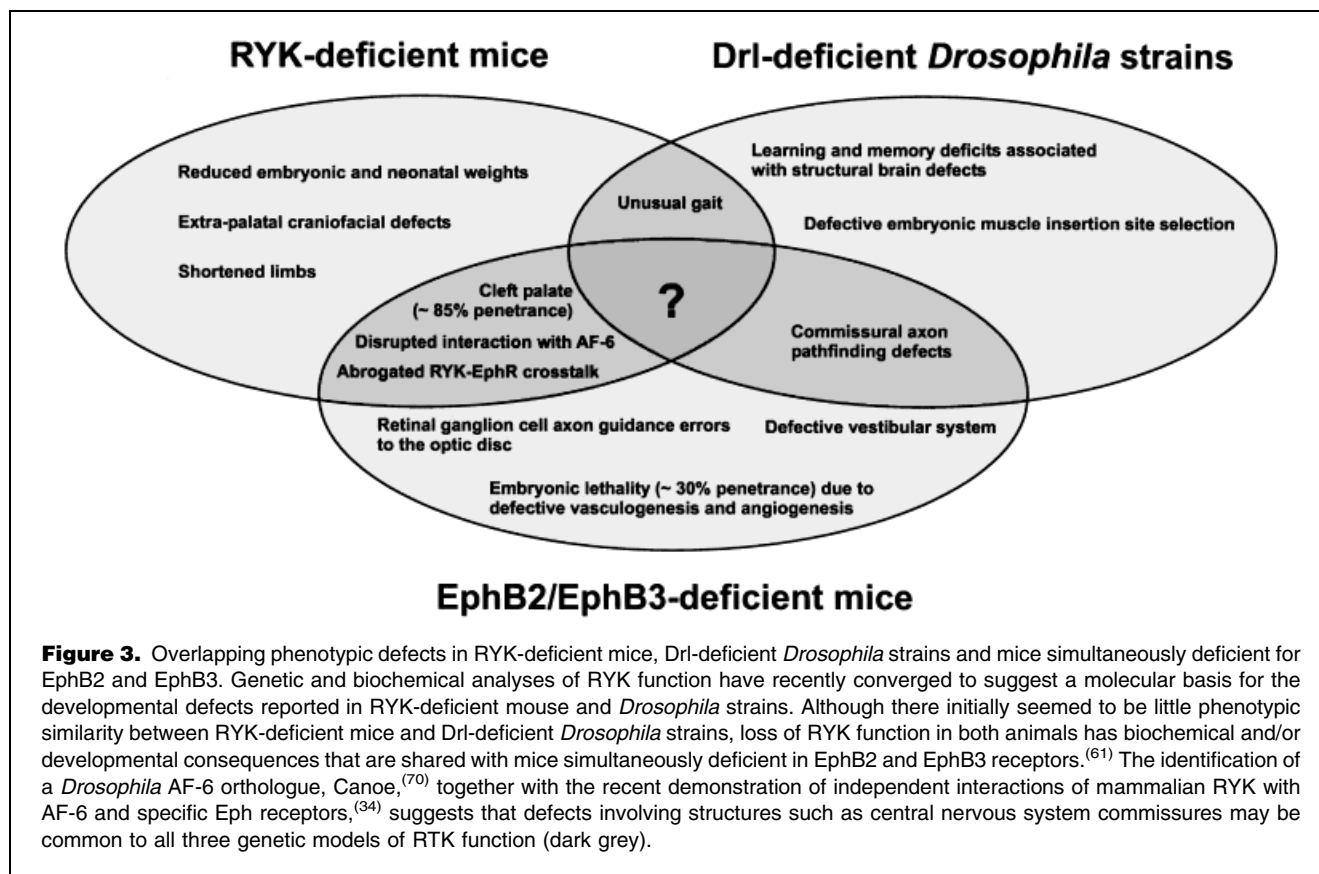
Genetic and biochemical approaches to mammalian RYK function have recently converged, allowing formulation of a molecular model of how RYK and its orthologues may function in vivo. A key finding was the identification of the RYK C terminus as a peptide ligand for the single PDZ domain of the cell-junction-associated scaffold molecule AF-6, both in vitro and in vivo.<sup>(34)</sup> This new information suggested a possible explanation for the overlapping defects observed in RYK-deficient mice, Drl-deficient *Drosophila* strains (see above) and mice simultaneously deficient in EphB2 and EphB3 (Fig. 3).<sup>(61)</sup>

