Development/Plasticity/Repair

A Role for Receptor Protein Tyrosine Phosphatase λ in Midbrain Development

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The mid-hindbrain boundary (MHB) harbors an important organizing center for the adjacent brain regions. Here, we present evidence that the *receptor protein tyrosine phosphatase* λ (*RPTP*λ) is part of the complex molecular network that maintains and shapes the MHB region. *RPTP*λ is expressed in a tight band of cells in the caudal midbrain, anterior to the transverse ring of *Wnt1* expression. Forced expression of *RPTP*λ across the mid-hindbrain region repressed expression of *Wnt1*, whereas RNA interference-mediated knock-down of *RPTP*λ resulted in expansion and distortion of the *Wnt1* domain. When ectopically expressed in the mesencephalon, *RPTP*λ specifically inhibited the induction of *Wnt1* expression after subsequent stimulation with *Fgf8*. Reduced *Wnt1* expression after *RPTP*λ transfection correlated with a decrease in Ras- mitogen-activated protein kinase activity at the MHB. We further show that in the embryonic midbrain, RPTPλ can bind to β-catenin, a central component of the canonical *Wnt* signaling pathway. Overexpression of *RPTP*λ suppressed the activity of a *β-catenin* responsive promoter in the midbrain and reduced progenitor cell proliferation. Cotransfection of *Wnt1* or of a stabilized form of *β-catenin* together with *RPTP*λ partially rescued the *RPTP*λ-mediated proliferation defect. Together, these data suggest that *RPTP*λ may play a dual role in the control of midbrain development: as a negative modulator of *Fgf8*-induced *Wnt1* expression at the MHB, which may help to confine the *Wnt1* domain to it characteristic tight ring at the MHB; and as an inhibitor of canonical *Wnt* signaling through interaction with and presumably sequestration of β-catenin.

Key words: RPTPψ; Fgf8; Wnt1; β-catenin; midbrain; chick

Introduction

The mid-hindbrain (MHB) (isthmic) organizer, located near the constriction between the developing mesencephalic and metencephalic vesicles, acts as a secondary organizer for the control of growth and pattern formation of the adjacent midbrain and hindbrain (Nakamura et al., 1986; Martinez and Alvarado-Mallart, 1990; Martinez et al., 1991). Its activity is regulated by an interdependent network of nuclear factors and signaling proteins, which includes the transcription factors *En1/En2*, *Pax2/Pax5*, *Lmx1b*, *Hes1/Hes3*, *iro1/iro7*, and members of the *Fgf* and *Wnt* families of secreted proteins (Bally-Cuif et al., 1992; Rowitch and McMahon, 1995; Araki and Nakamura, 1999; Shamim et al., 1999; Adams et al., 2000; Hirata et al., 2001; Itoh et al., 2002; Matsunaga et al., 2002; O'Hara et al., 2005).

Fibroblast growth factor 8 (*Fgf8*), expressed in the rostral hindbrain adjacent to the MHB, is of central importance for midbrain and hindbrain development. Beads soaked in recombinant Fgf8 and implanted in prosomeres p1, p2 of the diencephalon, in the midbrain or hindbrain can mimic the inductive capacity of isthmic transplants (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999; Irving and Mason, 2000). Conversely, zebrafish *ace* mutants, which lack functional *Fgf8*, and mice hypomorphic for *Fgf8* fail to maintain gene expression at the MHB and display massive defects in midbrain and cerebellar development (Meyers et al., 1998; Reifers et al., 1998). An important target of Fgf8 at the MHB is the secreted glycoprotein Wnt1. After isthmic organizer formation, *Wnt1* expression is restricted to a narrow band of cells in the caudal mesencephalon, anterior to the *Fgf8* expression domain (Bally-Cuif et al., 1995; Hollyday et al., 1995). *Wnt* signaling is critically involved in mid-hindbrain development, because targeted deletion or tissue-specific overexpression of *Wnt1* at the MHB lead to massive perturbation of mid-hindbrain growth (Thomas and Capecchi, 1990; McMahon et al., 1992; Panhuysen et al., 2004).

Reversible protein phosphorylation on tyrosine residues is a key mechanism underlying intracellular signal transduction. Protein tyrosine phosphatases (PTPs) antagonize the activity of protein tyrosine kinases (PTKs) and thereby limit the duration and intensity of the PTK signal (Stoker and Dutta, 1998; den Hertog, 1999; Paul and Lombroso, 2003; Tonks, 2006). *Receptor protein tyrosine phosphatase* λ (*RPTP*λ; also called *RPTPψ*), a member of the type IIb family of receptor tyrosine phosphatases, is expressed in a spatially and temporally dynamic manner in the embryo and has been shown recently to be necessary for somito-

Received July 4, 2007; revised March 21, 2008; accepted April 17, 2008.

This work was supported by the International Max-Planck Research School Program in Structure and Function of Biological Membranes (Frankfurt, Germany) (A.B.). We thank C. Tabin (Harvard Medical School, Boston, MA), C. Logan (University of Calgary, Calgary, Alberta, Canada), C. Krull (University of Michigan, Ann Arbor, MI), H. Rohrer (Max-Planck Institute for Brain Research, Frankfurt, Germany), F. Costantini (Columbia University Medical Center, New York, NY), and R. Dorsky (University of Utah, Salt Lake City, UT) for reagents; C. Ziegler for excellent technical assistance; G. O'Sullivan for critically reading this manuscript; and A. Vennemann, E. Dohle, and A. Poplawski for experimental help.

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DOI:10.1523/JNEUROSCI.5593-07.2008

 0270-6474/08/286152-13$15.00/0

genesis and convergent extension during gastrulation (Aerne et al., 2003; Aerne and Ish-Horowicz, 2004). We reported previously the developmental expression of *RPTPλ* in the CNS and have shown that its expression is particularly dynamic anterior to the isthmic constriction (Badde et al., 2005). Here, we have taken a gain-of-function and loss-of-function approach to show that *RPTPλ* can modulate mesencephalic development through a dual mechanism: it inhibits the induction of *Wnt1* expression by *Fgf8*, which may help to restrict the *Wnt1* domain to its characteristic tight ring at the MHB, and binds to β-catenin and appears to thereby negatively modulate canonical *Wnt* signaling.

Materials and Methods

Expression constructs and in ovo *electroporation.* cDNAs encoding full-length mouse *RPTPλ* [National Center for Biotechnology Information (NCBI) accession number U55057] or *RPTPλ-ΔIC* (corresponding to nucleotides 352–2701 of NCBI accession number U55057) were fused to a triple HA-tag and cloned into the expression vector pMES, which includes an internal ribosomal entry site (IRES)-*green fluorescent protein* (*GFP*) cassette (Swartz et al., 2001) or into the vector pMIWIII, which lacks IRES-*GFP* (Suemori et al., 1990). The coding sequence of chick *RPTPλ* was cloned using degenerate primers based on published sequences of the mouse, rat, and human orthologs and found to be mostly identical to NCBI accession number AY147868 (sequences are available on request). The coding sequences of chick *Fgf8b*, mouse *Wnt1*, or mouse *En1* were cloned into pMIWIII. For $dnRas^{N17}$-pMES, a dominant-negative form of the Ras protein, which contains a serine to asparagine mutation at residue 17, was inserted into pMES (Clontech). *Axin*-pMES carried the full-length coding region of chick *Axin*, a generous gift from F. Costantini (Columbia University Medical Center, New York, NY) (Zeng et al., 1997). For *caβ-catenin*-pMIWIII, a stabilized form of *β-catenin* was cloned into pMIWIII (Funayama et al., 1995). For the rescue experiment with *RPTPλ* and *Wnt1*, the coding sequence of *GFP* in *RPTPλ*-pMES was replaced by a single *Cla*I site (*RPTPλ*-pMEC). The coding region of *Wnt1* was then cloned into this *Cla*I site resulting in *RPTPλ-Wnt1*-pMEC. Unless otherwise noted, 1–2 μg/μl of each construct were injected into the neural tube of Hamburger–Hamilton stage 9–10 (HH9–HH10) chick embryos (White Leghorn) (Hamburger and Hamilton, 1992). Electroporation was performed as described, except that five pulses of 10 V for 50 ms were given to facilitate DNA uptake (Schulte et al., 1999). To visualize DNA uptake, 0.5–0.8 μg/μl of plasmids expressing *enhanced GFP* (*GFP*-pMIWIII), *Discosoma red fluorescent protein* (*dsRed*-pCMV), or a nuclear version of *red fluorescent protein* (*nRFP*-pCAGGS) were coelectroporated. To control for possible unspecific effects of the procedure, pMES (which carries IRES-*GFP*) or *GFP*-pMIWIII was introduced. For consecutive electroporation into the same region of the neural tube (see Fig. 3*A*), *GFP* with or without the full-length form of *RPTPλ* or the catalytically inactive allele *RPTPλ-ΔIC* were introduced into the mesencephalic vesicle at HH9–10. Six hours after initial transfection, a time window known to be sufficient for robust transgene expression (Momose et al., 1999), an expression plasmid carrying the coding sequence of *Fgf8b* together with a plasmid carrying *dsRed* were electroporated into the same area that had received the first electroporation 6 h earlier. Only embryos that exhibited extensive coexpression of both fluorescent markers, indicating that both transgenes were targeted to the same region of the neural tube, were chosen for additional analysis. For TOP:dgfp reporter analysis, 1 μg/μl of the TOP:dgfp reporter construct, which drives expression of *destabilized green fluorescent protein* off the TOPFLASH promoter/enhancer (Korinek et al., 1997; Dorsky et al., 2002), were electroporated into the mesencephalic vesicle alone or in combination with 1.5 μg/μl *RPTPλ*-pMIWIII or 1.5 μg/μl *RPTPλ-ΔIC*-pMIWIII. *GFP* expression was monitored and documented 24 h later.

Bead implantation. Heparin-coated acrylic beads (Sigma-Aldrich) were soaked in 0.015–0.15 mg/ml mouse recombinant Fgf8b protein (R&D Systems) overnight at 4°C, washed in sterile PBS, and split with forceps. Appropriately sized bead fragments were implanted into the lateral wall of the mesencephalic vesicle of HH11 chick embryos. The embryos were collected 24 h after bead implantation and processed for *in situ* hybridization.

RNA in situ *hybridization.* To ensure specificity of the probe, a partial sequence of chick *RPTPλ* was used for all *in situ* hybridization experiments (Badde et al., 2005). RNA probes for *cGrg4*, *cSprouty2*, and *cSef* were cloned from chick HH15–20 whole head total RNA by reverse transcriptase-PCR with gene-specific primers (primer sequences are available on request). The cDNAs used to generate *in situ* probes for *cEn1*, *cFgf8*, *cLmx1b*, *cOtx2*, *cPax2*, *cPax5*, or *cWnt1* were gifts from C. Tabin (Harvard Medical School, Boston, MA), C. Logan (University of Calgary, Calgary, Alberta, Canada), or H. Rohrer (Max Planck Institute for Brain Research, Frankfurt, Germany). *In situ* hybridization on whole embryos was performed as described previously (Schulte et al., 1999). For open book preparation, the mesencephalic vesicle was opened along the dorsal midline and flat-mounted. Two-color *in situ* hybridization was performed as described with the exception that the first transcripts were detected using 5-bromo-4-chlor-indolyl-phosphate (BCIP) and nitroblue–tetrazolium–chloride as chromophores, which results in a dark blue/purple precipitate, whereas the second transcripts were detected with BCIP alone, which results in a bright blue/turquoise precipitate (Schulte and Cepko, 2000). Specimens were photographed with a Zeiss StemiSV11 microscope and a Canon PowerShot G5 camera or with a Leica MZ12 microscope and a Leica MPS60 camera. Brightness and contrast were adjusted in Adobe Photoshop (Adobe Systems). In Figure 1, *C*, *D*, *D′*, *I′*, and *I″* were false colored in Adobe Photoshop for better visual contrast.

