DEVELOPMENTAL DYNAMICS 238:204–209, 2009

Samba, a *Xenopus* hnRNP Expressed in Neural and Neural Crest Tissues

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RNA binding proteins regulate gene expression at the posttranscriptional level and play important roles in embryonic development. Here, we report the cloning and expression of Samba, a *Xenopus* hnRNP that is maternally expressed and persists at least until tail bud stages. During gastrula stages, Samba is enriched in the dorsal regions. Subsequently, its expression is elevated only in neural and neural crest tissues. In the latter, Samba expression overlaps with that of Slug in migratory neural crest cells. Thereafter, Samba is maintained in the neural crest derivatives, as well as other neural tissues, including the anterior and posterior neural tube and the eyes. Overexpression of Samba in the animal pole leads to defects in neural crest migration and cranial cartilage development. Thus, Samba encodes a *Xenopus* hnRNP that is expressed early in neural and neural crest derivatives and may regulate crest cells migratory behavior. *Developmental Dynamics 238:204–209, 2009.* © 2008 Wiley-Liss, Inc.

Key words: Xenopus; hnRNP; Samba; Slug; neural crest migration; RNA binding protein (RBP)

Accepted 26 October 2008

INTRODUCTION

RNA binding proteins (RBPs) regulate RNA splicing, stability, nuclear export, subcellular localization and translation. They play important roles during early metazoan embryogenesis. One illustrative example is the establishment and maintenance of the mRNAs gradient that determines anterior–posterior axis in *Drosophila* by RNA binding (Minakhina and Steward, 2005). In *Xenopus*, several RBPs containing the heterogeneous ribonucleoprotein (hnRNP) domains modulate early development by means of spatial restriction of RNA localization. Vg1-RBP/Vera mediates vegetal localization of Vg1 RNA, which encodes a critical mesendodermal inducing factor (Havin et al., 1998, Choo et al., 2005). Similarly, 40LoVe binds to vegetal localization elements in vegetal-specific RNAs for polarized distribution of these transcripts in *Xenopus* oocytes (Czaplinski et al., 2005). These RBPs thus participate in maternal determination of animal–vegetal axis. In addition to setting up the early embryonic axis, RBPs also regulate late developmental processes. For example, hnRNP Hermes is expressed in the egg and throughout development, but is also present in the developing heart where its misexpression induces cardiac malformations (Gerber et al., 2002). Vg1-RBP is present maternally and is later restricted to neural folds, eyes, and brachial arches. In addition to its early role in partitioning Vg-1 mRNA, Vg1-RBP also modulates neural crest migration at late neurula stages (Yaniv et al., 2003).

RESULTS

In the course of our expression library screening for genes that regulate early *Xenopus* development, we identified a *Xenopus* hnRNP, which was named Samba after its effect on dissociated animal cap cells plated on fibronectin substrate. Samba overexpression causes dissociated animal cap cells to oscillate back and forth on the same spot as opposed to the migratory behavior displayed by the control cells (not shown). The cDNA for Samba encodes a protein with 313 amino acids and a calculated

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DOI 10.1002/dvdy.21826
Published online 17 December 2008 in Wiley InterScience (www.interscience.wiley.com).

