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Ras-dva, a member of novel family of small GTPases, is required for the anterior ectoderm patterning in the *Xenopus laevis* embryo

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Ras-like small GTPases are involved in the regulation of many processes essential for the specification of the vertebrate body plan. Recently, we identified the gene of novel small GTPase Ras-dva, which is specifically expressed at the anterior margin of the neural plate of the *Xenopus laevis* embryo. Now, we demonstrate that Ras-dva and its homologs in other species constitute a novel protein family, distinct from the previously known families of small GTPases. We show that the expression of *Ras-dva* begins during gastrulation throughout the anterior ectoderm and is activated by the homeodomain transcription factor Otx2; however, later on, *Ras-dva* expression is inhibited in the anterior neural plate by another homeodomain factor Xanf1. Downregulation of Ras-dva functioning by the dominant-negative mutant or by the antisense morpholino oligonucleotides results in severe malformations of the forebrain and derivatives of the cranial placodes. Importantly, although the observed abnormalities can be rescued by co-injection of the *Ras-dva* mRNA, they cannot be rescued by the mRNA of the closest Ras-dva homolog from another family of small GTPases, *Ras*. This fact indicates functional specificity of the Ras-dva signaling pathway. At the molecular level, downregulation of Ras-dva inhibits the expression of several regulators of the anterior neural plate and folds patterning, such as *Otx2, BF-1* (also known as *Foxg1*), *Xag2*, *Pax6*, *Slug* and *Sox9*, and interferes with FGF8 signaling within the anterior ectoderm. By contrast, expression of the epidermal regulator *BMP4* and its target genes, *Vent1, Vent2b* and *Msx1*, is upregulated. Together, the data obtained indicate that Ras-dva is an essential component of the signaling network that patterns the early anterior neural plate and the adjacent ectoderm in the *Xenopus laevis* embryos.

KEY WORDS: Small GTPase, Neural plate, Forebrain, Patterning of the neural plate, Neural crest, *Xenopus*

INTRODUCTION

Members of the superfamily of small GTPases regulate many aspects of cell behavior, including gene expression, reorganization of the cytoskeleton, and vesicle and nuclear-cytoplasmic transport (Hall, 2000; Takai et al., 2001).

Among the currently described seven families of small GTPases, members of the Ras family are particularly interesting for developmental biologists because they are responsible for the intracellular transduction of FGF signaling, which plays a prominent role in many processes during early development of the neural system, including neural induction, patterning of the neural plate and cranial placodes differentiation (Baird, 1994). During these processes, Ras GTPases transmit the signal from the receptor tyrosine kinases, activated by FGF binding, to the MAP kinase cascade, which in its turn transduces the signal to the specific genetic targets (Ribisi et al., 2000; Whitman and Melton, 1992).

Although in many cases expression domains of the receptor tyrosine kinases and their ligands occupy very restricted areas in the developing embryo, genes of the Ras-like small GTPases are expressed more ubiquitously (Ford-Perriss et al., 2001; Golub et al., 2000). Therefore, it is thought that the spatial specificity of FGF signaling is achieved by the spatially restricted expression of a particular ligand-receptor couple within the embryo, rather than by localized expression of a small GTPase responsible for the transduction of this signal.

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Recently, we identified the gene of a novel Ras-like GTPase Ras-dva, which, in contrast to *Ras*, is expressed during *Xenopus laevis* neurulation in a very restricted area surrounding the anterior margin of the neural plate (Novoselov et al., 2003). At the midneurula stage, this area includes non-neural ectoderm of the anterior and lateral neural folds, the prospective regions of the cranial placodes and the neural crest. Such a localized expression of *Ras-dva* indicates a possible role of this small GTPase as a factor directly ensuring spatial restriction of, probably, some FGF signaling. Therefore, further investigation of Ras-dva functioning would be very important to better understand the mechanisms responsible for anterior ectoderm patterning.

Now, we report that Ras-dva and its homologs in other species constitute a novel family of Ras-like small GTPases. We show that downregulation of the Ras-dva functioning by the antisense morpholino or by the dominant-negative mutant Ras-dvaT22N results in head development abnormalities, which include reduction of the forebrain, olfactory pits, otic vesicles, branchial arches and malformations of the head cartilages. Although these abnormalities can be rescued by co-injections of the *Ras-dva* wild-type mRNA, they cannot be rescued by co-injections of the mRNA of a small GTPase belonging to another family, *Ras*.

At the molecular level, downregulation of Ras-dva inhibits the expression of several regulators of the anterior neural plate and folds patterning, such as *Otx2*, *BF-1*, *Xag2*, *Pax6*, *Slug* and *Sox9*, and interferes with the FGF-8a signaling within the anterior ectoderm. By contrast, expression of the epidermal regulator *BMP4* and its target genes, *Vent1, Vent2b* and *Msx1*, is upregulated. Altogether, these data indicate that Ras-dva might be involved into a novel signal transduction pathway essential for vertebrate head development and early patterning of the anterior neural plate.

MATERIALS AND METHODS

Preparation of DNA constructs, synthesis and injections of mRNA and anti-sense morpholino oligonucleotides

CDNA templates encoding for Tre22Asn mutant of Ras-dva (DN-RasdvaT22N) and for S17N mutant of Ras were obtained from the full-length *Ras-dva* cDNA (Novoselov et al., 2003) and from EST clone AN: BG553860 of Ras cDNA, respectively, by PCR methodology. The resulting cDNAs were subcloned into pSP64T vector. The *XlFgf8a* cDNA was obtained by PCR with the following primers: forward 5′-AGAATTCCCACCATGAACTACATCACCTCC; reverse 5′-ACTCGAGTTACCGAGAACTTGAATATC. The *VP16-Xanf1-BDG*, *EnR-Xanf1-BDGR*, *Otx2*, *Otx2-BDGR* and *Noggin* constructs have been described previously (Ermakova et al., 1999; Martynova et al., 2004; Lamb et al., 1993). Capped mRNAs for microinjections were synthesized by mMESSAGE mMACHINE kit (Ambion). The following antisense morpholino oligonucleotide (MO) (Gene Tools) to the 5′ UTR of the *Ras-dva* mRNA was used to block its translation: 5′-GTGAGATTGCGCTTTCTTTTGTCTG (conservative in both pseudo-alleles of the *Xenopus laevis Ras-dva*). Besides the commercial control MO provided by Gene Tools, we also used, as a control, the following mis-anti-Ras-dva MO with four mismatches (underlined): 5′-GTGACATTGCTCTTTCTTTTGTGTT. A 0.25 mM solution of these MO was microinjected in most of the experiments into one dorsal blastomere of two- to four-cell stage embryos.

Embryo handling, RNA microinjection, lineage labeling

Xenopus embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). From 2 to 6 nl of the following samples (water solution) were microinjected into one blastomere of four- to 32-cell stage embryos by Eppendorf FemtoJet microinjector: *VP16-Xanf1-BDGR*, *EnR-Xanf1-BDGR*, *Noggin*, *Ras*, *flag-Ras-dva* (100 ng/μl); *DN-RasS17N* and *DN-Ras-dvaT22N* (150 ng/μl); *Otx2*, *Otx2-BDGR*, *Fgf8* (30 ng/μl). For cell lineage labeling, the mRNAs were mixed with Fluoresceine-Lysinated-Dextran amine (FLD) (Molecular Probes).

Whole-mount in situ hybridization and RT-PCR

The whole-mount in situ hybridization was performed as described (Ermakova et al., 1999). All the in situ hybridization experiments were repeated two or three times with a group of 10-17 embryos. More than 60% of the experimental embryos had the effects described in the results section, while the extent of the effects varied depending on local distribution of the injected material within embryos.