Immunohistochemical staining. Localization of ectopically expressed *RPTPλ-HA* or *RPTPλ-ΔIC-HA* was visualized on cryosections using a polyclonal anti-HA antibody (1:1000; Roche Diagnostics). *GFP* expression was confirmed using a polyclonal antibody (1:1000; Invitrogen). The mitotic index was determined with a polyclonal anti-phosphorylated Histone H3 (pH3) antibody (1:1000; Upstate Biotechnology). All antibodies were diluted in PBS containing 5% Chemiblocker (Millipore Bioscience Research Reagents) and 0.5% Triton X-100 before use. Microscope fields with a magnification of 40× were documented either with an Axioplan2 (Zeiss), a FluoView 1000 confocal microscope (Olympus), or a LSM5 Pascal confocal microscope (Zeiss). Immunohistochemical detection of diphosphorylated Erk1/2 with the monoclonal anti-dpErk antibody (clone M-8159 Sigma-Aldrich) was performed as described by Corson et al. (2003).

RNA interference. For RNA interference (RNAi)-mediated knockdown of *RPTPλ* or *Wnt1* expression, the psiSTRIKE-U6 Hairpin Cloning System was used (Promega). The short hairpin RNA target sequences directed against nonoverlapping regions of the *cRPTPλ* cytoplasmic domain were GTCAACATGACCAAAGCAA (*RPTPλ_si-a*) and GCTTCAAGCAGGAGTATGA (*RPTPλ_si-b*). The efficiency and specificity of the RNAi targeting constructs were assessed by luciferase reporter assay. Human embryonic kidney 293T (HEK293T) cells were cotransfected with the RNAi targeting constructs *RPTPλ_si-a*, *RPTPλ_si-b*, targeting constructs containing randomized sequences of *RPTPλ_si-a* or *RPTPλ_si-b*, respectively (GAACTACGTAAACCAGACA or GCATAGACGAATCGGTATG), or an unrelated targeting construct (GAAGGTACACGAATTATGT) together with a psiCHECK-2 reporter construct, which contained the intracellular portion of *cRPTPλ* fused to *Renilla* luciferase (1.5 μg of each construct/six-well plate; Promega). After 48 h, luciferase activity was measured using the Dual-Luciferase Reporter 1000 Assay System (Promega) in a GloMax96 plate Luminometer (Promega). For *in vivo* analysis, *RPTPλ_si-a* or *RPTPλ_si-b* was electroporated into the neural tube at a concentration of 1.5 μg/μl together with *GFP*-pMIWIII (0.8–0.5 μg/μl) at the developmental ages indicated. Short hairpin RNA target sequences against *Wnt1* were GGTCATCTACGGCAACAAA and GCGCCTCGAGGGTCATCTA. Simultaneous transfection of both RNA targeting constructs (1.5 μg/six-well dish) repressed the activity of a psiCHECK-2 reporter construct, which contained the coding region of chick *Wnt1* fused to *Renilla* luciferase, to 34% compared with a targeting construct carrying the randomized sequence CGAATCAGTCAGTCCAGAA (data not shown).

Immunoprecipitation. RPTPλ-HA-pMES or *RPTPλ-ΔIC-HA*-pMES was electroporated into the midbrain at HH10. After 24 h, the midbrain

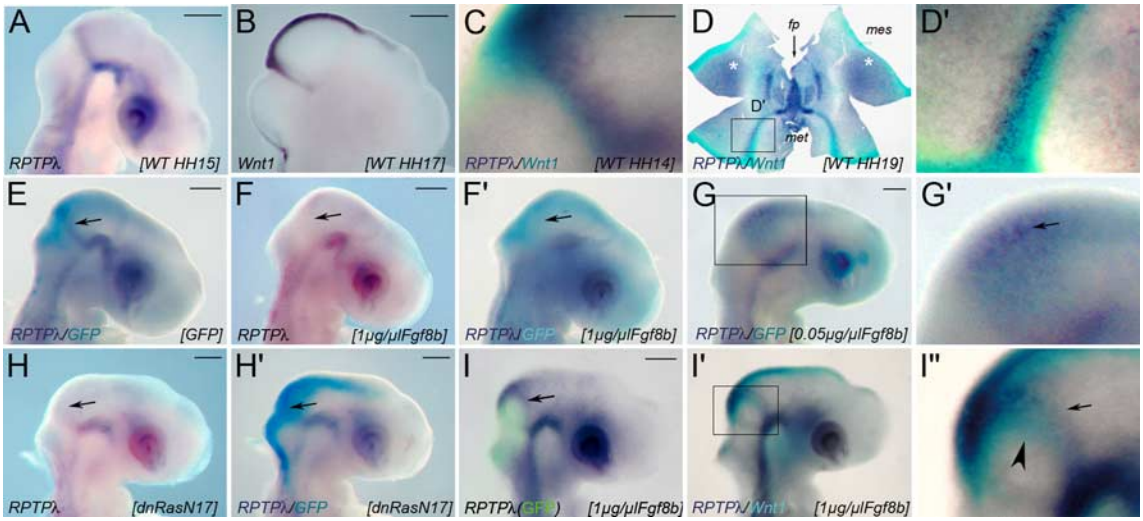

Figure 1. Expression and regulation of *RPTPλ* at the MHB. ***A***, Expression of *RPTPλ* in a WT chick embryo at HH15. ***B***, Expression of *Wnt1* at HH17. ***C***, Two-color *in situ* hybridization showing expression of *RPTPλ* in dark blue and *Wnt1* in turquoise at HH14. The ring of *Wnt1* expression at the MHB is bordered by *RPTPλ*-expressing cells at its rostral side. ***D***, ***D′***, Open book preparation of a HH19 (E3.5) chick midbrain showing *RPTPλ* expression in dark blue and *Wnt1* expression in turquoise. *RPTPλ* and *Wnt1* expression occurs in tightly associated, partially overlapping domains at the MHB; anterior is to the top, and posterior is to the bottom. ***D′***, Higher magnification of the boxed area in ***D***. ***E–I″***, Whole-mount *in situ* hybridizations on HH15–HH16 chick embryos, electroporated with different expression constructs. In all panels, detected transcripts are indicated in the bottom left corner, and misexpressed transgenes are indicated in brackets in the bottom right corner. ***E***, Expression of *RPTPλ* (dark blue) was not altered after misexpression of *GFP* (turquoise; 1.5 μg/μl). ***F***, Transfection of 1 μg/μl of the expression plasmid *Fgf8b*-pMIWIII together with *GFP*-pMIWIII effectively repressed *RPTPλ* expression at the MHB. ***F′***, Two-color *in situ* hybridization on the embryo shown in ***F*** for *RPTPλ* (dark blue) and *GFP* (turquoise). ***G***, ***G′***, Transfection of 0.05 μg/μl of *Fgf8b*-pMIWIII together with *GFP*-pMIWIII (turquoise) induced *RPTPλ* expression (dark blue) in the dorsal midbrain. ***G′*** is a higher magnification of the boxed area shown in ***G***. ***H***, *RPTPλ* expression at the MHB was lost after misexpression of the dominant-negative *RasN*^{17} together with *GFP*. ***H′***, Two-color *in situ* hybridization on the embryo shown in ***H*** (*RPTPλ*, dark blue; *GFP*, turquoise). ***I***, Forced expression of *Fgf8b* (together with *GFP*) in the MHB region shifted the ring of *RPTPλ* expression rostrally around the transfected area. The image is an overlay of the GFP fluorescence photographed immediately after harvesting of the embryo and the *RPTPλ* expression domain as detected subsequently by *in situ* hybridization (dark blue). ***I′***, ***I″***, Spatial relationship of *RPTPλ* (purple) and *Wnt1* (turquoise) expression in the embryo shown in ***I***. ***I″*** is a higher magnification of the boxed area in ***I′***. In all panels, upregulation of *Wnt1* is indicated by arrowheads, and expression of *RPTPλ* (or lack thereof) is indicated by arrows. The asterisks in ***D*** mark upregulation of *RPTPλ* expression in the mesencephalic alar plate after HH18. ***C***, ***D***, ***D′***, ***I′***, and ***I″*** are false colored for better visual contrast. fp, Floor plate; mes, mesencephalon; met, metencephalon. Scale bars: ***A***, ***B***, ***E–G***, ***H–I***, 500 μm; ***C***, 250 μm.

was dissected in ice-cold PBS and lysed in 150 mM NaCl, 10 mM Na-Phosphate buffer, pH 7.2, 1% NP-40, 1% Desoxycholate, 0.1% SDS, 50 mM Na-F, 0.2 mM Na-orthovanadate, and protease inhibitors (Complete tablets; Roche Diagnostics). Precipitation of the transgenes was achieved using a monoclonal, agarose-coupled anti-HA antibody (Sigma-Aldrich). Immunoprecipitation and Western blot analysis were performed as described previously (Mühleisen et al., 2006). Primary antibodies used for Western blot analysis were rat anti-HA high-affinity antibody (1:1000; Roche Diagnostics) or polyclonal anti-β-catenin (1:1000; Upstate Biotechnology). For stripping off the first set of antibodies, the blots were incubated in 0.1 M glycine, pH 2.5, for 30 min at 37°C.

Analysis of cell proliferation and programmed cell death. *RPTPλ-HA*-pMES or pMES were electroporated into the midbrain of HH9 chick embryos. After 24 h, bromodeoxyuridine (BrdU) was injected into the midbrain vesicle; labeling duration was 2 h. Detection of BrdU-positive cells was performed on 15-μm-thick cryosections using the BrdU Labeling and Detection Kit I (Roche Diagnostics) and colabeling with a GFP-specific antibody to visualize transfected cells. For analysis of programmed cell death, *RPTPλ*-pMIWIII together with *nRFP*-pCAGGS, or *nRFP*-pCAGGS alone, was electroporated into the midbrain vesicle. Detection of apoptotic cells was performed on cryosections using the Fluorescein *In Situ* Cell Death Detection Kit (Roche Diagnostics).