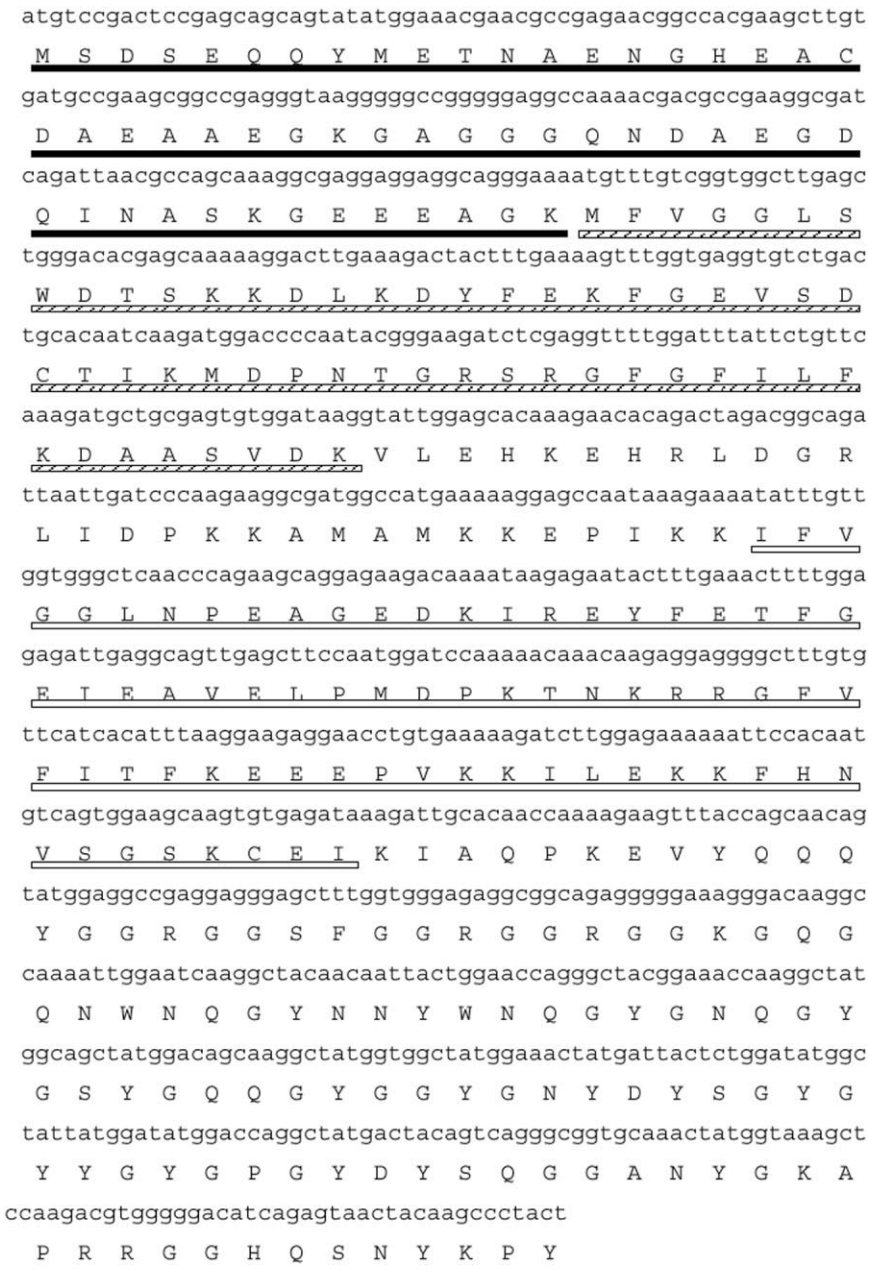

Fig. 1. Samba is homologous to hnRNPs. The amino-terminus of the predicted sequence shows high homology with CBFNT domains (black bar), immediately followed by two RNA recognition motifs: RRM1-like (hatched bar) and RRM2-like (white bar).

molecular weight of approximately 34 kDa (Fig. 1). Sequence analysis revealed several structural features that are characteristic of hnRNPs—namely, two RNA recognition motifs (RRMs) and a glycine rich-carboxyl terminus (Akindahunsi et al., 2005). RRM consists of 80–100 amino acids that are highly conserved among eukaryotes; it normally contains consensus RNA-binding sequences designated as RNP1 and RNP2, respectively, with other conserved hydrophobic residues interspersed in the motif (Burd and Dreyfuss, 1994; Merrill et al., 1988). In Samba, the first conserved RRM is found between amino acids 54–108. The second RRM is located between residues 138–208 (Merrill et al., 1988). The carboxyl terminal of Samba has a glycine-rich region that is common to previously characterized hnRNP-A/B proteins. Although Samba does not contain a traditional nuclear localization signal, nor is there one present in *Xenopus laevis* hnRNP A1/A2 carboxyl-terminus (Siomi and Dreyfuss, 1995), it does contain a conserved CBFNT domain at its amino terminus between residues 1–53. The CBFNT domain has been identified in the CARG-binding factor A protein as the domain that interacts with the promoter of immunoglobulin K (Bemark et al., 1998).

