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Spatiotemporal manipulation of retinoic acid activity in zebrafish hindbrain development via photo-isomerization

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SUMMARY

All-trans retinoic acid (RA) is a key player in many developmental pathways. Most methods used to study its effects in development involve continuous all-trans RA activation by incubation in a solution of all-trans RA or by implanting all-trans RA-soaked beads at desired locations in the embryo. Here we show that the UV-driven photo-isomerization of 13-cis RA to the trans-isomer (and vice versa) can be used to non-invasively and quantitatively control the concentration of all-trans RA in a developing embryo in time and space. This facilitates the global or local perturbation of developmental pathways with a pulse of all-trans RA of known concentration or its inactivation by UV illumination. In zebrafish embryos in which endogenous synthesis of all-trans RA is impaired, incubation for as little as 5 minutes in 1 nM all-trans RA (a pulse) or 5 nM 13-cis RA followed by 1-minute UV illumination is sufficient to rescue the development of the hindbrain if performed no later than bud stage. However, if subsequent to this all-trans RA pulse the embryo is illuminated (no later than bud stage) for 1 minute with UV light (to isomerize, i.e. deactivate, all-trans RA), the rescue of hindbrain development is impaired. This suggests that all-trans RA is sequestered in embryos that have been transiently exposed to it. Using 13-cis RA isomerization with UV light, we further show that local illumination at bud stage of the head region (but not the tail) is sufficient to rescue hindbrain formation in embryos whose all-trans RA synthetic pathway has been impaired.

KEY WORDS: Retinoic acid, Spatiotemporal control, Hindbrain, Zebrafish

INTRODUCTION

All-trans retinoic acid (RA) is a major player in early embryogenesis (Ross et al., 2000; Niederreither and Dollé, 2008; Dequéant and Pourquié, 2008). It has been shown to be required for the proper development of the anteroposterior axis (Diez del Corral et al., 2003), to be implicated in left-right asymmetry (Vermot and Pourquié, 2005; Sirbu and Duester, 2006; Vermot et al., 2003) and to act as a morphogen during hindbrain development (White et al., 2007) and somitogenesis (Niederreither et al., 1999; Grandel et al., 2002), as well as heart and appendage regeneration (Kikuchi et al., 2011; Blum and Begemann, 2012). All-trans RA and its 13-cis isomer have also been used in various cancer therapies (Elstner et al., 1998; Armstrong et al., 2005). RA is synthesized (Niederreither and Dollé, 2008) by retinaldehyde dehydrogenase (Raldh) from retinal, which is obtained from retinol (vitamin A). It exists in various isoforms. All-trans RA, the major active isomer, binds to the retinoic acid receptor (RAR). Another

isomer, 9-cis RA, targets mainly the retinoic X receptor (RXR), which is capable of binding many other ligands, termed rexinoids (Niederreither and Dollé, 2008). RAR and RXR form dimeric complexes that are capable of regulating the expression of hundreds of RA-responsive genes, some of them directly by binding to a regulatory element upstream of the gene. In developing embryos of zebrafish (Costaridis et al., 1996) and chicken (Maden et al., 1998), HPLC analysis has found evidence mostly for the trans-isomer. Other isomers, in particular 13-cis RA, have only been found in 10-day-old fish embryos.

Because of its involvement in so many developmental pathways, much effort has been devoted to control RA activity in developing embryos. The usual approach is to block endogenous RA synthesis and look for rescue of the investigated pathway when externally supplying all-trans RA. To impede RA synthesis, Raldh mutant lines or drugs (such as diethyl-aminobenzaldehyde, DEAB) that block Raldh can be used. To rescue normal development all-trans RA is usually dispensed continuously, either globally (Begemann et al., 2004) by adding it at a known concentration to the embryo medium or locally through the implantation of beads soaked in all-trans RA (White et al., 2007), which generates an unknown gradient of RA. However, to better investigate these issues it is essential to control the concentration, timing and location of all-trans RA, which is difficult using current techniques.