Results

RPTPλ is expressed in a domain tightly associated with the ring of *Wnt1* expression at the MHB and is regulated by *Fgf8*

We reported previously that between the 19 and 40 somite stages (HH13–HH19), *RPTPλ* is expressed in a tight, transverse ring anterior to the isthmic constriction and to the transverse domain of *Wnt1* expression (Fig. 1*A*,*B*) (Badde et al., 2005). To correlate the precise location of this ring to that of *Wnt1* at the organizer, *RPTPλ*- and *Wnt1*-specific transcripts were visualized together in the same specimen by two-color *in situ* hybridization. Expression of the two molecules was found in tightly associated domains at HH14 (Fig. 1*C*) and HH19 (Fig. 1*D*,*D′*). Although both expression domains appear to slightly overlap, in all specimens analyzed, *RPTPλ*-specific transcripts were found rostral to the ring of *Wnt1* expression that abuts the MHB. Therefore, during this developmental period, the *Wnt1* domain at the MHB is bordered by a ring of *RPTPλ*-expressing cells at its anterior side. Based on these expression data, the *RPTPλ* expression domain at the MHB may lie within the future preisthmic domain, the caudal-most aspect of the mesencephalic alar plate, which is characterized by coexpression of *Otx2*, *Pax2*, and possibly *Wnt1* (Hidalgo-Sánchez et al., 2005).

As a first step to understanding *RPTPλ* function at the MHB, we investigated whether its expression was regulated by the secreted protein Fgf8. *Fgf8* is expressed in rhombomere 1 adjacent to the isthmic organizer and can induce organizer characteristic gene expression and cell fate changes when ectopically applied in the caudal diencephalon or mid-hindbrain region (Crossley et al., 1996; Martinez et al., 1999). Transfection of 1 μg/μl to 0.1 μg/μl of an expression plasmid carrying the splicing isoform *Fgf8b* into the mid-hindbrain region of chick embryos has been shown previously to induce ectopic cerebellar development, whereas transfection of 0.01 μg/μl promoted tectal growth (Sato et al., 2001). We introduced an *Fgf8b* expression plasmid at different concentrations into the mid-hindbrain region together with an expression plasmid carrying *GFP* for visualization (Fig. 1*F–G′*). Expression of *Wnt1* and *RPTPλ* at the MHB were unaltered after transfection with the *GFP* expressing plasmid alone compared

with wild-type (WT) control embryos, demonstrating that *in ovo* electroporation per se does not affect gene expression at the MHB (Fig. 1*E*) (data not shown) ($n = 36$ of 37 for *RPTPλ*; $n = 12$ of 12 for *Wnt1*). Consistent with our observation that *RPTPλ* is not expressed in the hindbrain region during normal development, we found that widespread electroporation across the mid-hindbrain territory of 1 μg/μl of the *Fgf8b*-pMIWIII expression plasmid, a concentration known to stimulate cerebellar development, effectively repressed *RPTPλ* expression at the MHB ($n = 9$ of 10) (Fig. 1*F*,*F*′). Electroporation of 0.01 μg/μl of the *Fgf8b* expression plasmid, a concentration that enhanced midbrain growth in a previous study (Sato et al., 2001), did not alter *RPTPλ* expression ($n = 7$ of 7) (data not shown). However, *RPTPλ*-specific transcripts were upregulated in the midbrain vesicle at an intermediate concentration of 0.05 μg/μl *Fgf8b*-pMIWIII ($n = 3$ of 5) (Fig. 1*G*,*G*′). *Fgf8b* misexpression at either concentration was unable to stimulate *RPTPλ* expression in the hindbrain, supporting the idea that metencephalic tissue is not permissive for *RPTPλ* expression ($n = 18$ of 18) (Fig. 1*F–G*′) (data not shown). Together, these results suggest that *RPTPλ* expression at the isthmic organizer requires a distinct Fgf8 signaling level, which is below the level required for metencephalic development but above the level that stimulates tectal growth. Fgf8 signal transduction at the MHB appears to be mediated primarily through the Ras-mitogen-activated protein (MAP) kinase pathway (Corson et al., 2003; Sato and Nakamura, 2004). To confirm the dependency of *RPTPλ* expression on Fgf8 signaling at the MHB, we misexpressed a dominant-negative form of *Ras* ($dnRas^{N17}$), which was shown previously to effectively block Fgf8 signal transduction at the isthmic organizer (Sato and Nakamura, 2004). *RPTPλ* expression was consistently lost in cells forced to express $dnRas^{N17}$, demonstrating that *RPTPλ* expression at the MHB indeed requires *Fgf8*/Ras-MAP kinase pathway activity (Fig. 1*H*,*H*′) ($n = 8$ of 9).

Next, we targeted the *Fgf8b* expression plasmid (at a concentration of 1 μg/μl) to a narrow region surrounding the isthmic organizer and analyzed the expression of *RPTPλ* and *Wnt1* together in the same specimen 24 h later (Fig. 1*I–I*″). The domain of *RPTPλ* expression was shifted rostrally around the region targeted for *Fgf8* misexpression (recognizable by the fluorescence of the coelectroporated GFP; $n = 9$ of 10) (Fig. 1*I*, arrow), consistent with the idea that *RPTPλ* expression is inhibited by strong *Fgf8b* signals but induced at a distance from the *Fgf8b* source, where Fgf8b protein levels are expected to be lower. When *Wnt1*- and *RPTPλ*-specific transcripts were visualized together, both expression domains were arranged in concentric circles, with the *RPTPλ* domain (arrow) always located anterior to the *Wnt1* domain (Fig. 1*I*′,*I*″, arrowhead) ($n = 6$ of 6). *Fgf8*-induced upregulation of *Wnt1* and *RPTPλ* thus mimics their normal spatial relationship at the MHB, where the *Wnt1* domain separates the *Fgf8* and *RPTPλ* expression domains (Badde et al., 2005) (Fig. 2*G*). This raises the possibility that the threshold of Fgf8 signaling differs from the induction of *Wnt1* and *RPTPλ* expression at the MHB, and that *Wnt1* expression requires higher levels of Fgf8 signaling than expression of *RPTPλ*. To investigate this in more detail, we narrowed down the concentrations of *Fgf8* necessary for inducing *Wnt1* and *RPTPλ* expression, respectively, by implanting fragments of beads soaked in different concentrations of Fgf8b. As reported previously, *Wnt1* expression was induced around a bead fragment soaked in 0.15 mg/ml Fgf8b (Fig. 2*A*,*A*′, arrowheads) ($n = 12$ of 12) (Crossley et al., 1996). Induction of *RPTPλ* expression was also consistently observed, yet its expression domain was located at a distance to the Fgf8b soaked bead

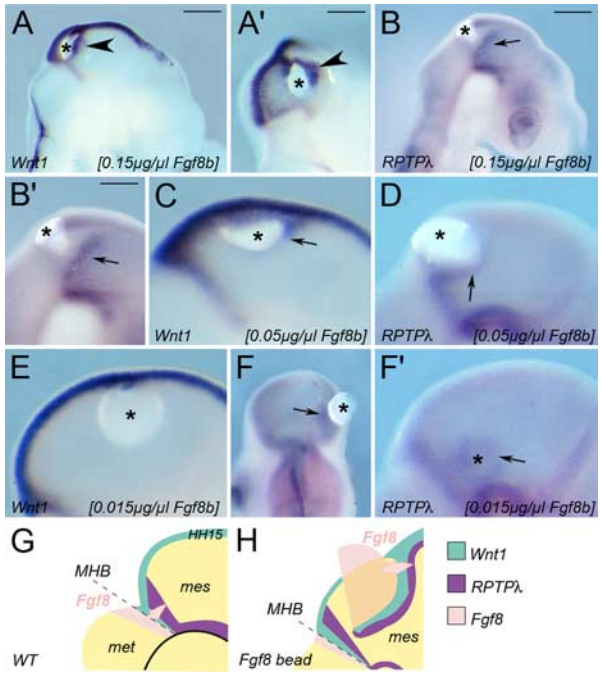

Figure 2. *Fgf8*-induced upregulation of *Wnt1* and *RPTPλ* mimics their normal spatial relationship at the MHB. ***A′–F′***, Expression of *Wnt1* (***A***, ***A′***, ***C***, ***E***) and *RPTPλ* (***B***, ***B′***, ***D***, ***F***, ***F′***) after implantation of beads soaked in different concentrations of recombinant Fgf8b. *Wnt1* expression (***A***, ***A′***) is ectopically upregulated in the mesencephalon closely around a partial bead soaked in 0.15 mg/ml Fgf8b, *RPTPλ* expression only at a distance to the bead (***B***, ***B′***). ***A′*** is a back view of the embryo shown in ***A***. ***C***, ***D***, Ectopic expression surrounding beads soaked in 0.05 mg/ml Fgf8b. ***E***, ***F***, ***F′***, Expression surrounding beads soaked in 0.015 mg/ml Fgf8b. In ***F′***, the Fgf8b-releasing bead was removed for better visual clarity. ***G***, Schematic drawing of the gene expression patterns of *Fgf8*, *Wnt1*, and *RPTPλ* at the MHB of a normal chick embryo (HH15). ***H***, Schematic drawing of *Wnt1* and *RPTPλ* expression around a Fgf8-releasing bead fragment (pink) implanted into the mesencephalic alar plate, which acts as ectopic Fgf8 source in the dorsal midbrain. The asterisks in all panels indicate the location of Fgf8b-releasing beads. Scale bar, 100 μm.

and must therefore lie distal of the ring of *Wnt1* expression, which generally surrounded the Fgf8 source (Fig. 2*B*,*B*′, arrows) ($n = 15$ of 15). When the Fgf8b concentration was lowered to 0.05 mg/ml, some ectopic *Wnt1* expression could still be observed surrounding the Fgf8 soaked bead, and the ring of *RPTPλ*-specific transcripts was located closer to the bead (Fig. 2*C*,*D*) ($n = 5$ of 8 for *Wnt1*; $n = 7$ of 7 for *RPTPλ*). Fgf8b at 0.015 mg/ml was not sufficient to induce *Wnt1* expression (Fig. 2*E*) ($n = 1$ of 3) but could still elicit robust ectopic *RPTPλ* expression (Fig. 2*F*,*F*′) ($n = 4$ of 4). Although additional work is clearly needed to fully understand *Fgf* receptor signaling dynamics at the MHB, these results strongly suggest that transcriptional activation of *Wnt1* and *RPTPλ* requires different levels of *Fgf8* signaling.