For the RT-PCR analysis, animal cap explants were excised from injected embryos at stage 8-9 and incubated in 0.5×MMR solution until sibling embryos reached stage 15. The total RNA was extracted (Chomczynski and Sacchi, 1987) from 10-12 explants of each type and RT-PCR was performed as described (Zaraisky et al., 1992) with the following pairs of primers: *EF1α*, 5′-GGAAAGGGTAACACCTAGATC and 5′-CAACGACGACCACAACCAC; *Xag2*, 5′-ATACCATGGAGACTGGCCTGTCACTTG and 5′-ATCCTTCTCGAGAAAGCTCAGTCTTCAGGAAAC; *Vent1*, 5′-GAACGGAAGAAATTGGCAACATC and 5′-ATATCCTAGAGTTACATATACTGAG; *Vent2*, 5′-TTAGTCGACTGAACACAAGGACTAATACA and 5′-TTACTCGAGAGGCCAGAGACTGCCCAA; *BMP4*, 5′-GCATGTACGGATAAGTCGATC and 5′-GATCTCAGACTCAACGGCAC; *BF-1*, 5′-AACAAGCAGGGCTGGCAGAA and 5′-CCGCTCTATCCATAAAGGTG; *Otx2*, 5′-GCAACAGCAGCAGCAGAATG and 5′-TGTAATCCAGGCAGTCAGTG; *NCAM*, 5′-GCGGGTACCTTCTAATAGTCAC and 5′-GGCTTGGCTGTGGTTCTGAAGG.

Three to five independent experiments, including microinjections and all of the following procedures, were carried out for each type of explant.

RESULTS

Ras-dva is a member of a novel family of Ras-like small GTPases

The small GTPase Ras-dva was previously identified as a result of differential screening for genes regulated in the anterior ectoderm of *Xenopus laevis* embryos by the homeodomain factor Xanf1 (Novoselov et al., 2003).

To identify possible homologs of Ras-dva in other animals, we screened available GenBank databases by the on-line BLAST tool. During this, we chose 50% amino acid identity as the crucial threshold, which is typical for different families of small GTPases (Takai et al., 2001). As a result, 11 homologs of Ras-dva were identified: one homolog in *Gallus gallus* (AY729886), two in *Xenopus tropicalis* (AY729885 and DQ278180), two in *Danio rerio* (DQ278181 and AY729884), three in *Takifugu rubripes* (DQ278182, DQ278183 and DQ278184), and one homolog in each *Gasterosteus aculeatus* (DQ278185), *Oncorhynchus mykkis* (DQ278186) and *Oryzias latipes* (DQ278187). Interestingly, no small GTPases, which demonstrate higher than 35% identity to Ras-dva, were found in all invertebrate and mammalian databases, including human.

To determine the systematic position of the identified Ras-dva proteins within the super-family of small GTPases, we aligned four of them (by one from each *Gallus gallus*, *Xenopus tropicalis*, *Danio rerio*, *Takifugu rubripes*) with 19 amino acid sequences of small GTPases, belonging to all known seven families of small GTPases (ClustalW version 1.83 at http://www.genebee.msu.ru). Based on the results of this alignment, a phylogenetic reconstruction of the superfamily of Ras-like small GTPases was implemented in MEGA software (http://megasoftware.net) using a neighbor-joining algorithm and p-distance model (Fig. 1). The phylogenetic tree shows that the Ras-dva proteins form a separate cluster of the same class as the clusters, which are formed by members of other already described families. The percentage of identical proteins inside the Ras-dva cluster is 56-94%, while the percentage identical proteins of the Ras-dva cluster and of any other cluster is 14-35%. We therefore concluded that the Ras-dva proteins form a new eighth family in the superfamily of small GTPases.

The primary structure of small GTPases is characterized by the typical G-domain, which consists of five consensus motifs (G1-5) (Paduch et al., 2001). These motifs include amino acid residues essential for GDP/GTP binding and GTP hydrolysis (Table 1, red). Importantly, all Ras-dva proteins have some specific features in the primary structure of functional regions in G2 and G3 motifs, which sharply distinguish them from other small GTPases. Thus, all Ras-dva proteins, like the α-subunit of heterotrimeric G protein (Cabrera-Vera et al., 2003; Hall, 2000), have one or two positively charged arginine residues in the crucial position near highly conserved threonine residue of the G2 motif (Table 1, blue). Besides, Ras-dva proteins have a Ser residue in G3 motif but not a conservative Gln like all other small GTPases. These features also confirm a distinction between Ras-dva and all other small GTPases, and indicate that these proteins might perform some specific molecular functions.

The homeodomain factors Otx2 and Xanf1 directly regulate the *Ras-dva* expression in the anterior ectoderm

In our previous paper, we investigated the *Ras-dva* expression pattern starting from the early neurula stage, when its expression forms a horseshoe shape domain surrounding the anterior margin of the neural plate (Novoselov et al., 2003). Now we studied the expression at earlier stages and found that the *Ras-dva* transcripts could be detected in *Xenopus* embryos by whole-mount in situ hybridization as early as at the midgastrula stage (stage 11). Interestingly, *Ras-dva* is expressed during gastrulation within a more broad territory, which includes not only the anterior non-neural ectoderm but also all the presumptive anterior neuroectoderm (Fig. 2A). However, by the late gastrula-early neurula stages, the *Ras-dva*

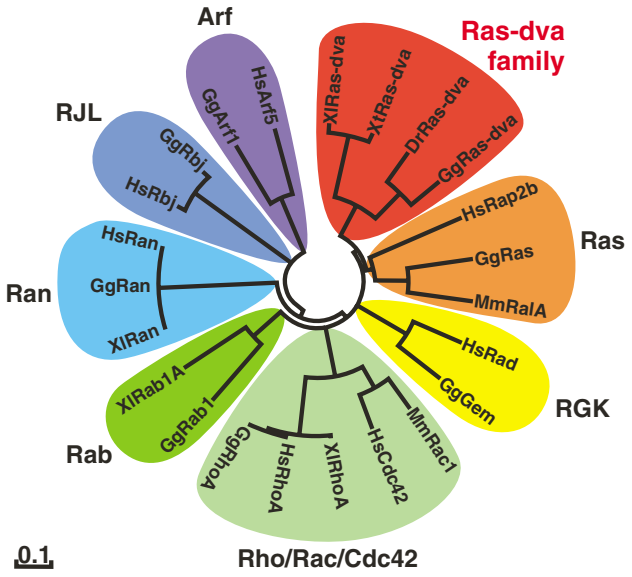

Fig. 1. Phylogenetic tree of the superfamily of small GTPases. Phylogenies were reconstructed using a neighbor-joining algorithm and p-distance model ($p=n_d/L$, where n_d=number of amino acids different between two aligned sequences and L=number of sites compared). The bar sets a value of p. Thus, p-distance value in the pairing of XlRas-dva: with another protein, e.g. with GgRas, is 0.65; with XlRab1A or XlRho is 0.69; with XlRan is 0.79; with GgArf1 is 0.85; with GgRbj is 0.78; with GgGem is 0.73; with GgRas-dva is 0.44; and with XtRas-dva is 0.06. Initials before the protein name represent respective organisms, as follows: Hs, *Homo sapiens*; Mm, *Mus musculus*; Gg, *Gallus gallus*; Xl, *Xenopus laevis*; Xt, *Xenopus tropicalis*; Dr, *Danio rerio*. Every colored area indicates an individual family of small GTPases.

expression weakens in the central part of this domain, in the area corresponding to the anterior neural plate (Fig. 2B). As a result of this inhibition, the *Ras-dva* expression at the beginning of neurulation appeared to be confined to the horseshoe-shape domain of ectoderm surrounding the neural plate from the anterior and lateral sides (Fig. 2C,J).