Sequence alignment between Samba and RNPs from different species showed that Samba is highly homologous to zebrafish hnRNP A/B (AAH48898), chick single stranded D-box binding factor (NP_990659, 89% identical), and human hnRNP A/B (AAH09359, 89% identical). In addition, we identified two *Xenopus* hnRNPs that show significant identity to Samba: BC074212.1, a gene identified through the *Xenopus* sequencing initiative (96% identical; Klein et al., 2002) and BC043814.1 (84% identical). The singular difference between Samba and these two hnRNPs is the absence of an amino-terminal insert in Samba, which is present in the other two proteins. The high sequence homology between Samba and BC074212.1 suggests that they are likely pseudoalleles in the *Xenopus* genome generated through partial genomic duplication during evolution (Hellsten et al., 2007).

To examine temporal and spatial expression of Samba, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) and in situ hybridization. Samba is expressed maternally, its zygotic expression peaks during neurula stages and is maintained until at least tail bud stages (Fig. 2A). Detailed analysis of Samba distribution at gastrula stages by tissue dissection showed that, although Samba is present in all embryonic regions, it is enriched in the dorsal and vegetal domains during gastrulation (Fig. 2B). Whole-mount in situ hybridization revealed that Samba transcripts are concentrated in the neural plate at early neurula stages (Fig. 3B). As development proceeds, Samba is localized to the neural as well as the neural crest tissues. By the time of neural tube closure, Samba is expressed both in the migratory neural crest and the neural tube (Fig. 3C). Double in situ hybridization for the neural crest marker Slug indicated that Samba indeed overlaps with Slug expression in the neural crest; however, Samba also

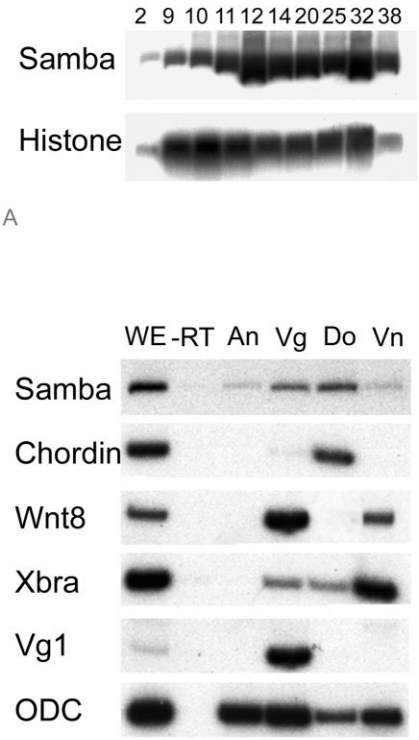

Fig. 2. Samba is expressed throughout early developmental stages. **A:** Reverse transcriptase-polymerase chain reaction for Samba with embryos at different stages shows that Samba is a maternal transcript that is expressed until late tail bud stages. Histone was used as a normalizing control. Numbers indicate the corresponding Nieuwkoop and Faber stages. **B:** Samba is enriched in the dorsal and the vegetal regions in stage 11 gastrula embryos. As controls for different regions, we used the markers Chordin for the dorsal mesoderm, Wnt8 for ventrolateral mesoderm, Xbra for mesoderm, Vg1 for the vegetal region. WE, whole embryo; −RT, samples without reverse transcriptase; An, animal pole; Vg, vegetal pole; Do, dorsal mesoderm; Vn, ventral mesoderm.

has additional expression in the neural tissues that is not seen in Slug expression pattern. At tail bud stages, Samba expression is maintained in neural and neural crest derivatives, including the brain, the spinal cord, eyes, and brachial arches (Fig. 3F–I).