To achieve local spatiotemporal control of all-trans RA activation, we have previously used photoactivation of caged all-trans RA (Neveu et al., 2008). However, owing to the poor water solubility of the caged compound, the concentration of the released all-trans RA is ill defined. Here we propose an alternative method to precisely control RA concentration, timing and location by UV illumination (at 365 nm) and isomerization of 13-cis RA or all-trans RA (see Fig. 1A). These stereoisomers exhibit widely different levels of biological activity (Allenby et al., 1993). Thus,

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illuminating the biologically inactive (Allenby et al., 1993) 13-cis RA form (see Fig. 1A) generates at steady state ~20-25% of the biologically active all-trans RA [in addition to 30-35% 9-cis and 20-25% 13-cis RA stereoisomers (Neveu et al., 2008)]. Conversely, illumination of the active all-trans RA reduces its concentration by a factor of four to five (Neveu et al., 2008) (supplementary material Fig. S1). This approach for controlling all-trans RA (and 9-cis RA) concentration *in vivo* is non-invasive, simple, quantitative, precise in both space and time and reproducible.

Before investigating the developmental response to a local activation (or deactivation) of all-trans RA, it is important to verify that uniform global activation/deactivation at a given time of a controlled concentration of all-trans RA in an embryo is feasible and results in well-characterized perturbations to its development. An experiment in which an embryo at a given developmental stage is transiently exposed to all-trans RA (either directly or through photoactivation) differs in principle from the more common experiments in which an embryo is exposed beyond a certain stage to a constant concentration of all-trans RA. In the case of transient exposure, all-trans RA might diffuse out of the embryo and may therefore not necessarily affect its development.

We show here that in zebrafish embryos whose endogenous RA synthetic pathway has been blocked by DEAB, a global transient exposure (pulse) to 1 nM all-trans RA (either directly or via the isomerization of 13-cis RA) reproduces the previously characterized rescue of hindbrain formation by continuous exposure to all-trans RA, suggesting that all-trans RA is sequestered in embryos that have been exposed to it. We show that isomerization of all-trans RA (if performed no later than bud stage) is enough to impair this rescue, suggesting that the critical window for hindbrain rescue by all-trans RA is at the end of gastrulation. We then use our approach to evaluate the concentration of endogenous all-trans RA at a given developmental stage. Finally, we show that activation of all-trans RA at the end of gastrulation, via the photo-isomerization of 13-cis RA, in the precursor cells of the head rescues hindbrain development, but fails to rescue it if activated in the precursors of the tail region, implying that the concentration of all-trans RA can be controlled, and have its effect, locally.

MATERIALS AND METHODS

Fish lines and maintenance

Fish were raised and bred according to standard methods (Westerfield, 2000). A transgenic line mp311+/+GFP was obtained from a large-scale enhancer trap screen (S.L., unpublished) using a Tol2 vector (Kawakami et al., 2004) containing a 249 bp zebrafish *gata2* minimal promoter (Meng et al., 1997) linked to a *gfp* reporter gene. *In situ* hybridization shows that the expression pattern and RA response of this transgene are similar to those of *krox20* (see Fig. 3), a known marker of rhombomeres 3 and 5. Hence, in some experiments we used this transgenic line to observe and analyze the development of these rhombomeres. The embryos were incubated at 28°C. Developmental stages were determined as hours post-fertilization (hpf).

Whole-mount *in situ* hybridization and RT-qPCR

Single whole-mount *in situ* hybridization with digoxigenin-labeled riboprobes was performed at the 1- to 2-somite stage as described previously (Prince et al., 1998), and double *in situ* hybridization with fluorescein-labeled probes was as described (Hauptmann and Gerster, 2000). Riboprobes (gift of P. Charnay, IBENS) were synthesized from template plasmids: *krox20*, *hoxb1a* (Oxtoby and Jowett, 1993) and *vhnf1* (Sun and Hopkins, 2001).

Total RNA was extracted using Trizol (Invitrogen) and the RNeasy Mini Kit (Qiagen) at the 1- to 2-somite stage from embryos incubated from sphere stage in 10 μM DEAB. Some of these embryos were also transiently

incubated (5 minutes) at 90% epiboly in 1 nM all-trans RA, 1 nM or 5 nM 13-cis RA and in some cases subsequently UV illuminated for 1 minute. cDNA was synthesized from these pools of total RNA (extracted from 30-40 embryos) using the Superscript II Reverse Transcription System for RT-qPCR (Invitrogen), and RT-qPCR was performed using these cDNAs with TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assay: Dr03144482_m1(*vhnf1*) and Dr03124995_m1(*hoxb1a*) (Applied Biosystems). The difference (ΔC_t) in the number of amplification cycles between these genes (in various conditions) and a reference gene (β -actin) was measured in triplicate PCR assays. The fold increase (f.i.) in expression with respect to incubation in DEAB (which inhibits the expression of *vhnf1* and *hoxb1a*) was computed as: $f.i. = 2^{\Delta C_t} / 2^{\Delta C_t(DEAB)}$ where the average ($\langle \dots \rangle$) is taken over five different experiments.