Speculating about possible factors that regulate the *Ras-dva* expression in cells of the anterior ectoderm, we supposed that one candidate could be the product of the homeobox gene *Otx2*, the expression of which in the anterior ectoderm follows that of *Ras-dva* very closely (Fig. 2D,E). Thus, as it is seen on the late gastrula embryos split into halves and hybridized separately with the *Otx2* and *Ras-dva* probes, the expression domains of these genes in the anterior ectoderm are noticeably overlapped, except for a small lateroposterior region of the *Ras-dva* expression domain, which is located outside the *Otx2* expression territory (Fig. 2G,H). Another fact indicating tight coupling of *Otx2* and *Ras-dva* expression is the revealed parallel inhibition of both these genes in the central part of the anterior neural plate by the early neurula stage (Fig. 2C,E,H,J).

As we showed previously (Novoselov et al., 2003), the latter area of inhibition of the *Ras-dva* expression corresponds well to the expression domain of a strong transcriptional repressor, the homeodomain factor Xanf1 (compare Fig. 2B,F with 2I,J). This, along with the fact that *Ras-dva* was previously revealed as the genetic target of Xanf1 (Novoselov et al., 2003), led us to suppose that the endogenous Xanf1 protein could be a factor responsible for the inhibition of the *Ras-dva* expression within the anterior neural plate.

To verify whether Otx2 and Xanf1 could actually regulate *Ras-dva* expression, we investigated the influence of *Otx2* and *Xanf1* mRNA microinjections upon the expression of *Ras-dva* by whole-mount in situ hybridization. During this and all other experiments, the microinjected mRNA was mixed with the fluorescent tracer, FLD. As a result, we observed significant expansion of the *Ras-dva* expression domain in embryos microinjected with the *Otx2* mRNA (Fig. 3A,A', red arrow). Interestingly, in all cases, the ectopic expression of *Ras-dva* was detected only in the non-neural ectoderm but not in the neuroectoderm. This may indicate the lack of a factor, synergizing with Otx2, in the anterior neuroectoderm. Alternatively, some inhibitory factor(s) operating in the neural plate cells could prevent activation of *Ras-dva* expression by Otx2 in these cells.

By contrast, overexpression of *Xanf1* mRNA elicited severe downregulation of the endogenous *Ras-dva* (Fig. 3B, black arrows). A similar effect was observed when mRNA encoding for dominant repressor version of Xanf1 (EnR-Xanf1) was microinjected (Fig. 3C, black arrows). However, ectopic expression of the dominant activator version, which encoded for Xanf1 fused with the activation domain of the herpes virus VP16 protein, resulted in an expansion of the *Ras-dva* expression area (Fig. 3D, red arrows).

To verify whether *Ras-dva* is the direct genetic target for Otx2 and Xanf1, we tested the abilities of the dexamethasone-inducible versions of these factors to influence the *Ras-dva* expression in conditions of total protein synthesis inhibition. To achieve this, we microinjected embryos with the mRNA encoding for a fusion of Otx2 or dominant-activator version of Xanf1 (VP16-Xanf1) with the binding domain of glucocorticoid receptor (BDGR). Owing to sequestration of BDGR by the hsp90 heat-shock protein complex, such fusion proteins appear to be inactivated within the embryonic cells. At the end of gastrulation (stage 12), the total protein synthesis was blocked by cycloheximide (CHX) solution. After that, dexamethasone (DEX) was added to the same incubation

Table 1. Comparison of G1-5 consensus motifs from different families of small GTPases and α subunit of heterotrimeric Gi1 protein

Protein	G1 GXXXXGKS/T	G2(switch1) XTX	G3(switch2) DXXGQ	G4 NKXD/E	G5 EXS/CA
Ras	GAGGVGKS	YDPTIED	ILDTAGQE	VGNKCD	YIETSAK
Rab	GDSGVGKS	YISTIGV	IWDTAGQE	VGNKC/SD	FLETSAK
Ras-dva	**GAAGVGKT**	**(H/Y)RRTVEE**	**II/LDTSGSY**	**V/IGNKXD/E**	**FV/LESSAK**
RhoA	GDGACGKT	YVPTVFE	LWDTAGQE	VGNKKD	YMECSAK
Ran	GDGGTGKT	YVATLGV	NVWDTAGQE	CGNKVD	YYDISAK
RJL	GNAEVGKS	YGVTKVQ	FYKDTQGVI	CANKID	YFETSAQ
Arf	GLDAAGKT	TIPTIGF	VWDVGGQD	FANKQD	IQATCAT
RGK	GDPGVGKT	V/LMDTWE	--------	VGNKAD	FIETSAT/A
Giα1	GAGESGKS	RVKTTGI	MFDVGGQR	FLNKQD	THFTCAT

Consensus sequences of G1-5 loops are given in the first line. The most conservative amino acid residues in G1-5 loops are in red. Amino acid residues unique to Ras-dva proteins are in blue; X indicates any residue; bold represents G1-G5 consensus motifs; underline represents conservative arginine residue in the G2 motif of the α subunit of heterotrimeric G protein.

solution, which resulted in the release of the previously accumulated proteins Otx2-BDGR or VP16-Xanf1-BDGR from the hsp90 complex (Ermakova et al., 1999). Under these conditions, only direct genetic targets of the inducible versions of Otx2 and Xanf1 could be activated. After 2 hours of incubation with CHX and DEX, the embryos were processed for the whole-mount in situ hybridization. As a result, an expansion of the *Ras-dva* expression area was observed in both cases (Fig. 3E,G, red arrows; 82% activated, 34 embryos total in two independent experiments for each construct). At the same time, no expansion of the *Ras-dva* expression domain was detected in embryos treated by CHX alone (Fig. 3F,H).

In further support of the direct mode of regulation of *Ras-dva* by Otx2 and Xanf1, we established that the promoter region of the *Xenopus tropicalis Ras-dva* contains at least three canonical binding sites for Otx2, TAATCC (Briata et al., 1999) located between positions –1581 and –1513 from the translation initiation codon, and so called P3 palindromic sites, TAATnnnATTA (between positions