As Samba is expressed in neural and neural crest cells, we examined whether increased expression of Samba affected the development of these cells. We co-injected Samba with beta-galactosidase in one blastomere in two-cell stage embryos and examined the expression of neural plate (Sox2, Sox3) and neural crest marker (Slug, Msx1, Twist) expression. We found that there was no change in Sox2, Sox3, Slug, Msx1, and Twist expression when Samba was overexpressed, suggesting that Samba did not alter neural plate or neural crest cell fate (Fig. 4). Thereafter, we analyzed the effect of Samba expression on neural crest behavior. Because Samba was expressed in migrating crest streams, we analyzed neural crest migration. At neurula stages, the neural fold from green fluorescent protein (GFP) -injected embryos was transplanted isotopically into a red fluorescent protein (RFP) -injected host embryo (Borchers et al., 2000). In this experimental paradigm, transplanted cells from control donors injected with GFP only showed the characteristic streams of migratory neural crest (Fig. 5A). In contrast, neural crest cells derived from donors that were co-injected with Samba and GFP did not migrate efficiently (83% with abnormal streams; n = 12). Most of the cells formed tight clusters around the dorsal neural tube; some of the cells that did migrate out did not reach beyond the ventral region of the eyes (compare Fig. 5B with 5A). Transplantation of GFP-expressing crest cells into Samba-overexpressing host embryos resulted in normal migratory patterns (0% with abnormal streams, n = 12; Fig. 5E), suggesting that Samba acts cell-autonomously in neural crest cells, but its level in tissues through which neural crest migrates is not critical for migratory behavior. Consistent with an inhibitory effect of Samba on neural crest migration, we observed that, when Samba was injected into the animal pole of a single blastomere of two-cell stage embryos, the resulting tadpoles displayed defects in neural crest-derived head cartilage without any obvious alterations in other embryonic tissues (Fig. 5I). Because overexpression of Samba did not affect neural crest specification as seen by marker expression, but clearly affected crest cell migration, these results suggest that Samba impairs neural crest migration, which ultimately leads to defects in the formation of cranial crest derivatives.

Other RBPs, such as Vg1-RBP, Hermes, Quaker (Xqua), and YY1, have been shown to affect anterior neural structure development as well (Zorn et al., 1997; Gerber et al., 2002; Yaniv et al., 2003; Morgan et al., 2004). Among these, only Vg1-RBP has been reported previously to affect neural crest migration (Yaniv et al., 2003). However, Vg1-RBP and Samba act differently on crest development. Whereas overexpression of Samba inhibits crest migration, it is Vg1-RBP knockdown that has this effect (Yaniv et al., 2003). This apparent contradiction should be considered in light of our current knowledge of the role of RBPs in cell migration. Vg1-RBP, and its homologues ZBP-1 and ZBP-2 are selectively enriched in migratory leading edges where they associate with actin mRNA and are required for its accumulation and increased translation (Gu et al., 2002; Oleynikov and Singer, 2003; Leung et al., 2006). These data indicate that Vg1-RBP levels and distribution control local cytoskeletal dynamics during cell migration. In contrast, another family of RBPs, hnRNP K and E1, play a functional role in protein complexes responsible for regulating the initial steps of cell spreading. In this setting, loss of RBP function increased cell spreading (de Hoog et al., 2004). Thus, it is clear that different RBPs play distinct roles in cell spreading and migration and alterations in their levels could affect both phenomena. In this scenario, we believe that endogenous Samba might act similarly to hnRNP K in regulating neural crest cell spreading required for proper migration.

EXPERIMENTAL PROCEDURES

Embryo Manipulations and RNA Preparation

Xenopus embryos were obtained and cultured as previously described (Wilson and Melton, 1994) and staged according to Nieuwkoop and Faber. Samba coding sequence was cloned in the pCS2++ vector, which was linearized with *Asc*I and transcribed using mMessage mMachine kit (Ambion) for RNA synthesis. Samba RNA was injected at 200-pg dose into the animal poles of two-cell stage embryos, with or without co-injection of 500 pg of GFP RNA.