Drug treatments

Wild-type and mp311+/+GFP embryos were incubated in 10 μM DEAB, 0.01-10 nM all-trans RA, 0.01-10 nM 9 cis-RA or 0.05-50 nM 13 cis-RA (all Sigma-Aldrich; all from a 10 mM stock in DMSO). Dilutions were made with embryo medium (EM), which was either Volvic water (Herbomel et al., 1999) or double-distilled water supplemented with 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄. Incubations were carried out in 10 ml plastic Petri dishes. All-trans RA, 9 cis-RA and 13 cis-RA treatments were carried out in the dark. Transient RA incubations were performed by transferring DEAB-incubated embryos into RA solution in EM for a set time (usually 5 minutes), followed by washing in EM and then back into DEAB solution. As controls, siblings were kept in EM.

UV illumination

UV isomerization

We used capillary electrophoresis (CE) to investigate the course of the photo-isomerization of all-trans RA into 13-cis RA and vice-versa under the UV illumination conditions used in our experiments on zebrafish embryos. Starting from a 25 μM solution of a pure retinoic acid stereoisomer (either all-trans RA or 13-cis RA), we measured the concentrations of all-trans RA and 13-cis RA as a function of the illumination duration by integrating their corresponding CE peaks.

Global illumination with UV lamp

Global one-photon illumination experiments were performed at room temperature with a benchtop UV lamp (Fisher VL-6-L) at 365 nm. This lamp has a strong line at 365 nm accompanied by a Gaussian spectral dispersion around 350 nm with a 40 nm width at half maximum, delivering on the illuminated sample a typical photon flux (Neveu et al., 2008) of $\sim 4.3 \times 10^4$ Einstein/s.m². This UV lamp was placed on top of a Petri dish containing the embryos. We checked that, when illuminated for up to 4 minutes under such conditions, the embryos developed normally. To inactivate all-trans RA (by isomerization into cis-isomers) the embryos previously incubated transiently in all-trans RA were illuminated for 1 minute with the UV lamp. Similarly, to activate all-trans RA (by photo-isomerization of 13-cis RA) the embryos previously incubated in 13-cis RA were illuminated for 1 minute with the UV lamp. Following illumination, all embryos were incubated at 28°C in 10 μM DEAB solution.

Local illumination with UV laser

A 40× 0.8 NA water-immersion objective (Olympus) was used to image the embryos on a CCD camera (Andor Luca) and locate the focal spot of the UV laser. For the UV illumination (375 nm, CW, from Crystal Laser) a beam of 1 mm diameter was coupled to the microscope without expansion. The incident UV power at the sample (~ 5 μW) was measured with a NOVA II power meter (Laser Measurement Instruments). For local 13-cis RA isomerization, embryos were kept in 10 μM DEAB from 4 hpf, and were exposed to 5 nM 13-cis RA for 5 minutes at the 75% epiboly stage. They were dechorionated and gently mounted in an array of agarose wells (Herrgen et al., 2009). They were orientated properly as shown in Fig. 5A with extended length tips, and were then illuminated with the UV laser at the 80-90% epiboly stage for 20 seconds in selected regions of the gastrula that give rise to the anterior or posterior part of the embryo. To demonstrate the localization of the UV illumination, embryos were injected at the one-cell stage with mRNA encoding the photoactivatable protein