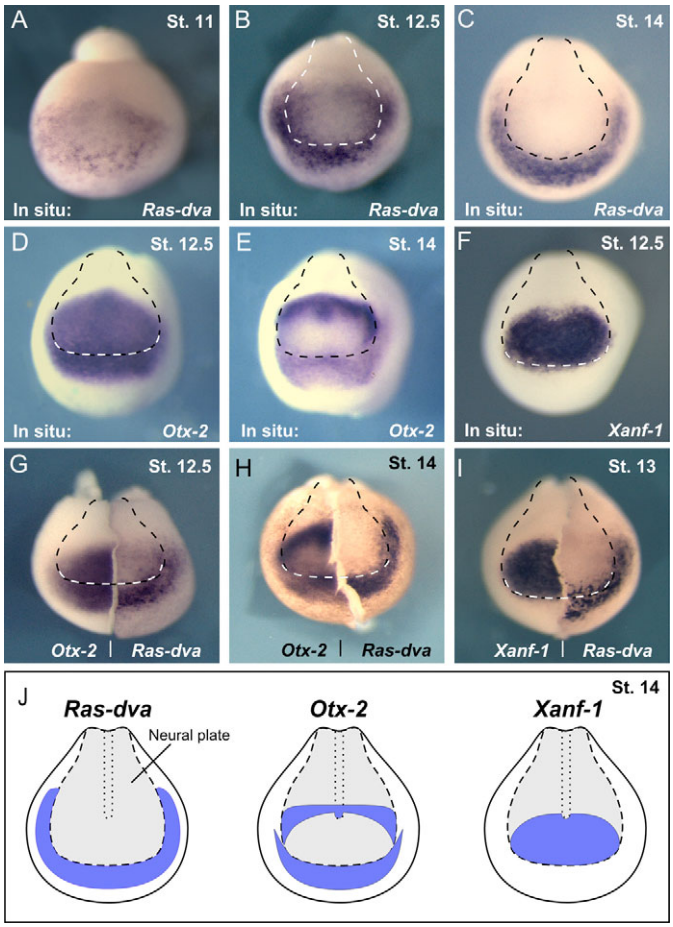

Fig. 2. Normal expression patterns of *Ras-dva* and its transcriptional regulators, the homeobox genes *Otx2* and *Xanf1*, in the gastrula and neurula stage *Xenopus* embryos as revealed by whole-mount in situ hybridization. All embryos are shown from the anterior, dorsal side upwards. Broken line indicates the neural plate border. (**A**) At midgastrula (stage 11) the *Ras-dva* is diffusely expressed within a broad territory that includes the presumptive anterior neural and non-neural ectoderm. (**B**) At late gastrula (stage 12.5) the *Ras-dva* expression weakens in the area corresponding to the anterior neural plate but it increases in the surrounding area. (**C**) At the midneurula (stage 14) *Ras-dva* is expressed in cells of the non-neural anterior ectoderm and in the lateral neural folds. (**D-F**) Normal expression patterns of the homeobox genes *Otx2* and *Xanf1*. Although *Ras-dva* and *Xanf1* are expressed in mutually excluding domains, the expression domains of Ras-dva and Otx2 are largely overlapping. (**G,H**) The results of in situ hybridization in halves of *Xenopus* embryos on the 12.5 and 14 stages. Left halves show the expression of *Otx2* gene, right halves show *Ras-dva* expression. (**I**) The *Xenopus* embryo (stage 13) in which the left half is stained for *Xanf1* expression and the right half is stained for *Ras-dva* expression. (**J**) The scheme of expression patterns of *Ras-dva, Otx2* and *Xanf1* at the midneurula stage (stage 14).

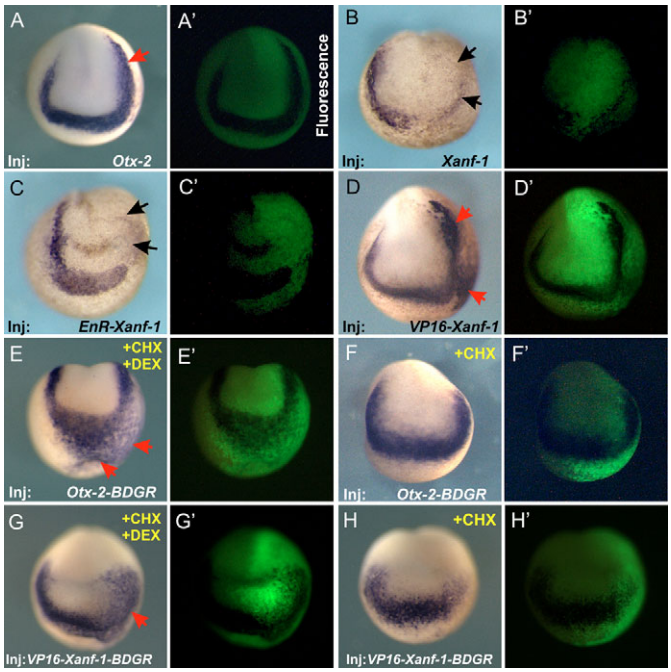

Fig. 3. Effects of *Otx2* and *Xanf1* over-expression on *Ras-dva* expression at the neurula stage (stage 14). (**A**,A′) Microinjection of the *Otx2* mRNA into the right side of the embryo results in lateral and ventral expansion (red arrow) of the *Ras-dva* expression area on this side. (**B**,B′) Overexpression of *Xanf1* leads to an inhibition of the *Ras-dva* expression (black arrows). (**C**,C′) The dominant repressor version of Xanf1 (EnR-Xanf1) inhibits *Ras-dva* expression at the microinjected side (black arrows). (**D**,D′) The dominant activator version of Xanf1 (VP16-Xanf1) induces expansion of the *Ras-dva* expression area at the microinjected side (red arrows). (**E**,E′) The dexamethasone-inducible version of Otx2 (Otx2-BDGR) elicits lateral expansion (red arrows) of the *Ras-dva* expression area when protein synthesis was completely inhibited by cycloheximide and dexamethasone treatment. This indicates that *Ras-dva* is the direct target of Otx2 in cells of the anterior ectoderm. (**F**,F′) No expansion of the *Ras-dva* expression domain is detected in embryos, microinjected with *Otx2-BDGR* and treated by the cycloheximide solution alone. (**G**,G′) The dexamethasone-inducible version of activating Xanf1 variant (*VP16-Xanf1-BDGR*) stimulates lateral expansion (red arrow) of the *Ras-dva* expression area when protein synthesis was completely inhibited by cycloheximide and dexamethasone treatment. This indicates that *Ras-dva* is the direct target of Xanf1 in cells of the anterior ectoderm. (**H**,H′) No expansion of the *Ras-dva* expression domain is detected in embryos microinjected with *VP16-Xanf1-BDGR* and treated by the cycloheximide solution alone.

–1600 and –1589; –496 and –485) that are suitable for binding of the Prd homeodomain transcription factors, including Xanf1 (Wilson et al., 1993).

Thus, on the basis of all these results, we concluded that Otx2 and Xanf1 are direct transcriptional regulators responsible for the patterning of *Ras-dva* expression in the anterior ectoderm.

Downregulation of Ras-dva results in severe abnormalities of the head structures

To understand a possible biological role of Ras-dva during embryonic development, we analyzed the consequences of inhibiting the translation of endogenous Ras-dva by microinjecting antisense morpholino oligonucleotides (MO) into early embryos.

As a result, severe head abnormalities were observed on the microinjected side of the developing tadpoles (compare Fig. 4A with 4B). These include a reduction of the cranial placodes derivatives, including the olfactory pits (41% of tadpoles from 156 total analyzed in three independent experiments), lenses (eye rudiments, 54%) and otic vesicles (62%). In addition, the frontal lobe of the telencephalon was frequently reduced in these tadpoles (35%) and the branchial arches were reduced or absent at the injected side, indicating that the cranial neural crest development was also impaired. Importantly, no abnormalities were detected in more caudal regions of the tadpoles.

The specificity of the effects obtained is confirmed by the fact that we saw neither head nor trunk abnormalities when the control mis-anti-Ras-dva MO, which contains four mismatches with anti-Ras-dva MO, was injected (not shown).

To prove the specificity of the MO effects in one more way, we performed the rescue experiment, in which anti-Ras-dva MO were co-injected along with the synthetic *Ras-dva* mRNA lacking the morpholino binding sequence. As a result, we observed a partial rescue of the head structures abnormalities (compare Fig. 4D and E). Thus, the percentage of tadpoles with the abnormalities described above was reduced from 63%, in the case of only anti-Ras-dva MO injection, to 30%, in the case of the co-injection of MO and *Ras-dva* mRNA (286 embryos in total analyzed in three independent experiments). At the same time, no rescue was observed when we co-injected the mRNA of a small GTPase of another family, *Ras*. The latter result indicates that the set of intracellular effectors regulated by Ras-dva may differ, at least partially, from that regulated by Ras.