Overexpression of *RPTPλ* suppresses *Wnt1* expression at the MHB

To examine the function of *RPTPλ* at the MHB, we misexpressed the murine homolog of *RPTPλ* in the area surrounding the isthmic constriction (Fig. 3*A*). By fusing the coding region of *RPTPλ* to a triple HA-tag, the ectopically expressed *RPTPλ* protein could be distinguished from its endogenous form by the use of an HA-specific antibody. As expected for a transmembrane protein, *RPTPλ-HA* staining was concentrated to cellular plasma membranes, suggesting that the recombinant protein was processed normally (Fig. 3*B*). When *GFP* alone was ectopically expressed in the MHB region, *Wnt1* expression was unaltered (Fig. 3*C*) ($n =$

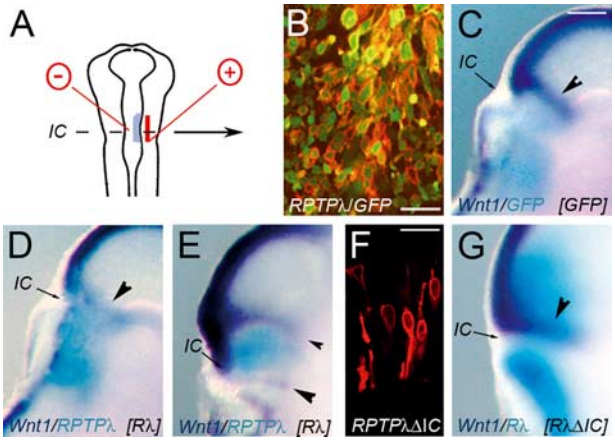

Figure 3. Altered *Wnt1* expression after *RPTP*λ misexpression at the MHB. ***A***, Schematic representation of the experimental protocol. ***B***, Double immunohistochemical labeling using antibodies against GFP (green) and the HA-epitope (red) on a frozen section through the MHB region of an embryo electroporated with *RPTP*λ*-HA*-pMIWIII and *GFP*-pMIWIII. HA-specific staining is concentrated to cellular plasma membranes. ***C***, Expression of *Wnt1* after misexpression of *GFP*. ***D***, ***E***, Different example of *Wnt1* expression after misexpression of *RPTP*λ*-HA* together with *GFP*. ***F***, Immunohistochemical analysis of *RPTP*λ*-*Δ*IC-HA* expression as detected by expression of the HA-fusion epitope. Expression is concentrated to cellular plasma membranes. ***G***, *Wnt1* expression after transfection with *RPTP*λ*-*Δ*IC-HA*. In ***C–E*** and ***G***, *Wnt1* expression is shown in dark blue, and *GFP* is shown in turquoise. The arrowheads in ***C–E*** and ***G*** mark the transverse *Wnt1* domain at the MHB. Scale bars: ***B***, ***F***, 20 μm; (in ***C***) ***C***, ***D***, ***E***, ***G***, 100 μm. IC, Isthmic constriction.

15 of 16). In contrast, after ectopic expression of *RPTP*λ*-HA*, the normal *Wnt1* expression pattern was markedly disturbed (Fig. 3*D*,*E*) ($n = 17$ of 20). In embryos in which *RPTP*λ*-HA* was misexpressed in a broad region surrounding the isthmic constriction, *Wnt1* expression was reduced or lost from the electroporated side of the embryo, suggesting that strong *RPTP*λ expression inhibits *Wnt1* expression at the MHB (Fig. 3*D*, arrowhead) ($n = 12$ of 20). In some embryos, particularly in those where *RPTP*λ*-HA* expression was directly targeted to the organizer (and just to the region where *Wnt1* would normally be expressed), a rostral displacement or split of the *Wnt1* expression domain around the *RPTP*λ*-HA* expressing cells was observed (Fig. 3*E*, arrowheads) ($n = 5$ of 20). Identical results were obtained when an untagged form of the protein was misexpressed, indicating that the presence of the triple-HA tag was not critical for *RPTP*λ function (data not shown). To test for the possible contribution of the cytoplasmic part of *RPTP*λ, the domain most likely to be essential for its phosphatase activity and the interaction with components of intracellular signal transduction pathways, we constructed truncated forms of *RPTP*λ in which a triple HA-tag replaced the juxtamembrane and phosphatase domains (*RPTP*λ*-*Δ*IC-HA*). When overexpressed in the mesencephalic vesicle, *RPTP*λ*-*Δ*IC-HA* staining was concentrated to cellular plasma membranes, indicating correct targeting of the truncated protein (Fig. 3*F*). In contrast to the full-length form of *RPTP*λ, electroporation of *RPTP*λ*-*Δ*IC-HA* did not perturb *Wnt1* expression (Fig. 3*G*) ($n = 21$ of 22), indicating that the cytoplasmic domain was required for *RPTP*λ function at the MHB.

To further characterize the potential role of *RPTP*λ in modulating gene expression at the MHB, we analyzed the expression of other MHB marker genes after electroporation of *RPTP*λ*-HA*. The expression domain of *Fgf8* was normal 24 h after misexpression of *RPTP*λ ($n = 6$ of 6) (supplemental Fig. 1*A*,*B*, available at www.jneurosci.org as supplemental material). The altered *Wnt1* expression that we observed 24 h after *RPTP*λ transfection can therefore not be a secondary effect of an altered *Fgf8* expression profile at the MHB. In addition, forced expression of *RPTP*λ did not affect expression of *Lmx1b* ($n = 16$ of 17), *En1* ($n = 8$ of 8), *Pax2* ($n = 9$ of 11), *Pax5* ($n = 9$ of 10), *Otx2* ($n = 4$ of 4), *Sef1* ($n = 7$ of 8), *sprouty2* ($n = 7$ of 7), or *Grg4* ($n = 5$ of 5) within 24 h after transfection (supplemental Fig. 1*C–K*, available at www.jneurosci.org as supplemental material) (data not shown).

*RPTP*λ interferes with *Fgf8*-mediated *Wnt1* induction

Next, we took advantage of the fact that *Fgf8* can mimic the organizer's activity when ectopically expressed in adjacent brain regions and performed two consecutive rounds of *in ovo* electroporation within the mesencephalic alar plate (Fig. 4*A*,*A*′). Electroporation of the *Fgf8b* expression plasmid into an embryo without previous manipulation reproducibly led to ectopic *Wnt1* expression (Fig. 4*B*,*B*′) ($n = 5$ of 5). Mock-transfection with *GFP* or *dsRed* did not alter *Wnt1* expression (Fig. 4*G*). Overexpression of *Fgf8b* in embryos already experiencing 6 h of *GFP* or *RPTP*λ*-*Δ*IC* expression also resulted in robust upregulation of *Wnt1* expression (Fig. 4*C*,*D*) ($n = 5$ of 5 for *GFP*; $n = 7$ of 8 for *RPTP*λ*-*Δ*IC*). In contrast, when full-length *RPTP*λ was misexpressed in the mesencephalic vesicle (Fig. 4*E*), only 22% of the transfected embryos exhibited robust upregulation of *Wnt1* after *Fgf8b* electroporation (Fig. 4*F*,*F*′) ($n = 4$ of 18). Because overexpression of *RPTP*λ at the MHB had specifically perturbed the expression of *Wnt1* but not that of MHB marker genes like *En1*, *Pax2*, or *Pax5* (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), we analyzed expression of these molecules after sequential *RPTP*λ*/Fgf8b* electroporation. In agreement with our previous results, expression of all three molecules was strongly induced by *Fgf8*, regardless of previous overexpression of *RPTP*λ (supplemental Fig. 1*L–O*, available at www.jneurosci.org as supplemental material) ($n = 6$ of 7 for *En1*; $n = 5$ of 5 for *Pax2*; $n = 5$ of 5 for *Pax5*). Together, these results suggest that strong *RPTP*λ expression specifically interferes with the ability of cells in the embryonic mesencephalon to initiate *Wnt1* expression in response to *Fgf8* signals. Considering that, during normal development (or in response to local *Fgf8* application), *RPTP*λ expression always extended over the *Wnt1* domain at its rostral side, our data suggest that *RPTP*λ may function to rostrally restrict the domain of *Wnt1* expression at the MHB.

If *RPTP*λ expression at the MHB indeed serves to restrict *Wnt1* expression, one would expect that reducing *RPTP*λ activity at the MHB should allow *Wnt1* expression to expand anteriorly. To test this idea, two RNAi constructs targeted to nonoverlapping sequences in the intracellular domain of *RPTP*λ were designed. The activity and specificity of the constructs were confirmed by luciferase reporter assays compared with the effects of an RNAi construct targeted to an unrelated protein or with that of RNAi constructs containing randomized targeting sequences (Fig. 5*A*). Repression by unrelated or randomized sequences was negligible (Fig. 5*A*). Electroporation of either *RPTP*λ*_si-a* ($n = 8$ of 9) (Fig. 5*D*) or *RPTP*λ*_si-b* ($n = 6$ of 9) (data not shown) but not of the randomized control targeting construct *random_a* ($n = 5$ of 5) (Fig. 5*C*) into the MHB region at HH10 markedly reduced the endogenous expression of *RPTP*λ. Knock-down of *RPTP*λ also led to an anterior expansion of *Wnt1*-positive cells into the domain where *RPTP*λ is normally expressed (Fig. 5*G–G*″,*I*,*J*) ($n = 9$ of 14). This effect was more pronounced near the dorsal midline than in the ventral midbrain, presumably because the domain of *RPTP*λ expression is broader and any effect of knock-down of its expression may therefore be more profound in the dorsal midbrain (Fig. 5*E*,*K*). *Wnt1* expression at the MHB,