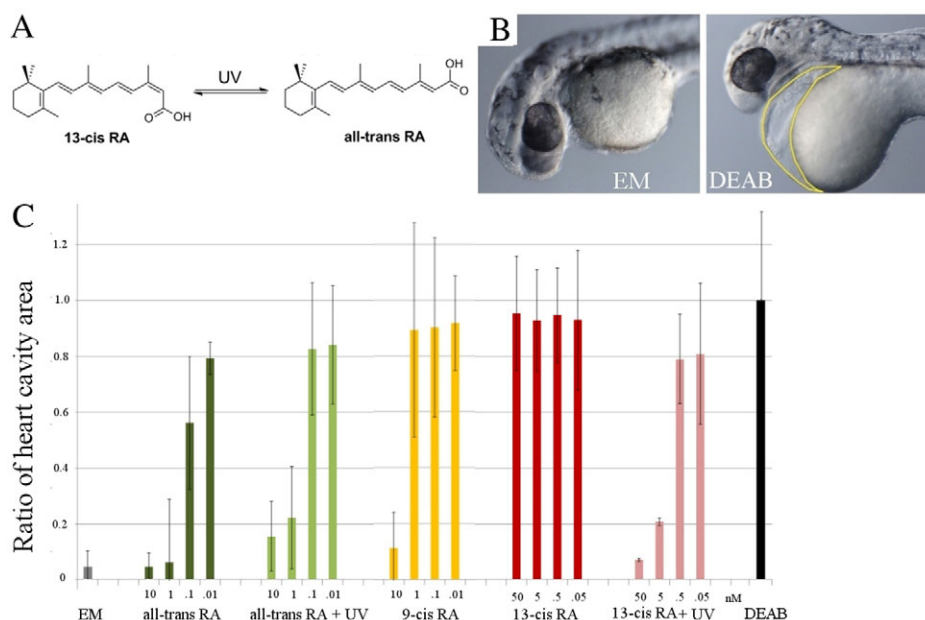


Fig. 1. Developmental response to various concentrations of different RA isomers. (A) Isomerization reaction of 13-cis RA into all-trans RA (and vice versa). (B) Typical phenotype of a zebrafish embryo incubated in embryo medium (EM) and in DEAB. Note the enlarged heart cavity (area enclosed by yellow line). (C) Developmental response of embryos incubated in DEAB to transient (5-minute) incubation in various concentrations of all-trans RA, 9-cis RA and 13-cis RA, subjected or not to UV illumination and fixed in 4% paraformaldehyde at 33 hpf. Since the heart cavity is enlarged in DEAB, we used the ratio of its transverse area (as enclosed by the yellow line in B) to the value in DEAB as a means to quantify the rescue (which is maximal when the ratio is minimal). Note that rescue of heart development requires ~1 nM all-trans RA, 10 nM 9-cis RA or 5 nM 13-cis RA plus UV illumination (1 minute). No rescue is observed in up to 50 nM 13-cis RA. The number of embryos used for each condition is ~20. Error bars indicate s.d.

Kaede (used as a label of photoactivation) and illuminated with the UV laser as described. Following UV photoactivation Kaede fluoresces in red, allowing identification of the illuminated regions at later developmental stages (see supplementary material Fig. S5).

Real-time GFP monitoring and data analysis

To monitor the development of rhombomeres 3 and 5 in different conditions, embryos under various treatments were dechorionated and gently mounted in an array of agarose wells (Herrgen et al., 2009). All the embryos that orientated properly were imaged overnight at the Imaging Core Facility of the California NanoSystems Institute (CNSI) using a time-lapse confocal microscope (STED, Leica). To ensure proper development of the embryos, their temperature was kept at ~28°C with a heating mat. To measure the GFP intensity from rhombomeres 3 and 5, the fluorescence from all the stacks of confocal images was added together. The ratio of the rhombomere fluorescence was then calculated after background subtraction and intensity averaging.

Capillary electrophoresis analysis following RA isomerization

Electrophoretic measurements were performed with a PACE/MDQ (Beckman Coulter) capillary electrophoresis system (Bempong et al., 1993). Migrations were performed at 20 kV in bare fused silica capillaries (Polymicro, Phoenix, AZ, USA), 50 µm internal diameter × 50 cm, filled with running buffer [30 mM Na₂B₄O₇ and 20 mM α-cyclodextrin containing 10% (v/v) acetonitrile] at 25°C. The analytes were detected by UV absorbance at 350 nm. In view of the similarity of the absorption spectra of the two analyzed retinoic acids at that wavelength, we assimilated the corrected areas of the peaks to the amounts of the corresponding retinoic acids.

RESULTS

A transient exposure to RA rescues zebrafish development

It has been shown (Begemann et al., 2004; Maves and Kimmel, 2005) that zebrafish embryos incubated in 5-10 µM DEAB, an