To investigate the consequences of the Ras-dva downregulation by an independent method, we used the dominant-negative version of Ras-dva (DN-Ras-dvaT22N), which had the point mutation Tre22Asn, by analogy with the well-known DN-RasS17N mutant (Ribisi et al., 2000). The tadpoles developed from embryos

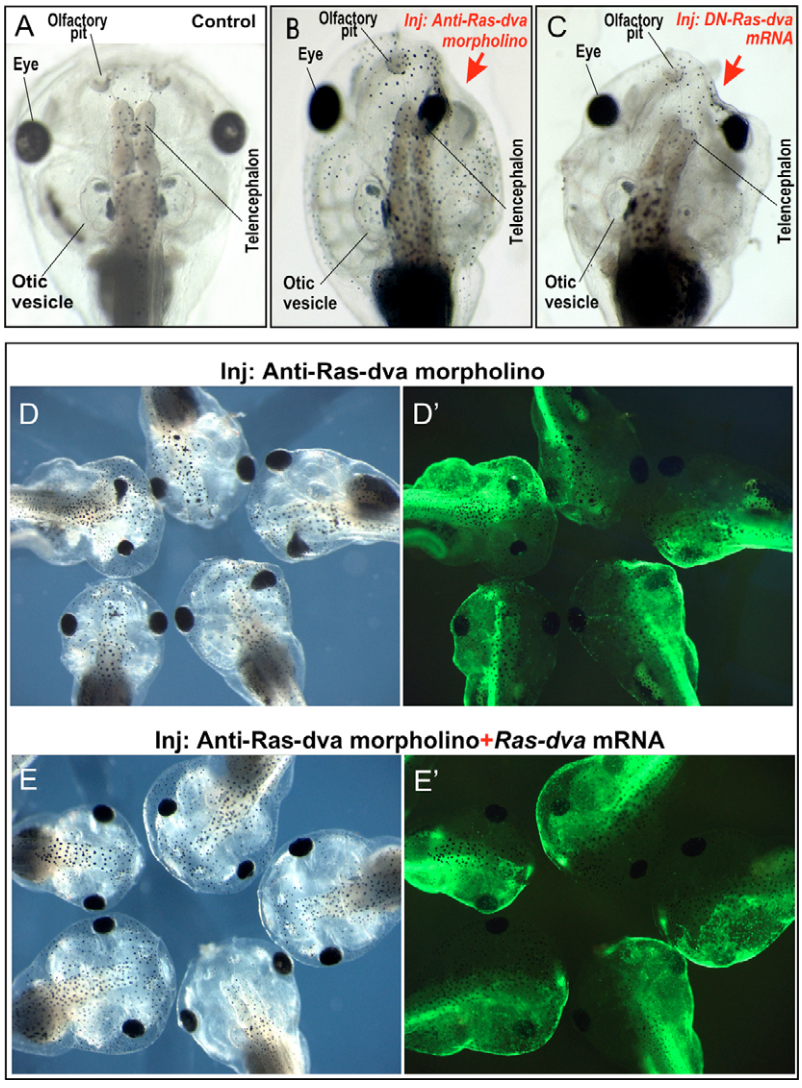

Fig. 4. Ras-dva functioning is essential for the development of the head structures. (**A**) The head of control tadpole, as seen from the dorsal side. (**B**) The head of a tadpole developed from the embryo microinjected with the anti-Ras-dva morpholino oligonucleotides (MO) into the right blastomere at the two-cell stage. As a result of inhibition of translation of the *Ras-dva* mRNA by the morpholino, the tadpole has a reduced eye, telencephalon, olfactory pit and otic vesicle. (**C**) The head of the tadpole developed from the embryo microinjected by *dnRas-dvaT22N* mRNA into the right blastomere at the two-cell stage. The malformations are similar to those described above that were caused by anti-Ras-dva MO microinjections. This indicates that the dnRas-dvaT22N construct works. (**D**,**E**) The rescue experiment. Co-injection of anti-Ras-dva MO with synthetic *Ras-dva* mRNA lacking the MO binding sequence (E) is able to rescue the effects of anti-Ras-dva morpholino oligonucleotides (D).

microinjected with the *DN-Ras-dvaT22N* mRNA into one out of two blastomeres had the same abnormalities of the head structures as were seen in the case of the anti-Ras-dva MO microinjections (Fig. 4C). Together, these experiments indicate a crucial role of Ras-dva during development of the embryonic head structures.

Ras-dva is necessary for anterior ectoderm patterning

To study the role of Ras-dva during the early patterning of the anterior ectoderm, we investigated changes in the expression of several genetic markers in embryos in which the Ras-dva functioning was impaired either by microinjections of the anti-Ras-dva MO or mRNA encoding for the DN-Ras-dvaT22N mutant. The expression of the following genetic markers was analyzed in these embryos at the middle neurula stage by whole-mount in situ hybridization: *BF-1* [also known as *Foxg1* (Kaestner et al., 2000)], *Otx2*, *Pax6* and *Xag-2*, as markers of the anterior neural plate, the anterior neural ridge and the cranial placodal ectoderm; *Slug* and *Sox9,* as markers of the neural plate border and the neural crest; and *HoxB9* as the marker of posterior neural ectoderm.

As a result, we observed significant inhibition of all these markers, except *Msx1* and *HoxB9,* in embryos microinjected both with the anti-Ras-dva MO or with the mRNA of the DN-Ras-dvaT22N mutant (Fig. 5A,B,E-J). At the same time, no changes were observed in the expression pattern of the posteriorly expressed *HoxB9* (Fig. 5D). By contrast, the expression area of the neural plate lateral borders and the neural crest marker, *Msx1*, the expression of which is normally upregulated by BMP4 signaling (Tribulo et al.,