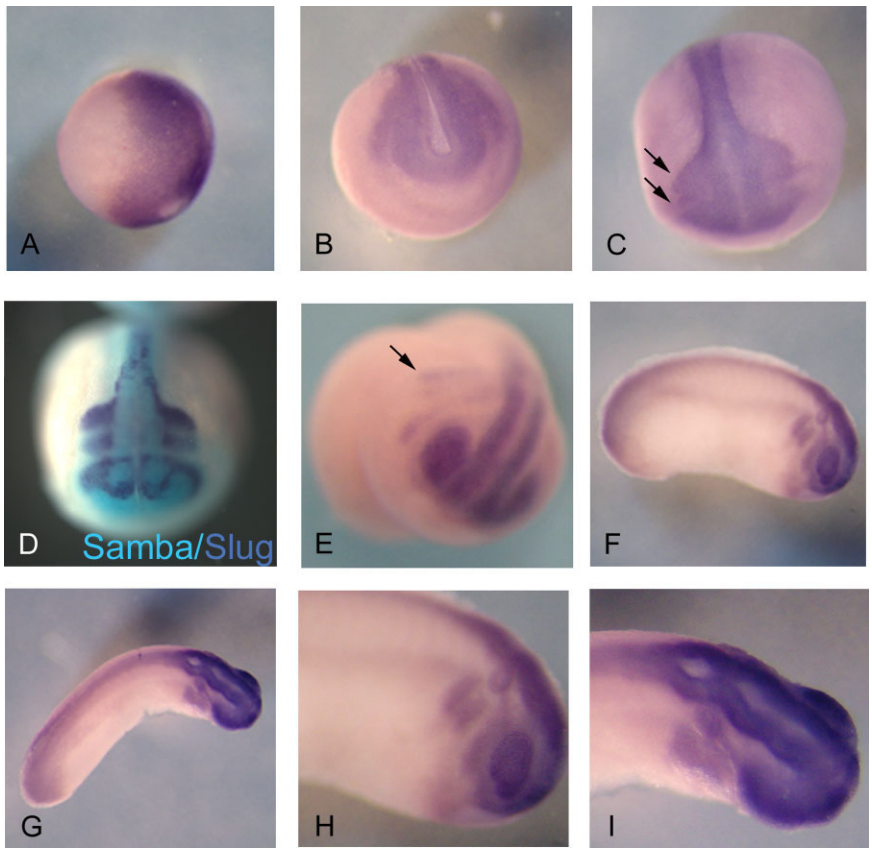

Fig. 3. Samba is expressed in the neural and neural crest derivatives as seen by in situ hybridization. **A:** Vegetal view of stage 10 embryos with dorsal side to the right. Samba expression is concentrated in the dorsal region. **B:** Neurula stage embryo. Samba is present in the neural plate and crest domain. **C:** Anterior–dorsal view of late neurula stage embryo. Arrows point to the outer edge of the forming migratory crest domain, which expresses Samba. **D:** Double in situ for Slug and Samba show that Samba overlaps with Slug-expressing migrating streams but is not restricted to neural crest domain. Slug probe was developed with a violet stain and Samba with blue stain. **E:** Anterior-lateral view of tail bud stage embryo indicates that Samba is expressed in migrating lateral streams of crest cells (arrow) and optic vesicles. **F:** Lateral view of tail bud stage embryo showing that Samba remains in the crest cells, optic vesicle, and now appears in the otic vesicle as well. **G:** Dorsal view of a late tail bud stage embryo. **H,I:** Higher magnification of cranial expression in Figures F and G.

RT-PCR

RT-PCR was performed as previously described (Wilson and Melton, 1994). The following primers were used in the PCR reaction: *Samba*: (U: GAT GCT GCG AGT GTG GAT AAG G; D: GTC ATA GCC TGG TCC ATA TCC); ODC (U: CCA AGG CTA AAG TTG CAG; D: AAT GGA TTT CAG AGA CCA); Chordin (U: CAG TCA GAT GGA GCA GGA TC; D: AGT CCC ATT GCC CGA GTT GC); Wnt-8 (U: GTT CAA GCA TTA CCC CGG AT; D: CTC CTC AAT TCC ATT CTG CG); *Xbra* (U: GGA TCG TTA TCA CCT CTG; D: GTG TAG TCT GTA GCA GCA); and *Vg1* (U: CCA TTG CTT AAT CCA AGC; D: GAC CAT ATG TGC CAG TAC).

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization was performed according to the method described previously (Harland, 1991). Double in situ hybridization was performed with the probes labeled with digoxigenin-UTP and fluorescein-UTP, respectively, and the chromogenic reaction was developed sequentially with BM purple and NBT-BCIP. For the in situ hybridizations analysis of the effect of Samba on neural plate and crest markers, 200 pg of Samba was co-injected with 100 pg of beta-galactosidase RNA. Beta-galactosidase activity was detected with Red-gal (Simonson et al.,