Neonatal mice doubly deficient in EphB2 and EphB3 phenocopy RYK-deficient mice in terms of cleft palate, but also exhibit commissural axon defects.<sup>(61)</sup> More recent work has also demonstrated defective vestibular function,<sup>(66)</sup> retinal ganglion cell guidance errors to the optic disc<sup>(67)</sup> and incompletely penetrant embryonic lethality in EphB2/B3-deficient mice due to defective vasculogenesis and angiogenesis.<sup>(68)</sup> EphB2 and EphB3 are expressed in a temporospatial pattern that overlaps with RYK in structures including axons, palatal shelves and the tongue. (Ref. 34; M.M.H., S.A.S. and C.M. Hovens, unpublished data; Ref. 61) The commissural axon defects seen in EphB2/B3-deficient mice are reminiscent of the *Drosophila drl* phenotype. Furthermore, AF-6 (alternatively known as Afadin,<sup>(69)</sup> or Canoe in *Drosophila*<sup>(70)</sup>) binds the C-termini of RYK and multiple Eph receptors including EphB2 and EphB3 through its single PDZ domain.<sup>(71,72)</sup>

These observations formed the basis for experiments that established the association of RYK with specific members of the Eph RTK subfamily. Although RYK is yet to be screened for association with all members of the Eph receptor subfamily, EphB2, EphB3 and EphA7, but not VEGFR-2, TIE2 or EGFR, coprecipitate with RYK from a transient transfection system.<sup>(34)</sup> Coprecipitation of EphB2 and EphB3 with RYK was also demonstrated from in vivo sources. Significantly, coexpression of Eph receptors B2 and B3, but not any of the above-mentioned RTKs including EphA7, with RYK in vitro resulted in the phosphorylation of RYK on tyrosine residues. Cotransfection of a catalytically inactive version of the EphB3 receptor kinase domain (K665R) demonstrated that the phosphorylation was strictly dependent on Eph kinase activity.

Intriguingly, the association of AF-6 with RYK versus Eph receptors is differentially regulated. While the PTK-inactive RYK can associate with full-length AF-6 in an apparently constitutive manner,<sup>(34)</sup> AF-6 must be phosphorylated on tyrosine residues by activated Eph receptors for its PDZ domain to become accessible to C termini of this RTK subfamily.<sup>(71,72)</sup> Although self-association of AF-6 has been predicted<sup>(73)</sup> and would represent a plausible mechanism for the RYK/Eph receptor interaction, such a property is apparently not responsible for the coclustering of RYK with EphB3 since the two receptors still coprecipitate when simultaneously uncoupled from AF-6 by C-terminal valine  $\rightarrow$  alanine substitutions (M.M.H. and S.A.S., unpublished data).

RYK may function in this context to mediate lateral interactions in the plane of the plasma membrane and/or fulfill a role as a low-affinity coreceptor analogous to that of ErbB2<sup>(74)</sup> or p75<sup>NTR</sup>.<sup>(75)</sup> Alternatively, as has been demonstrated for the *Drosophila* EGFR-associated Kekk1,<sup>(76)</sup> the ErbB2-associated ASGP2<sup>(77)</sup> and the CSF-1R-associated SHPS-1 (also known as SIRP $\alpha$ 1, BIT, p84 and MFR),<sup>(78,79)</sup> RYK may function as an accessory chain that modulates the characteristics of signals emanating from catalytically active partner RTKs such as the Eph receptors.



How such modulation can be achieved is best understood in the case of SHPS-1, which is constitutively associated with the CSF-1R in macrophages. Upon CSF-1R activation, SHPS-1 becomes tyrosyl phosphorylated and functions as a scaffold for the recruitment of the protein phosphotyrosyl phosphatase (PTPase) SHP-1, a negative regulator of signalling by its substrate, the activated CSF-1R.<sup>(78)</sup> Furthermore, the activity of components of the SHPS-1 scaffold that are recruited through phosphopeptide-independent means (e.g. SKAP55hom/R, FYB/SLAP-130 and Pyk2) is also controlled by SHP-1-mediated PTPase activity.<sup>(79)</sup> Our speculative model of RYK function (Fig. 4) is based on a similar scaffolding role for RYK when complexed with specific Eph receptors.