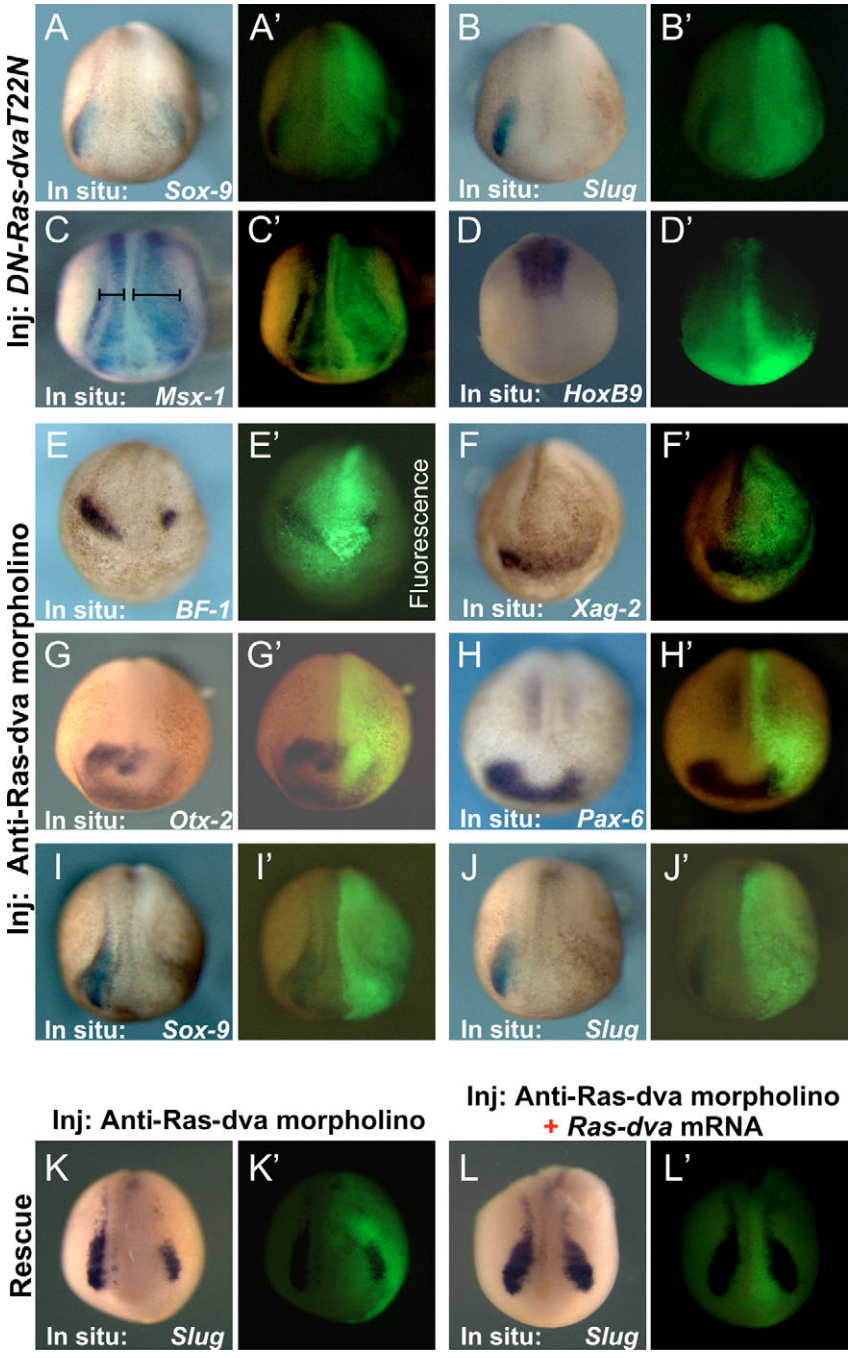

Fig. 5. Downregulation of Ras-dva functioning by the anti-Ras-dva morpholino or Ras-dva dominant-negative mutant, DN-Ras-dvaT22N, leads to an inhibition of anterior ectoderm markers expression. (**A**,**B**) The expression of the neural crest markers *Sox9* and *Slug* is inhibited on the side of embryos microinjected with *DN-Ras-dvaT22N* mRNA. (**C**) By contrast, the microinjection of *DN-Ras-dvaT22N* mRNA resulted in a broadening of the expression domain of the neural border marker *Msx1*. (**D**) No effect on the expression of posterior neural marker *HoxB9* was observed on the side microinjected with *DN-Ras-dvaT22N* mRNA. (**E-J**) The expression of markers of the neural plate (G, *Otx2*; H, *Pax6*), the anterior neural ridge (E, *BF-1*), the cranial placodes (G, *Otx2*;H, *Pax6*; F, *Xag2*) and the neural crest (J, *Slug*; I, *Sox9*) is inhibited on the side of embryos microinjected with the anti-Ras-dva morpholino. (**K**,**L**) Rescue of anti-Ras-dvaMO effects by co-injection of synthetic *Ras-dva* mRNA. The inhibited expression of the *Slug* marker, caused by a break of endogenous *Ras-dva* mRNA translation (K), can be restored by co-injection of a synthetic *Ras-dva* construct lacking the MO binding site (L). This result confirms the specificity of anti-Ras-dva MO effects. All embryos are shown from the anterior, dorsal side upwards. The 'primed' counterpart pictures show the location of progenies of the microinjected blastomeres labeled by FLD tracer.

2003), was significantly expanded within the territory populated by cells with downregulated Ras-dva (compare bars length on Fig. 5C). By contrast, no changes in the marker genes expression patterns were observed when the control mis-anti-Ras-dva MO was injected (not shown). Obviously, the results obtained indicate involvement of Ras-dva in the signaling pathways that regulate early patterning of both the anterior neural plate and its borders.

To confirm specificity of the anti-Ras-dva MO effects, we performed the rescue experiment, in which anti-Ras-dva MO were co-injected along with the synthetic *Ras-dva* mRNA lacking the morpholino binding sequence. At the midneurula stage, these embryos were collected and processed for whole-mount in situ hybridization with the probe to *Slug* mRNA, as a marker, the expression of which was shown to be most sensitive to the Ras-dva downregulation.

As a result, we revealed that while the expression of *Slug* was severely inhibited in embryos microinjected with anti-Ras-dva MO (64% of embryos; 28 embryos total in two independent experiments; Fig. 5K,K′), the embryos co-injected with the anti-Ras-dva MO and Ras-dva mRNA demonstrated obvious rescue of the *Slug* expression (71% of embryos, 28 total in two independent experiments; Fig. 5L,L′).

In another set of experiments, we also investigated the influence of the Ras-dva downregulation on the expression of the anterior neural and non-neural ectodermal markers by RT-PCR in the animal cap assay. In these experiments, we induced the development of the anterior neuroectoderm in the animal cap explants by microinjecting the embryos with mRNAs encoding either for the homeodomain factor Otx2 or for the secreted factor Noggin (Gammill and Sive, 2001; Lamb et al., 1993). Our preliminary experiments showed that microinjections of the *Otx2* or *Noggin* mRNAs induced in these explants the expression of *Ras-dva* (Fig. 6A and not shown).

In agreement with the in situ hybridization data, the RT-PCR analysis revealed inhibition of the anterior neural plate markers (*BF-1* and *Otx2*) and the anterior non-neural ectoderm marker (*Xag-2*) in animal cap explants from embryos microinjected with the mixture of the *Otx2* or *Noggin* mRNAs with anti-Ras-dva MO or the *DN-Ras-dvaT22N* mRNA (Fig. 6A,B). Additionally, we observed upregulation of the epidermal regulator *BMP-4* and its genetic targets, *Vent1* and *Vent2b* (Fig. 6A,B). At the same time, expression of pan-neural marker, *NCAM* (Fig. 6B), and posterior neural markers, *HoxD1* and *HoxB9* (not shown), did not change. These results are particularly interesting as BMP signaling has recently been shown to be crucial for the precise positioning of the neural/non-neural ectoderm boundary, the neural crest specification, and for determination of the actual sizes of the neural plate. Together, the results obtained demonstrate an essential role of Ras-dva for the normal patterning of the anterior ectoderm.

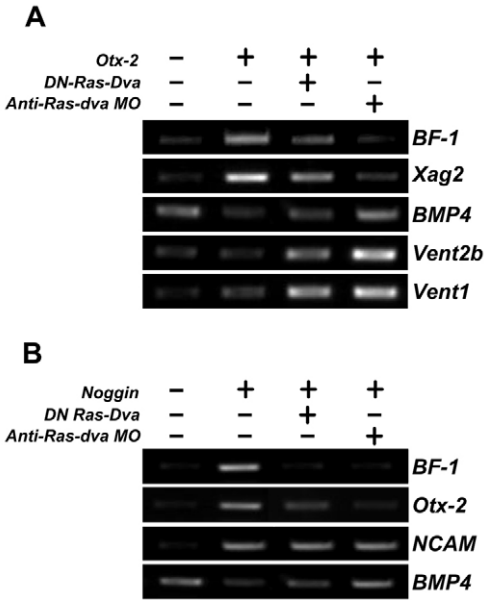

Fig. 6. Representative RT-PCR analysis of marker gene expression in animal cap explants induced to anterior neural differentiation by microinjection of the *Otx2* or *Noggin* mRNA in conditions of Ras-dva functioning downregulation. (**A**,**B**) Ras-dva downregulation by co-injection of the anti-Ras-dva MO or *DN-Ras-dvaT22N* mutant mRNA inhibits induction by Otx2 (A) or Noggin (B) of the neural plate and cranial placodal markers (*BF-1, Otx2 and Xag-2*) but promotes expression of the neural inhibitors (*BMP-4, Vent-1, Vent-2B*). Expression of the pan-neural marker *NCAM* does not change when Ras-dva is downregulated.