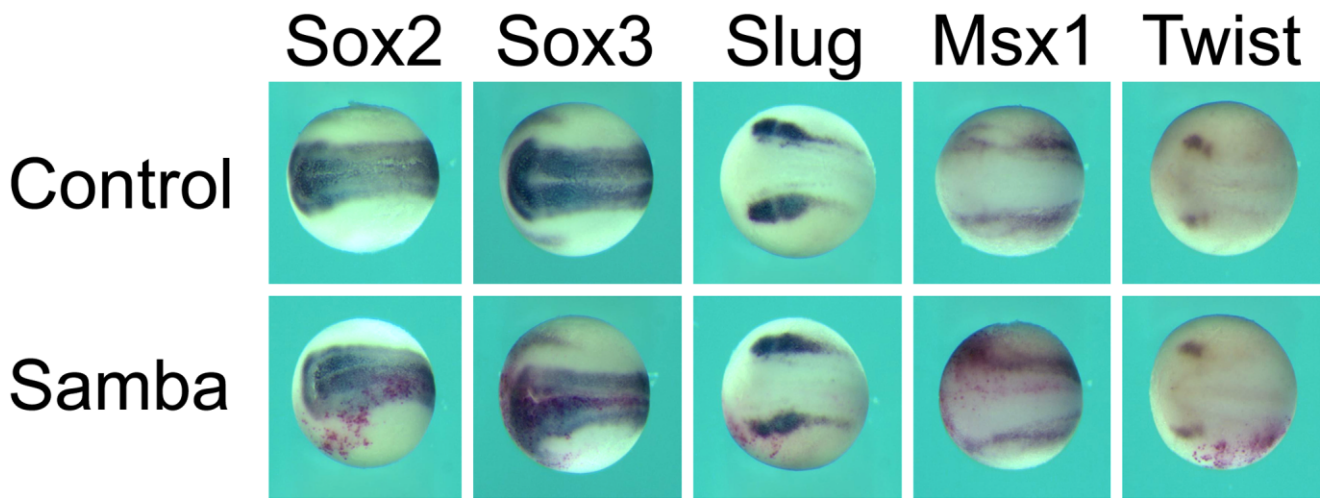

Fig. 4. Overexpression of Samba does not affect neural and crest markers. Embryos co-injected with 200 pg of Samba and 100 pg of beta-galactosidase in one blastomere at two-cell stage were processed for in situ hybridization for the neural markers Sox2 and Sox3 and the crest markers Slug, Msx1, and Twist. None of the markers altered their expression pattern in the presence of excess Samba.