At sites of cell–cell contact, RYK is believed to function as a transmembrane adaptor in recruiting AF-6 to specific Eph receptors (Fig. 4). Upon ligation and activation of Eph receptors by cognate ephrins, AF-6, its binding partners and RYK are then available as substrates for tyrosyl phosphorylation by the Eph receptor. Phosphorylation of AF-6 induces a stable PDZ domain-mediated association with the Eph receptor,<sup>(71,72)</sup> freeing RYK to recruit more AF-6 to the activated complex. It remains unclear whether phosphorylation of RYK by the activated Eph receptor triggers specific

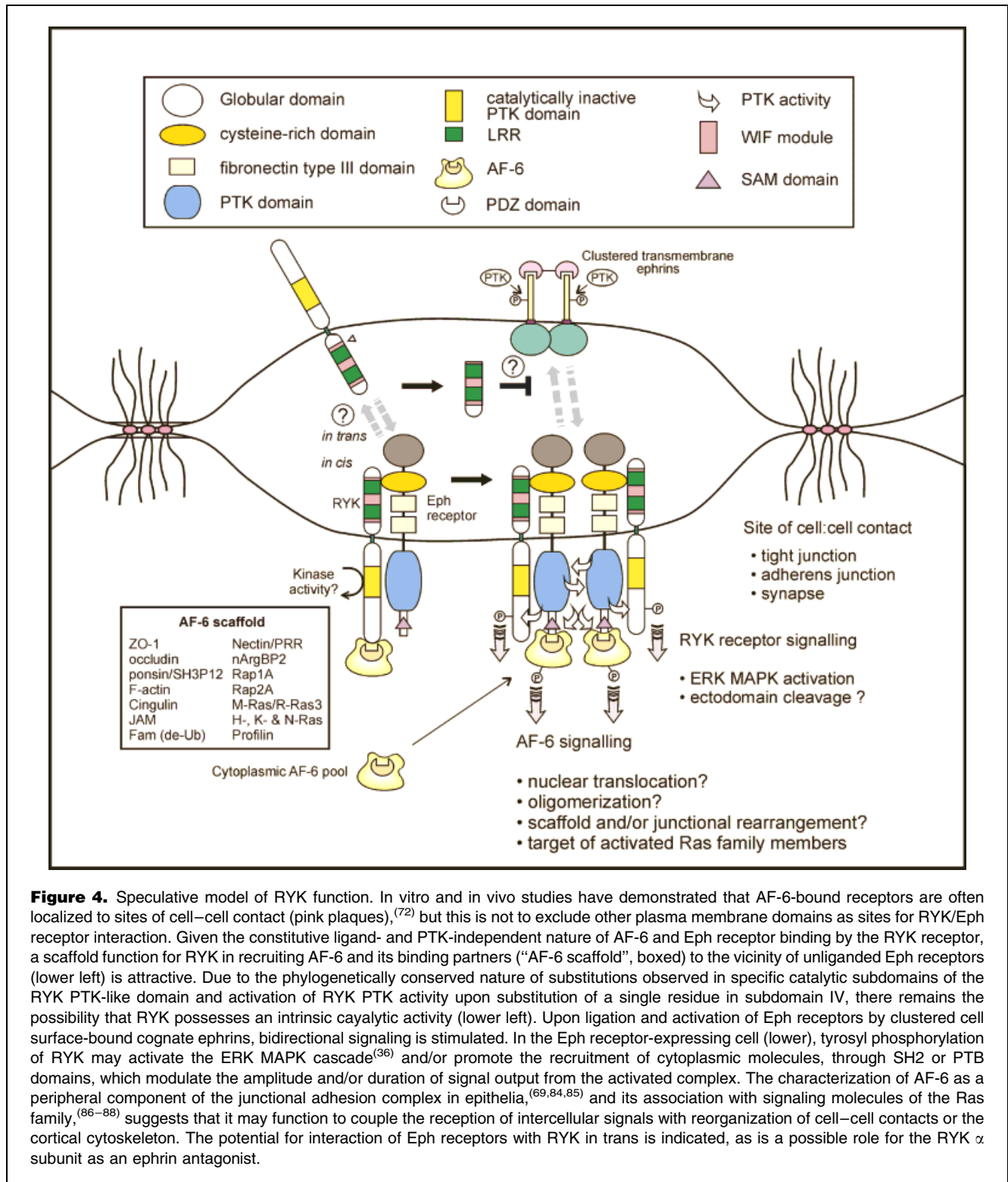
downstream signaling pathways, such as the ERK MAPK cascade.<sup>(36)</sup> In such a context, RYK could conceivably modulate the affinity or specificity of the partner Eph receptor for ligand (analogous to the action of p75<sup>NTR</sup> on the Trk receptor),<sup>(75)</sup> recognize a distinct ligand (likely to be membrane associated, as in the case of the SHPS-1 ligand, CD47)<sup>(80)</sup> or potentially function independently of interaction with ligand in trans, as in the case of ErbB2.<sup>(74)</sup>