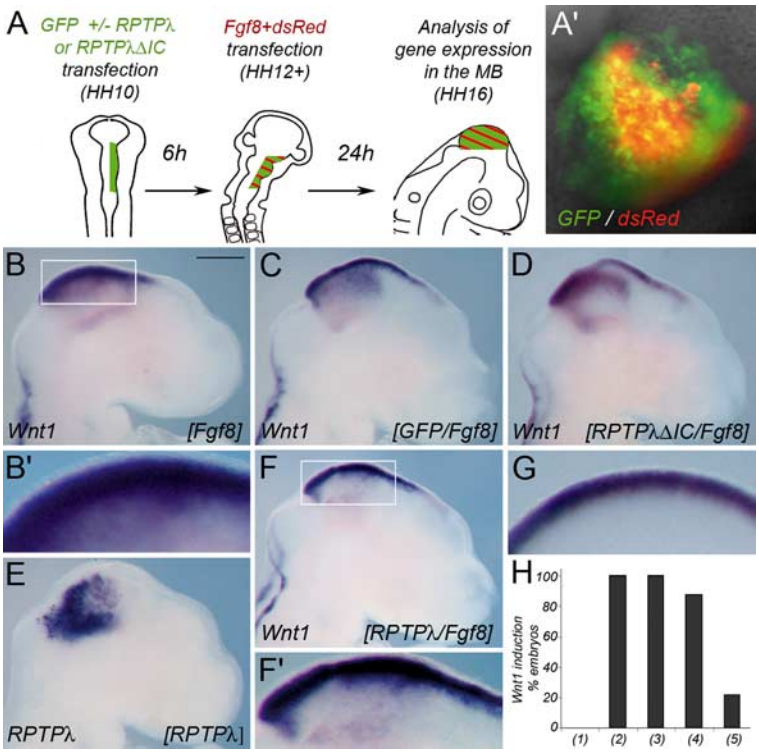

Figure 4. Misexpression of *RPTP*λ interferes with transcriptional *Wnt1* activation after ectopic *Fgf8* expression. ***A***, Schematic drawing of the experimental procedure. At HH10, *RPTP*λ plus *GFP*, *RPTP*λ-Δ*IC* plus *GFP*, or *GFP* alone were introduced into the right side of the midbrain vesicle. After 6 h (at HH12+), two expression plasmids carrying *Fgf8* and *dsRed*, respectively, were electroporated into the same side of the midbrain vesicle. The embryos were allowed to develop for an additional 24 h. ***A′***, Example of an embryo chosen for additional analysis, based on the massive coexpression of the red and green fluorescent proteins in the midbrain vesicle. ***B***, ***B′***, Ectopic expression of *Fgf8* in the midbrain without previous manipulation leads to widespread upregulation of *Wnt1*. ***C***, ***D***, *Fgf8* induced *Wnt1* expression is not affected by previous introduction of *GFP* (***C***) or *RPTP*λ-Δ*IC* (***D***). ***E***, Example of an embryo electroporated with *RPTP*λ-pMES indicating mosaic expression of the transgene. ***F***, ***F′***, Transcriptional upregulation of *Wnt1* is mostly prevented after ectopic expression of *RPTP*λ 6 h before *Fgf8* transgene introduction. ***B′***, ***F′***, Higher magnification of the boxed areas shown in ***B*** and ***F***. ***G***, Expression of *Wnt1* in an embryo electroporated with *GFP* followed by misexpression of *dsRed* 6 h later demonstrating normal expression in the mesencephalic roof plate under these experimental conditions. ***H***, Quantification of the results: (1), Percentage of embryos exhibiting strong ectopic expression of *Wnt1* 24 h after transfection with *GFP* or *dsRed* alone; (2), percentage of embryos exhibiting strong ectopic expression of *Wnt1* 24 h after transfection with *Fgf8b* (together with *dsRed*) without previous manipulation; (3), percentage of embryos exhibiting strong ectopic expression of *Wnt1* 24 h after transfection with *GFP* followed by *Fgf8b* (+*dsRed*); (4), percentage of embryos exhibiting strong ectopic expression of *Wnt1* 24 h after transfection with *RPTP*λ-Δ*IC-HA* (+*GFP*) followed by *Fgf8b* (+*dsRed*); (5), percentage of embryos exhibiting strong ectopic expression of *Wnt1* 24 h after transfection with *RPTP*λ-*HA* (+*GFP*) followed by *Fgf8b* (+*dsRed*). Scale bar, 500 μm.

however, was not only expanded rostrally, but expression was also scattered within the rostral half of its normal domain in some embryos (Fig. 5*F–J*). We do not know the molecular basis for this effect at present. A possible explanation may come from the fact that *RPTP*λ and *Wnt1* expression at the MHB appear to slightly overlap during normal development (Figs. 1*C-D′*, 5*E*). It is therefore possible that *RPTP*λ stabilizes the *Wnt1* expression border in the small region where both proteins are normally coexpressed. In addition, because the extracellular part of *RPTP*λ contains multiple cell adhesion-like motives, reduced *RPTP*λ expression at the MHB may alter cell adhesive properties critical for the maintenance of the coherent cellular organization within its normal expression domain. It is worth pointing out that disorganization of the *Wnt1* expression domain was also observed after targeted deletion of Fgf receptor 1 from the MHB territory (Trokovic et al., 2003).

At least three signaling pathways, the Ras-MAP kinase, phosphatidyl inositol 3 kinase, and phospholipase Cγ pathway, can be activated after Fgf receptor stimulation, and of the three, the Ras-MAP kinase pathway has been demonstrated to be important for midhindbrain development (Corson et al., 2003; Sato and Nakamura, 2004; Dailey et al., 2005). Because signal transduction in each of these pathways requires protein phosphorylation on tyrosine residues, it is possible that *RPTP*λ, because of its tyrosine phosphatase activity, interrupts signal transduction through dephosphorylation of intermediate signaling components. To test whether RPTPλ impinges on Ras-MAP kinase signaling, we visualized phosphorylation of the pathway intermediate Erk1/2 with the phosphorylation-specific antibody dpErk1/2 (Corson et al., 2003). Six hours after transfection of 2 μg/μl RPTPλ-pMES but not after transfection of pMES alone, dpErk1/2 labeling intensity was slightly reduced in the right electroporated side of the electroporated embryos (Fig. 5*N–P*) ($n = 6$ of 9). Repression of *Wnt1* expression by *RPTP*λ could also be detected 6 and 8 h after *RPTP*λ transfection (Fig. 5*Q–T*) ($n = 10$ of 18). In contrast to *Wnt1* but in agreement with our previous results (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), we did not observe any major disturbance of *En1* expression 6 or 8 h after *RPTP*λ transfection (Fig. 5*U*,*V*) (data not shown) ($n = 11$ of 11). These findings indicate that the influence of *RPTP*λ on *Wnt1* expression at the MHB may, at least in part, result from modulation of Ras-MAP kinase signaling intensity by *RPTP*λ.

RPTPλ interacts with β-catenin in the embryonic midbrain and inhibits the activity of a β-*catenin* responsive promoter

*RPTP*λ expression in the developing midbrain is highly dynamic during the first 4 d of chick development (Figs. 1, 6*A–C*) (Badde et al., 2005). Before HH12 (16 somites), *RPTP*λ is expressed throughout the mesencephalic vesicle (Fig. 6*A*) (Badde et al., 2005). Between HH12 and HH19, *RPTP*λ expression is lost from the midbrain alar plate but is maintained in a tight transverse domain rostral to the *Wnt1* domain (Figs. 1*A*,*C*, 6*B*) (Badde et al., 2005). Thereafter, *RPTP*λ expression is induced once again in the mesencephalic vesicle and covers the entire mesencephalic alar plate from late E4 onwards, without crossing into the isthmic organizer region (Figs. 1*D*, 6*C*, arrowhead) (Badde et al., 2005). Because of this prominent "on-off" expression in the midbrain alar plate, we speculated that *RPTP*λ may contribute to midbrain development by mechanisms in addition to restricting *Wnt1* expression at the MHB organizer.

The juxtamembrane domain of *RPTP*λ exhibits a high degree of homology to the cytoplasmic domain of *E-cadherin* (Wang et al., 1996). Moreover, the human homolog of RPTPλ, PCP2, colocalizes with β-catenin at sites of cell-to-cell contact in human adenocarcinoma cells and coprecipitates with β-catenin in PC-12 cells overexpressing both proteins (Yan et al., 2002). The interaction of both proteins occurs independently of the phosphorylation state of β-catenin, but β-catenin can be dephosphorylated on tyrosine residues by PCP2 (Yan et al., 2002). We therefore investigated whether β-catenin and RPTPλ may physically inter-

act in the developing mesencephalon. We overexpressed either *RPTPλ-HA* or *RPTPλ-ΔIC-HA* in the mesencephalic vesicle for 24 h and subsequently immunoprecipitated RPTPλ bound proteins using an antibody directed against the HA-epitope (Fig. 6*D*,*E*). Overexpressed RPTPλ-HA was detected as a discrete band of ~185 kDa recognized by the HA-specific antibody (Fig. 6*D*, top panel, lysate). After stripping and reprobing of the blot, endogenous β-catenin expression could also be readily detected (Fig. 6*D*, bottom panel, lysate). Precipitation with an unspecific antibody or with agarose-coupled protein-G did not deplete RPTPλ-HA or β-catenin from the extracts (Fig. 6, preclear). After immunoprecipitation with the HA-specific antibody, the 185 kDa band was cleared from the supernatant (Fig. 6*D*, top panel, sup) and enriched in the immunoprecipitate (Fig. 6*D*, top panel, IP) ($n = 5$ of 5). β-Catenin was also present in the immunoprecipitates (Fig. 6*D*, bottom panel, IP) ($n = 5$ of 5). Neither β-catenin nor RPTPλ could be precipitated with an antibody not directed against HA or β-catenin (data not shown). When the truncated allele RPTPλ-ΔIC-HA, which lacks the cytoplasmic part of the protein and thus the putative interaction domain with β-catenin, was electroporated into the mesencephalic vesicle, a specific band of 105 kDa was detected (Fig. 6*E*, top panel, lysate). After immunoprecipitation with the HA-specific antibody, RPTPλ-ΔIC-HA could be successfully immunoprecipitated but failed to enrich β-catenin (Fig. 6*E*, IP) ($n = 5$ of 5). These data demonstrate that RPTPλ can coprecipitate β-catenin from mesencephalic extracts via its intracellular domain.