Downregulation of Ras-dva leads to the inhibition of FGF8a signaling within the anterior ectoderm

Assuming high homology of Ras-dva with members of the Ras family of small GTPases, one may hypothesize that, like the latter, Ras-dva could be also involved in the transmission of some FGF signaling in cells at the anterior margin of the neural plate. We supposed that the most probable FGF ligand responsible for such signaling might be FGF8a, the activity of which at the anterior margin of the neural plate was implicated as a key factor in anterior neural plate patterning (Eagleson and Dempewolf, 2002; Shimamura and Rubenstein, 1997). In further support of this, we established that two crescent-shaped stripes of the *FGF8a* expression in the anterior ectoderm of the *Xenopus* neurula exactly coincided with the anterior and posterior borders of the *Ras-dva* expression domain (Fig. 7A). Therefore, we used *FGF8a* mRNA microinjections into *Xenopus* embryos to study the possible involvement of Ras-dva in the FGF8a signaling.

An important target of the FGF8a signaling in the anterior neuroectoderm is the gene encoding for the winged-helix transcription factor BF-1, the activity of which is essential for the forebrain specification (Hardcastle and Papalopulu, 2000). As has been shown previously in mouse and *Xenopus* (Eagleson and Dempewolf, 2002) and confirmed by us in *Xenopus* early stage embryos, *FGF8a* overexpression was able to induce ectopic expression of *BF-1* in the anterior ectoderm (Fig. 7B, 93%, 30 embryos total in two independent experiments). If at the same time Ras-dva function was impaired by co-injection of *DN-Ras-dvaT22N* mRNA, the *BF-1*-inducing effect of FGF8a was blocked (Fig. 7C; 81%, 32 embryos total in two independent experiments). Obviously, this result coincides well with the hypothesis of the involvement of Ras-dva in the transmission of FGF8a signaling.

To verify the specificity of this function of Ras-dva in cells of the anterior ectoderm, we investigated whether the dominant-negative mutant of a small GTPase from the closest family, Ras, could also block activation of *BF-1* by FGF8a. As a result, we established that, in contrast to DN-Ras-dvaT22N, DN-RasS17N was unable to prevent ectopic *BF-1* expression in embryos microinjected with *FGF8a* mRNA (Fig. 7D, 80%, 30 embryos total in two independent experiments). Conversely, DN-RasS17N effectively interrupted FGF signaling in posterior regions of the embryo, which was confirmed by inhibition of a mesoderm marker *Brachyury* (*Bra*) around the blastopore at the midgastrula stage (Fig. 8A black arrowheads; 93%,

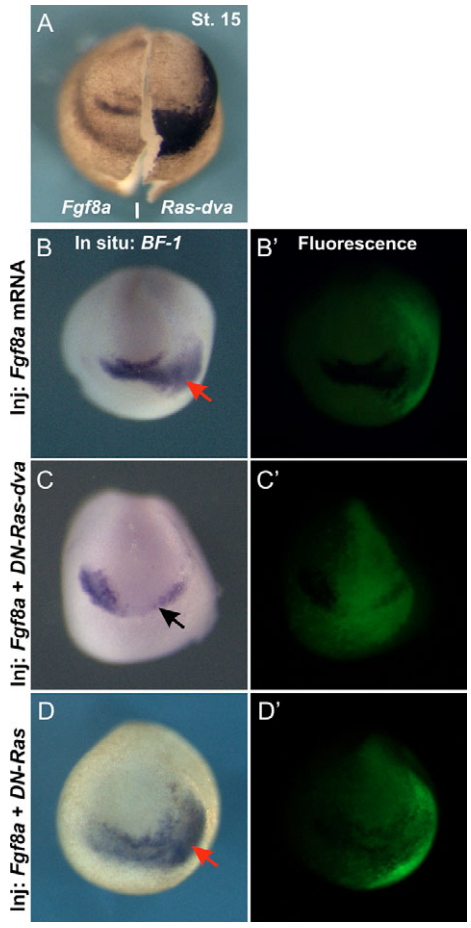

Fig. 7. Ras-dva downregulation, in contrast to Ras, can interrupt FGF8a signaling in the anterior ectoderm. (**A**) Two crescent-shaped stripes of the *FGF8a* expression in the anterior ectoderm of the *Xenopus* neurula (left half) are exactly coincident with the anterior and posterior borders of the *Ras-dva* expression domain (right half). (**B-D**) Ras-dva downregulation, in contrast to Ras, interferes with FGF-8a signaling in anterior ectoderm in *Xenopus* embryos. (B,B′) *FGF-8a* mRNA injection induces ectopic *BF-1* expression (red arrow). (C,C′) Co-injection of *FGF-8a* and *DN-Ras-dvaT22N* RNAs results in inhibition of *BF-1* expression in the injected area (black arrow). (D,D′) Co-injection of *DN-RasS17N* with *FGF8a* did not affect the *BF-1* activating signal from FGF8a (red arrow). These results were revealed by whole-mount in situ hybridization in neurula stage *Xenopus* embryos. All embryos are shown from the anterior, dorsal side upwards.

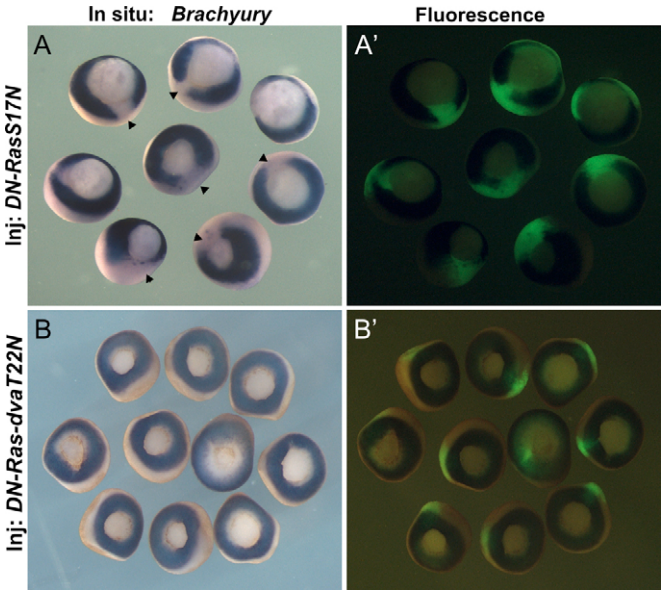

Fig. 8. Ras-dva downregulation, in contrast to Ras, is unable to interrupt FGF signaling in the posterior regions of the embryo. (**A**,A′) Microinjection of DN-RasS17N results in inhibition of the mesoderm marker *Brachyury* (*Bra*) around the blastopore at the midgastrula stage (black arrowheads). (**B**,B′) By contrast, DN-Ras-dvaT22N has no influence upon *Bra* expression.

30 embryos total in two independent experiments). In turn, the dominant-negative Ras-dva was unable to inhibit the *Bra* expression in a similar assay (Fig. 8B; 100%, 34 embryos total in two independent experiments).

In summary, these results indicate that downregulation of Ras-dva can interfere with FGF-8a signaling within the anterior ectoderm and that Ras-dva activity is distinct from the activity of its close homolog, Ras.

DISCUSSION

Ras-dva family of small GTPases

In the present work, we report that the *Xenopus* Ras-like small GTPase Ras-dva and its 11 homologs currently identified in other vertebrates constitute a novel family of small GTPases.

Seven families of proteins were distinguished hitherto within the whole superfamily of small GTPases: Ras, Rab, Rho/Rac/Cdc42, Ran, Arf/Sar, RGK and RJL (Hall, 2000; Nepomuceno-Silva et al., 2004; Pan et al., 2000; Takai et al., 2001). The subdivision of small GTPases into families is implemented in accordance with the following formal criterion: proteins belonging to the same family share more then 50% identity, while identity between members of two different families is around or less than 30% (Hall, 2000; Takai et al., 2001).