Although perinatal lethality, craniofacial and limb defects are the prominent features of RYK-deficient mice, we have observed other phenotypes that may underlie the incomplete correlation between cleft palate and perinatal death.<sup>(34)</sup> The biochemical link established with Eph receptors B2, B3 and A7 suggests a role for RYK in developmental events such as axon guidance and vasculogenesis/angiogenesis as the basis for these observations.

### Conclusion

The mammalian RYK receptors clearly represent orthologous members of a catalytically inactive subset of RTKs. While the biochemical properties and in vivo functions of one member of this subset, the ErbB3 receptor, are well established, there are indications that this paradigm will not apply to all of the catalytically inactive RTKs. RYK in particular displays the





**Figure 4.** Speculative model of RYK function. In vitro and in vivo studies have demonstrated that AF-6-bound receptors are often localized to sites of cell–cell contact (pink plaques),<sup>(72)</sup> but this is not to exclude other plasma membrane domains as sites for RYK/Eph receptor interaction. Given the constitutive ligand- and PTK-independent nature of AF-6 and Eph receptor binding by the RYK receptor, a scaffold function for RYK in recruiting AF-6 and its binding partners (“AF-6 scaffold”, boxed) to the vicinity of unliganded Eph receptors (lower left) is attractive. Due to the phylogenetically conserved nature of substitutions observed in specific catalytic subdomains of the RYK PTK-like domain and activation of RYK PTK activity upon substitution of a single residue in subdomain IV, there remains the possibility that RYK possesses an intrinsic catalytic activity (lower left). Upon ligation and activation of Eph receptors by clustered cell surface-bound cognate ephrins, bidirectional signaling is stimulated. In the Eph receptor-expressing cell (lower), tyrosyl phosphorylation of RYK may activate the ERK MAPK cascade<sup>(36)</sup> and/or promote the recruitment of cytoplasmic molecules, through SH2 or PTB domains, which modulate the amplitude and/or duration of signal output from the activated complex. The characterization of AF-6 as a peripheral component of the junctional adhesion complex in epithelia,<sup>(69,84,85)</sup> and its association with signaling molecules of the Ras family,<sup>(86–88)</sup> suggests that it may function to couple the reception of intercellular signals with reorganization of cell–cell contacts or the cortical cytoskeleton. The potential for interaction of Eph receptors with RYK in trans is indicated, as is a possible role for the RYK  $\alpha$  subunit as an ephrin antagonist.

potential for a unique mechanism of activity, exemplified by its combinatorial association with members of an unrelated subfamily of RTKs, the Eph receptors.

Data from diverse sources is slowly building a picture of RYK subfamily function. Detailed analysis of *Drosophila* strains deficient for a *Ryk* orthologue, *derailed/linotte*, has defined an essential role for this receptor in transducing an extracellular signal contributing to the pathfinding decisions made by a subset of navigating neurons in the developing central nervous system. RYK-deficient mice have demonstrated an essential role for RYK in the control of craniofacial and limb development. Convergent biochemical analysis of RYK has uncovered an interaction with particular Eph receptors and the cell junction-associated Ras effector AF-6.

Although, for the first time, molecules that interact with and/or phosphorylate RYK have been identified, much work remains to be done to test our current model of RYK function. Given the wide-ranging role of the Eph receptor/ephrin system as an effector of metazoan morphogenesis,<sup>(81)</sup> future studies of RYK function in vivo are likely to encompass areas such as axon pathfinding, limb morphogenesis and, potentially, vascular biology. Revealing the details of RYK activity in these systems may provide functional insight into the actions of other catalytically inactive RTKs.

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