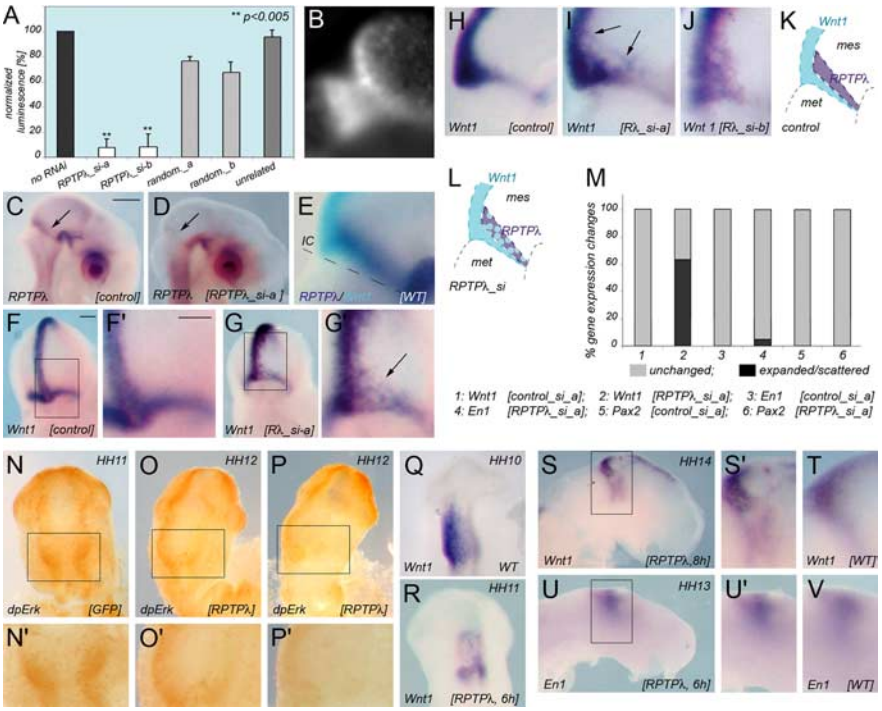

Figure 5. RNAi-mediated knock-down of *RPTPλ* and Ras-MAP kinase pathway activation after RPTPλ overexpression. ***A***, Efficiency and specificity of the RNAi targeting constructs as assessed by luciferase reporter assays. Experimental details are described in Material and Methods. *Renilla* luciferase activity of the reporter construct psiCHECK2-*RPTPλ* (Promega) without cotransfection of an RNAi targeting construct served as reference and was set at 100%. Cotransfection of either *RPTPλ_si-a* or *RPTPλ_si-b* together with psiCHECK2-*RPTPλ* reduced *Renilla* luciferase activity to <10%. Cotransfection of randomized targeting sequences or of an unrelated targeting construct did not significantly affect *Renilla* activity. Error bars represent SD. $^{**}p < 0.005$, Student's *t* test. ***B***, Representative example of GFP fluorescence 24 h after transfection of *RPTPλ_si-a* together with *GFP* across the MHB. ***C***, ***D***, ***F–J***, Analysis of gene expression after RNAi-mediated knock-down of *RPTPλ* expression. ***C***, *RPTPλ* expression after transfection with the randomized RNAi targeting construct *random_a*. ***D***, Expression of *RPTPλ* after transfection with *RPTPλ*_si-a. ***E***, Two-color *in situ* hybridization on a wild-type embryo showing expression of *RPTPλ* (dark blue) and *Wnt1* (turquoise). Note that the *RPTPλ* expression domain is broader in the dorsal than in the ventral midbrain. ***F***, ***H***, Expression of *Wnt1* in embryos transfected with the control targeting construct *random_a*. ***G***, ***I***, ***J***, Expression of *Wnt1* after transfection with *RPTPλ*_si-a (***G***, ***I***) or *RPTPλ*_si-b (***J***). ***F′*** and ***G′*** are higher magnifications of the boxed areas in ***F*** and ***G***. ***K***, Schematic drawing of the normal gene expression patterns of *Wnt1* (turquoise) and *RPTPλ* (purple) at the MHB. ***L***, Schematic drawing of *Wnt1* expression after *RPTPλ*_si transfection. ***M***, Quantification of the results. Gray bars indicate unchanged expression, and black bars indicate expanded and/or scattered expression of *Wnt1*. ***N–P***, DpErk1/2 staining at the MHB 6 h after electroporation of *GFP* (***N***, ***N′***) or *RPTPλ* (***O–P′***). ***N′***, ***O′***, and ***P′*** are higher magnifications of the boxed areas in ***N***, ***O***, and ***P***. ***Q–T***, *Wnt1* expression 6 h (***R***) and 8 h (***S***, ***S′***) after *RPTPλ* transfection and in age-matched controls (***Q***, ***T***). ***U***, ***V***, *En1* expression 8 h after *RPTPλ* transfection (***U***, ***U′***) and in an age-matched control (***V***). Scale bars: ***C***, 500 μm; ***F***, ***F′***, 100 μm. IC, Isthmic constriction; mes, mesencephalon; met, metencephalon.

We next investigated whether the observed interaction of β-catenin with RPTPλ was biologically relevant for canonical *Wnt* signaling. *β-Catenin* plays an essential role in the structural organization of cells by linking the cytoplasmic domain of type I *cadherins*, such as *E-cadherin*, to the actin cytoskeleton and, in addition, functions as a key component of the canonical *Wnt* signaling pathway. Loss of *E-cadherin*-mediated cell adhesion increased canonical *Wnt* signaling, presumably because, in the absence of *E-cadherin*, more *β-catenin* is available for *Wnt* signal transduction (Orsulic et al., 1999; Stockinger et al., 2001). We therefore wondered whether overexpression of RPTPλ in the mesencephalon may sequester a portion of β-catenin at the plasma membrane, making it unavailable for *Wnt* signaling. To test this idea, we examined the activity of the β-catenin responsive reporter TOP:dgfp in the midbrain, alone or in the presence of ectopically expressed *RPTPλ*. TOP:dgfp contains four consensus Lef-binding sites and a minimal c-Fos promoter-driving expression of a destabilized form of *GFP* (dGFP) (Dorsky et al., 2002). Transgenic zebrafish that stably express dGFP under control of this promoter exhibit intense GFP expression throughout the entire midbrain alar plate, demonstrating that the canonical *Wnt/β-catenin* pathway is active in this region of the neural tube (Dorsky et al., 2002). When the TOP:dgfp reporter was introduced into the MHB region of HH11 chick embryos by *in ovo* electroporation, strong GFP expression in the midbrain alar plate could be observed 24 h later (at HH15–16), demonstrating that the midbrain alar plate is a domain of active *Wnt/β-catenin* signaling in the chick at this developmental age (Fig. 6*F–H*,*N*) ($n = 8$ of 8). Therefore, at HH15–HH16, neither *RPTPλ* nor *Wnt1* are expressed in the mesencephalic alar plate, but the *Wnt/β-catenin* pathway is active. We therefore reasoned that misexpression of *RPTPλ* in the midbrain alar plate at HH15–H16 should allow us to analyze a possible influence of *RPTPλ* on canonical *Wnt* signal transduction that is independent of its ability to influence *Wnt1* expression at the MHB. When *RPTPλ* was coelectroporated with TOP:dgfp, GFP fluorescence intensity was reduced severely (Fig. 6*I–K*,*N*) ($n = 10$ of 11). Transfection of *RPTPλ-ΔIC*, which lacks the intracellular domain critical for coprecipitation of *β-catenin*, did not suppress

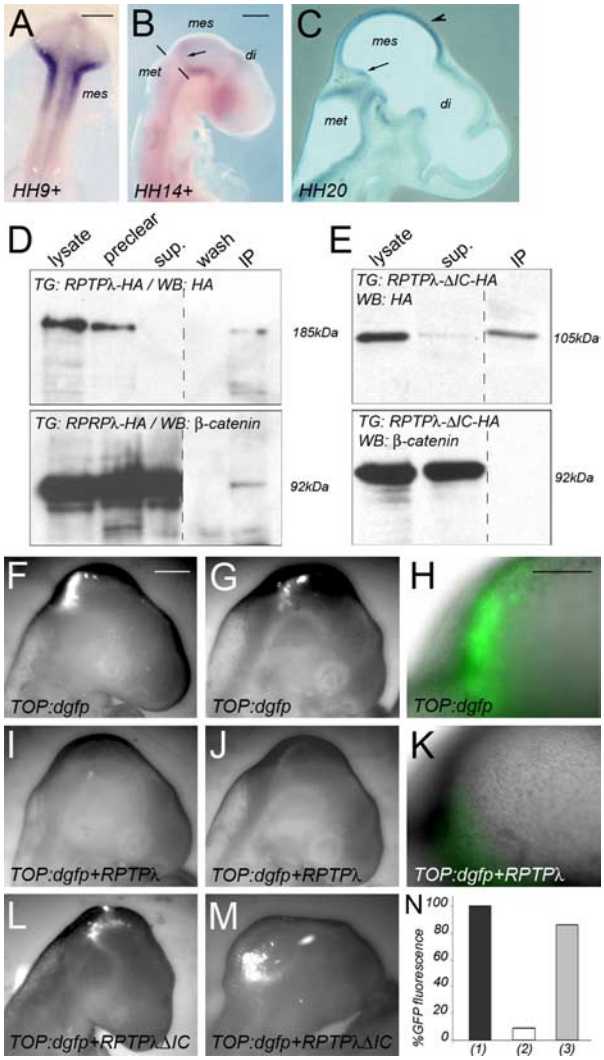

Figure 6. RPTPλ binds to β-catenin in the mesencephalon and inhibits the activity of a β-catenin responsive promoter *in vivo*. ***A–C***, On-off-on expression of *RPTPλ* in the mesencephalic vesicle between HH9+ and HH20 as described by Badde et al. (2005). The dashed line in ***B*** marks the isthmic constriction, the arrow in ***B*** and ***C*** marks the location of the transverse ring of *RPTPλ* expression at the MHB, and the arrowhead in ***C*** marks the rostral-to-caudal upregulation of *RPTPλ* in the midbrain alar plate. di, Diencephalon; mes, mesencephalon; met, metencephalon. ***D***, Immunoprecipitation using an HA-specific antibody on RPTPλ-HA transfected HH16 midbrain tissue demonstrating coprecipitation of β-catenin with RPTPλ-HA. ***E***, As in ***D***, except tissue transfected with RPTPλ-ΔIC-HA was used. No coprecipitation with β-catenin was observed. Top, Western Blot analysis with the HA-specific antibody detecting RPTPλ-HA (***D***) or RPTPλ-ΔIC-HA (***E***), respectively. Bottom, Detection of β-catenin on the blots shown above after stripping and reprobing with a β-catenin-specific antibody. The blots were cut to remove the marker lane. Preclear, Supernatant after precipitation with an unspecific antibody or with agarose-coupled protein-G; sup, supernatant after precipitation with the HA-specific antibody; IP, immunoprecipitate. ***F–M***, GFP fluorescence 24 h after transfection of a TOP:dgfp reporter into the MHB region. ***F–H***, Different examples of GFP expression in embryos 24 h after electroporation with TOP:dgfp. ***I–K***, Different examples of GFP expression after electroporation of TOP:dgfp together with *RPTP*λ-pMIW. ***L***, ***M***, Examples of GFP expression after electroporation of TOP:dgfp together with *RPTP*λ-Δ*IC*-pMIW. ***F***, ***G***, ***I***, ***J***, ***L***, and ***M*** are monochrome images. ***N***, Quantification of the results: (1), percentage of embryos exhibiting GFP fluorescence 24 h after transfection with TOP:dgfp alone; (2), percentage of embryos exhibiting GFP fluorescence 24 h after transfection with TOP:dgfp together with *RPTPλ-HA*; (3), percentage of embryos exhibiting GFP fluorescence 24 h after transfection with TOP:dgfp together with *RPTPλ-ΔIC-HA*. Scale bars: ***A***, ***B***, 200 μm; ***F***, 500 μm; ***H***, 250 μm.

GFP fluorescence (Fig. 6*L–N*) ($n = 6$ of 7). These results demonstrate that *RPTPλ* can suppress the activation of this *β-catenin* responsive reporter *in vivo*.

The normal growth and development of the mid-hindbrain region depends on activation of the canonical *Wnt* signaling pathway (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Brault et al., 2001; Panhuysen et al., 2004). To investigate whether ectopic *RPTPλ* expression or RNAi-mediated knock-down of expression altered midbrain growth, *RPTPλ* expression was targeted to the right side of the mesencephalic vesicle by *in ovo* electroporation into HH9 embryos, and the consequence of this manipulation on midbrain growth was assessed 24 h later (Fig. 7). We chose HH9 embryos for these experiments, because this developmental time is before the downregulation of endogenous *RPTPλ* expres-