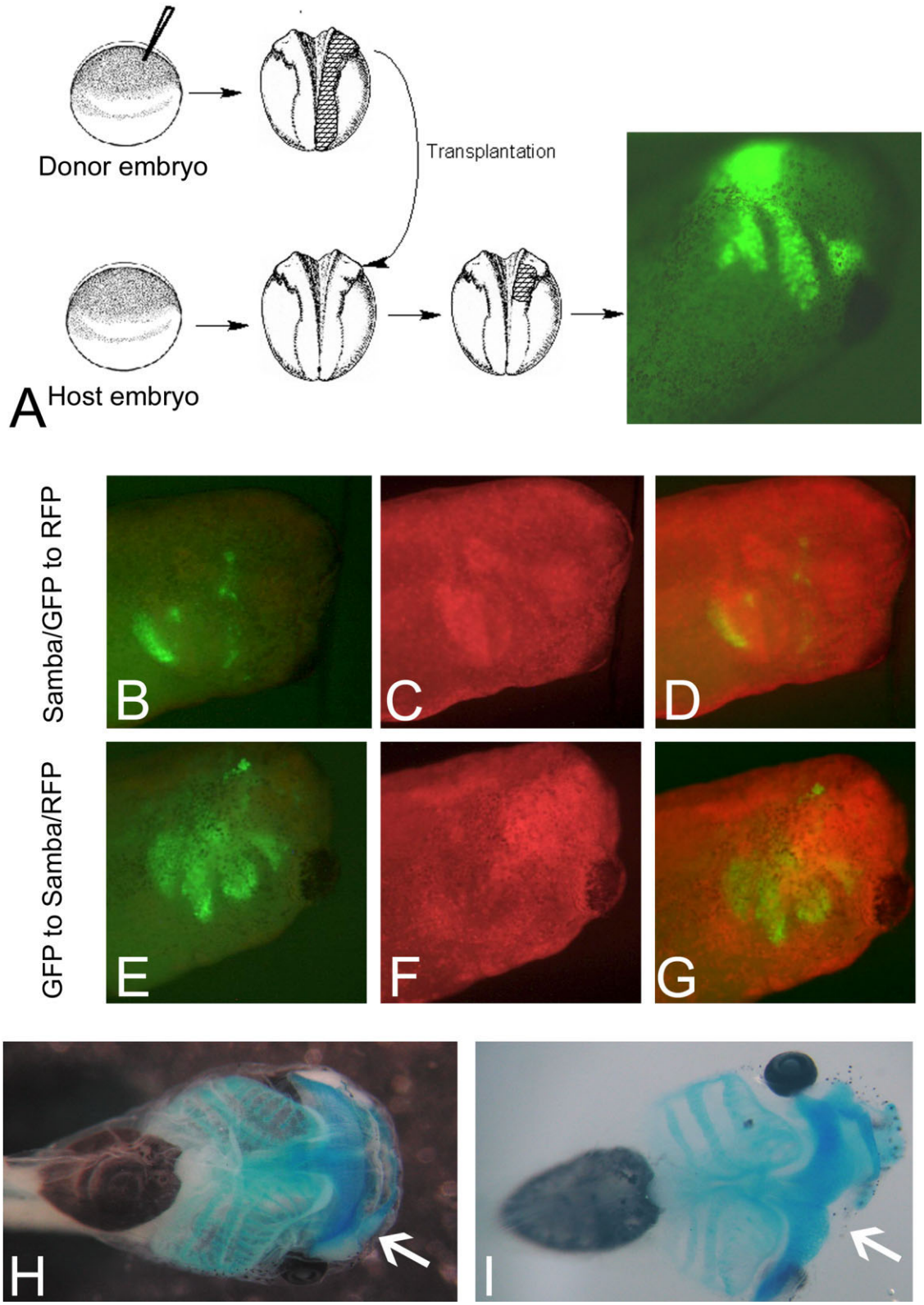

Fig. 5. Samba inhibits migration of crest cells and formation of crest derivatives. **A:** Diagram of the cranial neural crest transplantation procedure and the resulting embryo. Green fluorescent protein (GFP) -positive crest cells migrating from the transplant generated the characteristic streams of migration in control embryos injected with 500 pg of GFP (for details see the Experimental Procedures section). **B–D:** Crest cell migration was limited in red fluorescent protein (RFP) embryos that received transplants from donors co-injected with 200 pg of Samba and GFP. Green channel showing GFP-positive transplanted cells (B); red channel showing RFP-positive host cells (C) and overlay of green and red channels (D). **E–G:** In contrast, when the transplants expressed only GFP and were inserted into RFP-Samba–overexpressing embryos, crest cell migration was normal. Green channel showing GFP (E); red channel showing RFP (F) and overlay of green and red channels (G). **H:** Ventral view of an Alcian-blue–stained embryo injected with GFP on the left animal pole. Arrow points to the left maxillary cartilage. F: Embryo injected with 200 pg of Samba lacks the left maxillary cartilage (arrow).

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1995) before whole-mount in situ procedures.

Neural Crest Transplantation

Neural crest transplantation was performed using a modified version of Borchers' protocol (Borchers et al., 2000). Briefly, the donor embryos were injected in the animal pole of one blastomere at two-cell stage with 500 pg of GFP RNA and the host embryos with 200 pg of RFP. To verify the effect of Samba on migrating cells, the donor embryos were co-injected with 200 pg of Samba and GFP, whereas in the converse experiment, the donor embryos were injected with GFP only and the host embryos with Samba and RFP RNA. When the embryos reached stage 14, the donor embryos were positioned alongside with host siblings in embryo-sized troughs made of nontoxic modeling clay. The neural fold epidermis of the host embryo was peeled back and the neural crest region was carefully removed from the host embryo. The same region was removed from the donor embryo and transplanted into the host embryo. The pliability of the clay allowed us to adjust the borders so as to cover the embryo after transplantation to fix the transplant in position. The embryos were released from the troughs after 30 min and cultured normally until tail bud or tadpole stages for assessment of crest migration and differentiation.

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