inhibitor of Raldh, the enzyme responsible for all-trans RA synthesis, from 5 hpf exhibit abnormal development affecting organs such as the anteroposterior axis, brain and heart. These severe phenotypes (Fig. 1B) can however be rescued with addition to the embryo medium before the end of gastrulation of 1-10 nM all-trans RA (Begemann et al., 2004; Maves and Kimmel, 2005). In order to test whether a short transient pulse of all-trans RA could also rescue the development of embryos incubated in DEAB, we investigated the rescue of zebrafish embryos incubated at 90% epiboly for 5 minutes in increasing external concentrations (0.01-50 nM) of all-trans RA, 9-cis RA and 13-cis RA. To quantify the rescue we measured the area of the heart cavity that is expanded in the absence of RA (Fig. 1B) (Niederreither et al., 1999). A normal heart cavity is indeed observed when embryos are transiently incubated in as little as 1 nM all-trans RA (Fig. 1C). This rescue was also observed in embryos transiently incubated in 9-cis RA but required 10-fold higher concentrations and was not observed in 13-cis RA even at concentrations as high as 50 nM. Hence, possible endogenous isomerization of cis-RA into all-trans RA is negligible at the developmental stages studied here. However, rescue was observed in the presence of 5 nM 13-cis RA if the embryos were exposed for 1 minute to UV light, thereby isomerizing 20-25% of 13-cis RA to all-trans RA (Fig. 1A; supplementary material Fig. S1). When isomerizing with UV light we chose to tune the initial concentration of 13-cis RA rather than the illumination time to control the final concentration of all-trans RA. Indeed, the isomerization reaction proceeds quickly to photostationary state (~10 seconds) (Neveu et al., 2008) (supplementary material Fig. S1) and it is therefore more difficult to tune the final concentration via the illumination time.

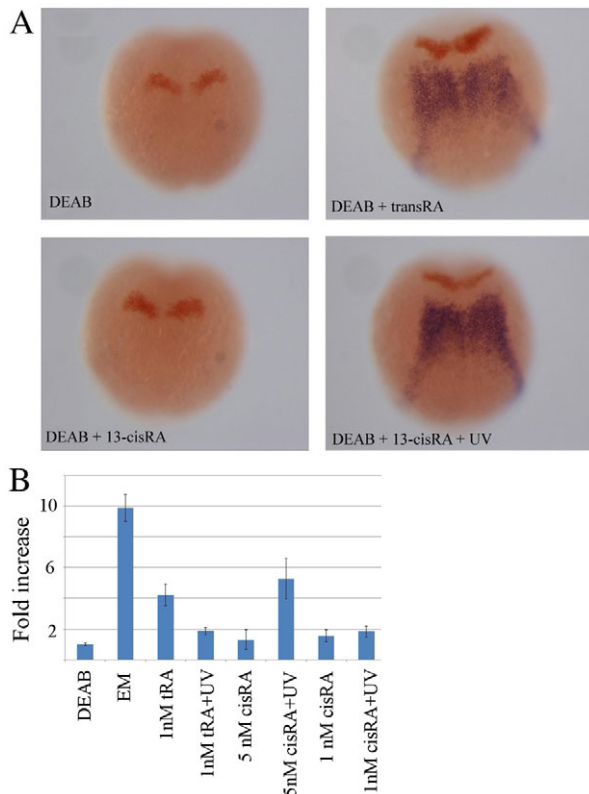


Fig. 2. Response of *vhnf1* to transient exposure to all-trans RA and 13-cis RA. Zebrafish embryos were incubated from sphere stage in 10 μ M DEAB (except for the control incubated in embryo medium, EM), exposed at 90% epiboly for 5 minutes to various concentrations of all-trans RA (tRA) and 13-cis RA (cisRA) and UV illuminated (for 1 minute) or not. (A) In situ hybridization at the 1- to 2-somite stage for *vhnf1* (blue) and *krox20* (red) in embryos incubated in DEAB and 5 nM 13-cis RA (no *vhnf1* is visible) or DEAB plus 1 nM all-trans RA and DEAB plus 5 nM 13-cis RA plus 1-minute UV illumination (rescue of *vhnf1* expression is visible). (B) Quantification of the expression of *vhnf1* by RT-qPCR in various conditions. Note that the expression of *vhnf1* in 1 nM all-trans RA is similar to that in 5 nM 13-cis RA plus UV. Also, the expression of *vhnf1* is similar in 1 nM all-trans RA plus UV and 1 nM 13-cis RA plus UV, as expected because in both cases the concentration of all-trans RA after UV illumination is the same. Error bars indicate s.e.m. from five experiments (for each experiment, RT-qPCR for the various genes was performed in triplicate).