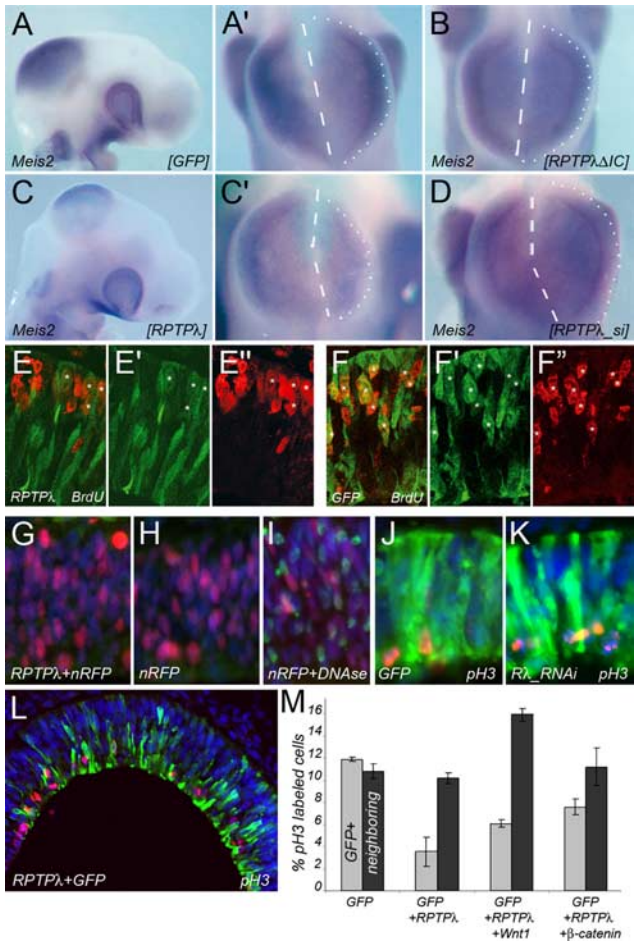

Figure 7. *RPTPλ* modulates mesencephalic growth. ***A–D***, Whole-mount *in situ* hybridization with a *Meis2*-specific probe on HH15–HH16 embryos after electroporation with pMES (***A***, ***A′***), *RPTPλ*-Δ*IC*-pMES (***B***), *RPTPλ*-pMES (***C***, ***C′***), or *RPTPλ_si-a* (***D***). In ***A′***, ***B***, ***C′***, and ***D***, the embryos are shown from the top. The dashed lines define the location of the dorsal midline as observed by the lack of *Meis2* expression, and the dotted lines mark the perimeter of the right side of the mesencephalic vesicle. ***E***, ***F***, Cross sections through HH16 midbrains showing BrdU incorporation after ectopic expression of *RPTPλ* together with *GFP* (***E–E″***) or *GFP* alone (***F–F″***). ***E***, ***F***, Overlaid channels of ***E′*** and ***E″*** or ***F′*** and ***F″***. ***E′***, ***F′***, *GFP* expression. ***E″***, ***F″***, BrdU incorporation. ***G–I***, TUNEL labeling of HH16 midbrains electroporated with nRFP together with *RPTPλ* (***G***) or nRFP alone (***H***). ***I***, DNase treatment before TUNEL labeling as control for the validity of the experimental procedure. ***J***, ***K***, Cross sections through HH16 midbrains labeled for pH3 (red) and GFP (green) and counterstained with DAPI (blue). ***J***, Misexpression of *GFP*. ***K***, Misexpression of *RPTPλ*_si-a together with *GFP*. ***L***, Cross section through an HH16 midbrain electroporated with *RPTPλ* together with *GFP* and stained for GFP (green), pH3 (red), and DAPI (blue). Fewer transfected (GFP+) cells are labeled for pH3 than nontransfected, neighboring (GFP−) cells. For more examples, see supplemental Figure 2 (available at www.jneurosci.org as supplemental material). ***M***, Quantification of the percentage of pH3-positive cells transfected with combinations of the gene products indicated (gray bars) and the percentage of pH3-positive cells among the nontransfected, neighboring cell population under each condition (black bars). Error bars indicate SD. Scale bar (***A***), 500 μm.

sion in the mesencephalon, which occurs after HH12 (Fig. 6*A–C*) (Badde et al., 2005). Introduction of *RPTP*λ-pMES into the HH9 mesencephalic vesicle therefore maintains *RPTP*λ expression in the midbrain beyond HH12, whereas introduction of an RNAi targeting construct leads to precocious loss of *RPTP*λ expression. The homeodomain *Meis2* was used as a marker for the tectal anlage, because its transcripts are excluded from the roof plate, allowing us to unambiguously determine the location of the dorsal midline and estimate the size of the left and right halves of the mesencephalic vesicle. After forced expression of *GFP* or of the truncated allele *RPTP*λ-Δ*IC-HA* together with *GFP*, the right and left halves of the tectal anlage appeared to have normal size (Fig. 7*A*,*B*) ($n = 5$ of 5 for *GFP*; $n = 6$ of 6 for *RPTP*λ-Δ*IC-HA*). However, in embryos that had been electroporated with an expression plasmid containing *RPTP*λ, the size of the right electroporated tectal anlage was reduced severely when compared with the left, nonelectroporated side of the same embryo (Fig. 7*C*,*C*′) ($n = 15$ of 16). Conversely, the right tectal anlage was slightly enlarged in embryos transfected with the specific RNAi targeting construct *RPTP*λ*_si-a* (Fig. 7*D*) ($n = 6$ of 7).

To determine whether this growth reduction was the result of decreased cell proliferation and/or enhanced cell death, BrdU pulse-labeling experiments were performed. We delivered *RPTP*λ*-HA* using the electroporation vector pMES, which contains an IRES-*GFP* cassette allowing us to visualize individual *RPTP*λ*-HA* transfected cells by their GFP fluorescence (Swartz et al., 2001). Two hours after BrdU injection, 51% (±1.85 SEM) of the cells forced to express *GFP*, but only 27.2% (±1.41 SEM; $p < 0.002$, Student's *t* test) of *RPTP*λ*/GFP* expressing cells had incorporated the BrdU label (Fig. 7*E–F*″). Similar to mock transfected or nontransfected embryos, apoptotic cell nuclei were rarely observed 24 h after misexpression of *RPTP*λ, indicating that *RPTP*λ electroporation does not compromise cell survival within this time frame (Fig. 7*G–I*) ($n = 3$). Knock-down of *RPTP*λ expression at HH9 slightly increased the percentage of mitotic cells, visualized by detecting pH3 from 11.7% (±1.29 SEM) after introduction of *GFP* alone to 14.2% (±1.45 SEM) after transfection of the RNAi targeting construct *RPTP*λ*_si-a* together with *GFP* (Fig. 7*J*,*K*) (supplemental Fig. 2*E*, available at www.jneurosci.org as supplemental material) ($n = 4$). In conclusion, these results suggest that *RPTP*λ functions as negative modulator of mesencephalic growth primarily through modulation of progenitor cell proliferation.

Because RPTPλ could precipitate (and possibly sequester) the *Wnt*-signaling intermediate β-catenin from mesencephalic extracts (Fig. 6*D*,*E*), we wondered whether cotransfection of *β-catenin* or *Wnt1* together with *RPTP*λ would rescue the proliferation defect observed after introduction of *RPTP*λ alone. To test this idea, we introduced either pMES (expressing only *GFP*), *RPTP*λ-pMES (expressing *RPTP*λ together with *GFP*), *RPTP*λ-*Wnt1*-pMEC together with *GFP*-pMIW (which results in coexpression of *RPTP*λ, *Wnt1*, and *GFP*), or *RPTP*λ-pMES together with *caβ-catenin*-pMIW (which results in coexpression of *RPTP*λ, *GFP*, and a stabilized form of *β-catenin*) into the mesencephalic vesicle at HH9 and determined the mitotic index of midbrain progenitor cells 24 h later (Fig. 7*L*,*M*) (supplemental Fig. 2*A–D*, available at www.jneurosci.org as supplemental material) ($n = 9$ for pMES, *RPTP*λ-pMES; $n = 3$ for *RPTP*λ-*Wnt1*-pMEC, *RPTP*λ-pMES plus *β-catenin*-pMIW). Transfection of *RPTP*λ together with *GFP* significantly reduced the percentage of mitotic cells compared with *GFP* transfected controls. Cotransfection of *Wnt1* or stabilized *β-catenin* together with *RPTP*λ and *GFP* partially restored the mitotic index (Fig. 7*M*, gray bars). Therefore, the negative influence of *RPTP*λ on midbrain progenitor cell proliferation could, at least in part, be alleviated by cotransfection of *Wnt1* or of the *Wnt1*-signaling intermediate *β-catenin*. To determine whether *RPTP*λ modulates midbrain progenitor cell proliferation in a cell-autonomous or noncell-autonomous manner, we compared the percentage of pH3-positive cell nuclei in the transfected (*GFP* expressing) cell population under each of these experimental conditions with the percentage of pH3+ cells in nontransfected (*GFP*-negative) neighboring cells (Fig. 7*M*, black bars). The mitotic index of cells that were located in close proximity to cells transfected with *RPTP*λ plus *GFP* did not differ from that of the neighbors of *GFP* transfected cells, suggesting that *RPTP*λ acts cell autonomously on midbrain progenitor cell proliferation. We also did not observe a significant difference in the percentage of pH3+ cell nuclei in neighbors of *RPTP*λ*/GFP/β-catenin* transfected cells compared with neighbors of *GFP*-expressing cells. In contrast, the percentage of pH3+ nuclei in cells located near *RPTP*λ*/Wnt1/GFP* transfected cells was clearly increased. This observation is consistent with the notion that the secreted molecule *Wnt1* may influence cell proliferation in a cell-nonautonomous manner.

Our finding that overexpression of *RPTP*λ interfered with *Fgf8*-mediated induction of *Wnt1* expression at the MHB as well as with the activation of the *Wnt/β-catenin*-dependent TOP:dgfp reporter in the mesencephalon raises the question of whether the reduced TOP:dgfp reporter activity in *RPTP*λ transfected embryos may be a secondary consequence of their diminished *Wnt1* expression or, as suggested above, may reflect a more direct inhibition of *Wnt/β-catenin* signaling, presumably through interaction of *RPTP*λ with *β-catenin*. To distinguish between these possibilities, we first decreased *Wnt1* expression at the MHB through RNAi-mediated knock-down and monitored TOP:dgfp activity in the mesencephalic vesicle 24 h later (supplemental Fig. 3*A–H*′, available at www.jneurosci.org as supplemental material) ($n = 15$). Although electroporation of *Wnt1*-specific RNAi targeting constructs reduced *Wnt1* expression *in vivo*, GFP expression of the TOP:dgfp reporter appeared normal. Second, we blocked *Wnt* signaling in the midbrain through forced expression of the known signaling antagonist *Axin* (Zeng et al., 1997). *Wnt1* expression at the MHB was normal 24 h after transfection (supplemental Fig. 3*I*,*J*, available at www.jneurosci.org as supplemental material) ($n = 10$). Based on these observations, we suggest that *RPTP*λ may modulate *Wnt1* expression and signal transduction in the midbrain through two separate mechanisms.

The midbrain alar plate undergoes massive expansion between HH12 and HH19 when *RPTP*λ expression is absent from this tissue. Cells around the MHB in embryonic day 10.5 (E10.5) to E11.5 mouse embryos are less mitotically active than cells in the midbrain and rhombomere 1 (Trokovic et al., 2005). A similar domain of reduced proliferation also exists at the chick MHB at HH15 and HH20 (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Although the ring of *RPTP*λ expression seen during this developmental period does not cover this domain entirely, *RPTP*λ expression nevertheless appears to localize within this domain.

Discussion

Here, we present evidence based on gain-of-function and knockdown experiments that *RPTPλ* can counteract transcriptional activation of *Wnt1* in the embryonic midbrain. In addition, we show that RPTPλ can physically interact with β-catenin in the midbrain, inhibit the activity of a β-catenin responsive promoter, and suppress mesencephalic cell proliferation. *RPTPλ* therefore emerges as a potential modulator of *Wnt1* expression and signaling at the MHB organizer.