According to this criterion, Ras-dva GTPases constitute the eighth family of small GTPases. Thus, all known Ras-dva proteins have significantly higher homology with each other (56-94%) than with small GTPases from other known families (14-35%). Consistently, all Ras-dva proteins are grouped into one cluster by the same Clustal algorithm, which firmly reveals all previously described families of small GTPases. The Ras-dva GTPases are most homologous to members of the Ras family of small GTPases.

Another feature sharply distinguishing Ras-dva proteins from all other known small GTPases is a specific pattern of the amino acid residues within their G2 and G3 motifs, which are involved in GTP hydrolysis and therefore play a crucial role in temporary regulation of small GTPase functioning. In particular, the presence of positively charged arginine residues in the Ras-dva G2 motif indicates that these GTPases can probably hydrolyze GTP without the external help of GAP proteins (Hall, 2000; Paduch et al., 2001). However, further biochemical study is necessary to verify this possibility.

Key regulators of the anterior ectoderm development, Otx2 and Xanf1, control early expression of *Ras-dva*

On the basis of the results obtained, we propose a basic model, explaining the observed dynamics of the *Ras-dva* expression pattern in the anterior ectoderm during gastrulation and

neurulation. At the gastrula stage, the homeodomain factor Otx2 might activate *Ras-dva* expression throughout the anterior ectoderm (Fig. 2A), except the most lateroposterior portions of its expression domain, where *Ras-dva* may be regulated by some other factors (compare Fig. 2B with 2D; see Fig. 2G). Later on, the expression of *Ras-dva* is inhibited in the central part (presumptive anterior neural plate) of this territory under the influence of another homeodomain factor, Xanf1 (compare Fig. 2B with 2F; see Fig. 2I). As a result, by the early neurula stage *Ras-dva* is expressed anteriorly and laterally to the *Xanf1* expression domain, in a horseshoe shaped area (Fig. 2C,I, J). The anterior part of this area also expresses *Otx2* (Fig. 2H,J).

The role of Otx2 for *Ras-dva* upregulation in cells of the anterior ectoderm is consistent with the previously established function of this transcription factor as a key anterior regulator (Boncinelli and Morgan, 2001; Gammill and Sive, 2001). Interestingly, our present data demonstrating that Ras-dva in its turn is necessary for *Otx2* expression indicate that both these genes could be part of the same positive regulatory feedback loop. Therefore, assuming an important role for Otx2 in the anterior ectoderm development, we suppose that the observed anterior malformations in embryos with downregulated Ras-dva were elicited, at least partially, just by downregulation of Otx2 through this regulatory feedback loop. Another fact confirming tight coupling of Otx2 and Ras-dva into the same regulatory cascade is our finding demonstrating their direct inhibition by Xanf1 in cells of the anterior neuroectoderm.

A remarkable difference between expression patterns of *Otx2* and *Ras-dva* during neurulation is the lack of the *Ras-dva* expression in cells of the presumptive midbrain, which is located just posterior to the *Xanf1* expression domain. By contrast, *Otx2* is strongly expressed in these cells (Fig. 2E,H,J). Obviously, this difference may occur as a result of inhibition of the *Ras-dva* expression in cells of the presumptive midbrain by some transcriptional repressor(s) expressed in these cells or due to the lack of some co-factors, synergizing with Otx2 in the anterior neural plate.

Interestingly, early activation of the *Ras-dva* expression throughout the anterior ectoderm followed by its inhibition in the posterior part of this territory is consistent with the prediction of Nieuwkoop's activation-transformation model of neural induction. According to this model, the entire neuroectoderm is initially specified to the anterior fate, but later on its more caudal regions are transformed into posterior fates (Nieuwkoop and Nigtevecht, 1954). Although this model was initially proposed for the neuroectoderm as a whole, our data indicate that it could be also correct even in respect to the anterior part of the dorsal ectoderm. In this case, Otx2 could be considered a pan-anterior activator of the anterior specific marker, *Ras-dva* and Xanf1, which inhibits the *Ras-dva* expression in the posterior part of this territory, in a zone corresponding to the anterior neuroectoderm, as a transformation regulator.

Ras-dva mediates FGF8a signaling within the anterior ectoderm

As we showed, downregulation of Ras-dva resulted in a decrease in the expression of key anterior regulators followed by severe malformations of the forebrain, cranial placodes and the anterior neural crest derivatives. Assuming close homology between Ras-dva proteins and members of Ras family of small GTPases, which are involved in transduction of the FGF-signal from FGFR to MAP kinases, we suppose that the observed developmental abnormalities could be a result of the FGF signaling violation in embryos with downregulated Ras-dva.

Intracellular signaling transmitted by members of the FGF family plays a prominent role in many processes, including neural induction, patterning of the neural plate and cranial placodes differentiation (Baird, 1994). A major FGF signaling pathway involves activation of the small GTPase Ras followed by the MAPKinase cascade. In turn, the MAP kinase activity was shown to be necessary for the normal development of the neurectoderm and, in particular, for the expression of the key anterior regulator *Otx2* (Sater et al., 2003). FGF3 and FGF8 are essential components of the regulatory signals that induce otic placode development (Kwak et al., 2002). In addition, FGF8 activity is necessary for the maintenance of *BF-1* expression at the anterior margin of the neural plate and accordingly for the early forebrain development and tissue patterning in regions adjacent to the mid-hindbrain junction (Crossley and Martin, 1995; Shimamura and Rubenstein, 1997). Consistent with the suggested role of Ras-dva as an intracellular transducer of FGF signaling, we observed inhibition of the FGF-regulated forebrain and placodal markers when Ras-dva was downregulated. Moreover, we demonstrated that DN-Ras-dvaT22N mutant could interfere with the activation by FGF8a of one of the early forebrain markers, *BF-1*.

Interestingly, although Ras-dva was shown to be crucial for transmission of the FGF8a signaling in the anterior ectoderm, it was unable to influence the Ras-mediated FGF signaling cascade responsible for the neural and mesodermal tissue patterning in the posterior region of the *Xenopus* embryos (Whitman and Melton, 1992). Conversely, DN-RasS17N in our experiments could not prevent activation of *BF-1* by the ectopic FGF8a in the anterior ectoderm. The latter result is consistent with the data of other authors, which suggest that the Ras-mediated signaling is crucial for the early patterning of the posterior but not anterior part of the *Xenopus* neuroectoderm (Ribisi, 2000). Moreover, it was recently shown that Ras-ERK pathway is involved in signal transduction from FGF8b, which is important for mesen/metencephalic development of the chick embryo, but not from FGF8a (Sato and Nakamura, 2004). In summary, all this indicates significant differences in the content of proteins that interact with small GTPases during transmission of FGF signal in the anterior and posterior regions of embryo.

The FGF signaling pathway is known to cooperate with that of BMP and Wnt during regulation of the early development of the neural crest (Deardorff et al., 2001; McGrew et al., 1997; Glavic et al., 2004; Streit and Stern, 1999). In particular, an intermediate level of BMP signaling at the lateral borders of the neural plate is crucial for neural crest induction (Aybar and Mayor, 2002), and this intermediate level is achieved through phosphorylation by the FGF signaling pathway of the BMP effector Smad1 (Pera et al., 2003). Consistent with the possible role of Ras-dva as a transducer of FGF signaling during neural crest specification, we observed inhibition of the neural crest markers (*Slug* and *Sox9*) and upregulation of genes activated through BMP signaling pathway (*BMP4*, *Msx1*, *Vent1* and *Vent2*) when Ras-dva was downregulated.

Despite our results indicate Ras-dva as a probable component of the FGF signaling cascade during the anterior ectoderm development, further efforts are necessary to investigate in depth the molecular mechanism of the Ras-dva functioning.

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