RPTPλ is part of the complex molecular interactions that maintain and shape the MHB region

A complex spatiotemporal pattern of gene expression has been described at the developing mid-hindbrain boundary. The MHB is bordered by a narrow, transverse ring of *Wnt1* expression at its anterior side and by a ring of *Fgf8* expression at its posterior side. Several proteins, including *Pax2/5* and *En1/2*, are expressed in nested, symmetric gradients across the MHB region. *Fgf8* signaling induces activation of the Ras-MAP kinase pathway, which is required for isthmic organizer activity (Sato and Nakamura, 2004). Fgf feedback inhibitors, such as *Spry2*, *Sef*, and *Mkp3*, interfere with Ras-MAP kinase signaling downstream of receptor tyrosine kinases at different steps within the signal transduction pathway and are thought to limit the lateral expansion of the Fgf signal at the MHB (Gross et al., 2001; Nutt et al., 2001; Fürthauer et al., 2002; Lin et al., 2002; Tsang et al., 2002; Kawakami et al., 2003; Echevarria et al., 2005). Maximal Ras-MAP kinase activation occurs within the domain of *Fgf8* expression but tapers off into the mesencephalic and metencephalic vesicles (Corson et al., 2003; Sato and Nakamura, 2004). The shapes of the *Pax2/5* and *En1/2* expression domains reflect the gradient of Ras-MAP kinase activity at the MHB. In contrast, *Wnt1* expression is confined to a tight ring of cells in the mesencephalon and is absent from the metencephalon. Metencephalic tissue is not permissive for *Wnt1* expression, explaining the asymmetric nature of the *Wnt1* expression domain (Kikuta et al., 2003). It is yet unclear, however, as to why *Wnt1* expression is restricted to such a discrete band of cells and does not, like that of *Pax2/5* or *En1/2*, taper off into the mesencephalic vesicle. As we show here, the receptor tyrosine phosphatase λ is expressed in a ring of cells rostral to the *Wnt1* domain and inhibits *Wnt1* expression when overexpressed. *RPTPλ* thus has the potential to restrict *Wnt1* expression to its characteristic tight ring at the MHB.

RPTPλ expression itself is induced by *Fgf8b* in a concentration-dependent manner. When *Fgf8*-induced expression of *Wnt1* and *RPTPλ* was monitored successively in the same specimen, ectopic expression of *RPTPλ* could always be observed anterior to the ring of *Wnt1* expression. Induction of *Wnt1* and *RPTPλ* expression by *Fgf8* thus mimics their normal spatial relationship at the MHB, where the *Wnt1* expression also separates the domain of *RPTPλ* expression from that of *Fgf8*. In addition, after implantation of Fgf8b releasing beads, ectopic *RPTPλ* expression could be observed at a Fgf8b concentration that was not sufficient to induce *Wnt1* expression. We conclude from these results that different dosages of the Fgf8 signal from the MHB are required for transcriptional induction of *RPTPλ* and *Wnt1*, respectively. Examples for dosage dependency of Fgf function during embryogenesis have been described previously and include telencephalic and cerebellar development, patterning of the foregut, and skeletal development (Sato et al., 2001; Storm et al., 2003, 2006; Hajihosseini et al., 2004).

RPTPλ specifically inhibited *Wnt1* expression but not that of other MHB marker genes when overexpressed in the MHB region. In addition, when cells in the midbrain were forced to express *RPTPλ*, they failed to upregulate *Wnt1* expression after subsequent stimulation by *Fgf8*, yet they readily expressed *En1*, *Pax2*, or *Pax5* under identical experimental conditions. *RPTPλ* therefore appears to specifically interfere with *Fgf8*-induced transcriptional activation of *Wnt1* at the MHB. How can this high degree of functional specificity be achieved mechanistically? Expression of many MHB marker genes, including *Otx2*, *Gbx2*, *Pax2/5*, *En1/2*, and *Wnt1*, is negatively affected by overexpression of a dominant-negative form of Ras (Sato and Nakamura, 2004). Yet, we observed recently after transfection of different concentrations of a constitutive active form of *Ras* (*caRas*) that transcriptional activation of *Wnt1* at the MHB could only be induced at a narrow concentration range of *caRas*, whereas transcriptional activation of *En1* was observed over a concentration range spanning more than an order of magnitude (Vennemann et al., 2008). Induction of *Wnt1* at the organizer thus appears to be more sensitive to changes in the strength of Ras-MAP kinase pathway activation than other MHB marker genes. This raises the possibility that tight, local regulation of Ras-MAP kinase activity may be a prerequisite for *Wnt1* expression at the MHB and that *RPTPλ* may contribute to this regulation through modulation of Ras-MAP kinase signaling. Notably, in mouse embryos in which *Fgf8* expression was specifically lost in the MHB territory from the five somite stage onwards, the transverse ring of *Wnt1* expression in the caudal mesencephalon was also absent, whereas the expression domains of *Fgf8*, *Otx2*, *Pax2*, or *En1* appeared normal at early stages of embryogenesis (Chi et al., 2003). Genetic loss of *Fgf8* at the MHB, however, caused massive cell death, an effect we did not observe within 24 h after *RPTPλ* transfection. We cannot rule out that *RPTPλ* inhibits *Wnt1* expression by interfering with still unknown factor(s) that are specifically required for transcriptional *Wnt1* activation in *Fgf8* responding cells. Regardless of the exact molecular nature of this inhibition, the experiments described here suggest a model in which *Fgf8* induces *Wnt1* and *RPTPλ* expression in the midbrain at different signaling thresholds and consequently at different distances to the MHB, so that the *Wnt1* domain is always bordered by *RPTPλ*-expressing cells at its rostral side. One function of *RPTPλ* at the MHB may thus be to act as a Ras-MAP kinase feedback inhibitor to restrict the anterior expansion of the transverse *Wnt1* domain (Fig. 8).

RPTPλ binds to *β-catenin* and modulates canonical *Wnt1* signaling at the MHB

In addition to restricting *Wnt1* expression at the MHB, *RPTPλ* also seems to play a role in modulating *Wnt1* signal transduction. In the canonical or *Wnt/β-catenin* pathway, *Wnt* binding to *frizzled receptors* and *LRP* coreceptors inhibits degradation of the cytoplasmic pool of *β-catenin*, allowing it to translocate to the nucleus, where it acts as coactivator for *LEF/TCF* transcription factors to promote target gene expression (Logan and Nusse, 2004). In addition to its role in *Wnt* signaling, *β-catenin* is a structural link between *cadherins/α-catenin* and the actin cytoskeleton. *Cadherin* bound *β-catenin* can be freed to participate in *Wnt* signaling and *vice versa*, a switch that appears to be regulated by phosphorylation of *β-catenin* on tyrosine residues (Brembeck et al., 2004; Nelson and Nusse, 2004; Schambony et al., 2004; Lilien and Balsamo, 2005). It was shown previously that *β-catenin* interacts with the juxtamembrane domain of the human homolog of *RPTPλ* and can be dephosphorylated by this protein *in vitro* (Yan et al., 2002). As shown here, a similar interaction of *RPTPλ* and

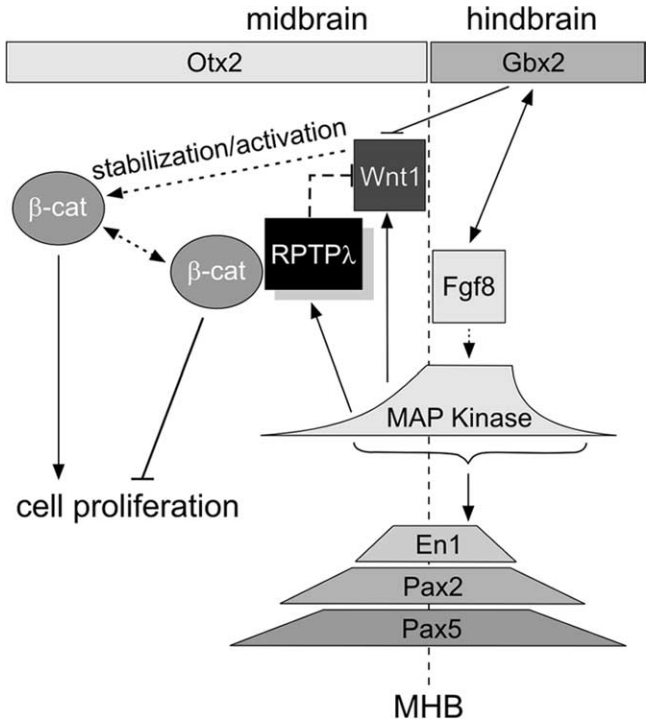

Figure 8. Proposed model for the function of *RPTP*λ at the MHB. The MHB (dashed vertical line) is located at the border between the expression domains of *Otx2* (rostral) and *Gbx2* (caudal). Once the MHB is established during early somitogenesis, *Fgf8*, *Wnt1*, *En2*, *Pax2*, and *Pax5* function in an interdependent, positive feedback loop, which is necessary for organizer maintenance. *RPTP*λ expression is induced by *Fgf8* at a signaling threshold that differs from that required for *Wnt1* induction, resulting in a band of *RPTP*λ expression anterior to the *Wnt1* domain. *RPTP*λ counteracts *Fgf8*-mediated transcriptional activation of *Wnt1*, which constrains the *Wnt1* domain to its typical tight ring anterior to the *Otx2-Gbx2* boundary. Presumably, as a consequence of the high degree of homology between the juxtamembrane domain of *RPTP*λ with *cadherins*, *RPTP*λ can bind to and sequester *β-catenin* and thereby negatively influence canonical *Wnt* signaling.

β-catenin also occurs in the embryonic chick midbrain. In addition, when full-length *RPTP*λ was overexpressed, we found that two functional readouts of canonical *Wnt* signaling in the midbrain, activation of a *β-catenin* responsive promoter and neuroepithelial cell proliferation, were reduced. This proliferation defect could be partially reversed by cotransfection of *Wnt1* or of a stabilized form of *β-catenin*. It is intriguing to speculate that *RPTP*λ may antagonize canonical *Wnt* signaling through binding and potentially dephosphorylation of *β-catenin* and ultimately serve as a negative regulator of cell proliferation in the developing midbrain (Fig. 8). Decreasing *Wnt1* expression at the MHB through transfection of *Wnt1*-specific RNAi targeting constructs did not notably reduce *β-catenin* responsive reporter activity in the midbrain, nor did misexpression of *Axin*, a known inhibitor of the *Wnt* signaling pathway, in the mesencephalon disturb the transverse ring of *Wnt1* expression at the organizer. These observations suggest that *RPTP*λ acts on *Wnt1* expression and signal transduction through two independent mechanisms.

How can this dual effect be explained? Protein tyrosine phosphatases antagonize the activity of protein tyrosine kinases and influence a broad spectrum of physiological processes. For instance, *RPTP*λ (also termed *RPTP*ψ) is required for Delta/Notch-dependent oscillatory gene expression in the presomitic mesoderm as well as for convergent extension during gastrulation through an as yet unknown mechanism (Aerne and Ish-Horowicz, 2004). It is therefore possible that *RPTP*λ impinges on multiple signaling pathways through dephosphorylation of different signaling components. In addition, the ring of *RPTP*λ expression at the MHB may coincide with that of another receptor tyrosine phosphatase, *RPTPζ/β*, a keratan sulfate modified protein, which has been suggested to modify signal transduction at the MHB through influencing the diffusion of morphogenetic signals of the Wnt and Fgf families (Canoll et al., 1993; Hamanaka et al., 1997). Although the precise role of *RPTP*λ in any of these processes will only be fully understood once its substrates are identified, our results suggest that *RPTP*λ is part of the complex molecular network that governs development of the midbrain region.

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