Rescue of hindbrain development upon transient exposure to all-trans RA

The rescue of zebrafish development by all-trans RA has been particularly studied in the context of the hindbrain where its absence affects the development of the more posterior rhombomeres. We have studied quantitatively the effect of a short pulse of all-trans RA, either through a transient (5 minute) incubation in all-trans RA or via UV illumination of 13-cis RA, on the rescue of hindbrain development in embryos incubated in DEAB. We performed in situ hybridization against RA-responsive genes that are expressed in early development of the hindbrain [*krox20* (*egr2* – Zebrafish Information Network), *vhnf1* (*hnf1ba* – Zebrafish Information Network), *hoxb1a*] and are known to differ in expression in DEAB-treated embryos versus normally developing controls (Begemann et al., 2004). A short pulse of 1 nM all-trans RA given at 90% epiboly is sufficient to rescue the known

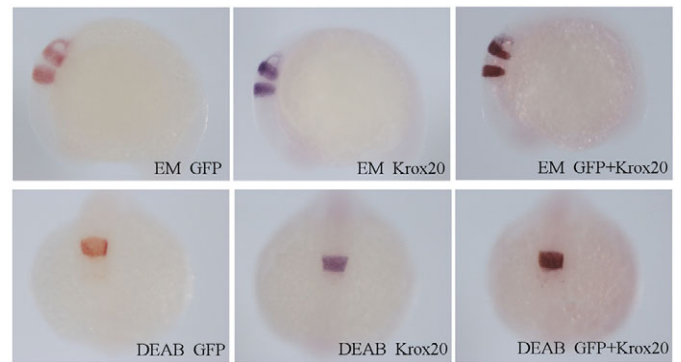


Fig. 3. Response of the GFP transgenic embryos to DEAB and RA treatments. *gfp* expression in r3 and r5 in transgenic zebrafish embryos incubated in EM or 10 μ M DEAB (from sphere stage). (Top row) In situ hybridization of embryos grown in EM at the 12- to 14-somite stage for *gfp* (red), *krox20* (a known marker of r3 and r5, blue) and both *gfp* and *krox20*. Side view. (Bottom row) In situ hybridization of embryos grown in DEAB for *gfp*, *krox20* and both *gfp* and *krox20*. Dorsal view. *gfp* expression reproduces the response of *krox20*. Since *gfp* allows the monitoring of live embryos, we have used it to quantify their response to all-trans RA.

normal expression pattern of these RA-responsive genes, as identified by in situ hybridization (Fig. 2, supplementary material Fig. S2). To better quantify this rescue we used RT-qPCR to estimate the change in expression levels of two RA-responsive genes, the expression of which is blocked in presence of DEAB: *vhnf1* and *hoxb1a*. Partial quantitative (~50%) rescue of the expression levels of these genes was observed following a pulse of 1 nM all-trans RA, either through transient incubation in all-trans RA or via transient incubation in 5 nM 13-cis RA followed by UV illumination for 1 minute (Fig. 2, supplementary material Fig. S2). Note that 1-minute UV illumination of 1 nM all-trans RA or 1 nM 13-cis RA yields similar, but much reduced, rescue of these genes (~15%). This is to be expected because UV illumination of all-trans RA or 13-cis RA yields a similar final concentration of the active all-trans RA isomer, at ~20-25% of the initial concentration (supplementary material Fig. S1) (Neveu et al., 2008).

To further investigate the possible rescue of hindbrain development upon exposure to an all-trans RA pulse, we used a transgenic zebrafish line expressing GFP in rhombomere (r) 3 and r5 of the hindbrain only (see Materials and methods). We first verified that the GFP response to all-trans RA in these embryos was similar to that of RA-responsive genes by performing in situ hybridization of *gfp* and *krox20* (a known RA-responsive gene expressed in r3 and r5). The expression patterns of *gfp* and *krox20* are similar whether the embryos are grown in normal conditions or incubated in DEAB (Fig. 3). The advantage of this line is that one can monitor in living embryos the expression pattern of GFP and thus observe in real time the response of DEAB-treated embryos to pulses of all-trans RA. The rate of GFP fluorescence increase in r5 in embryos past the 6-somite stage varied in different conditions. It is maximal (similar to r3) in normal conditions (embryo medium), minimal in DEAB and rescued in embryos exposed to 5 nM 13-cis RA and UV illuminated for 1 minute (Fig. 4A). To quantify the response to all-trans RA we have thus used the ratio (Rf) of GFP expression (fluorescence) in r5 versus r3 measured in 24 hpf embryos. For normally developing embryos $Rf=1.25\pm 0.05$, whereas for embryos incubated in DEAB $Rf=0.25\pm 0.05$ (Fig. 4B).

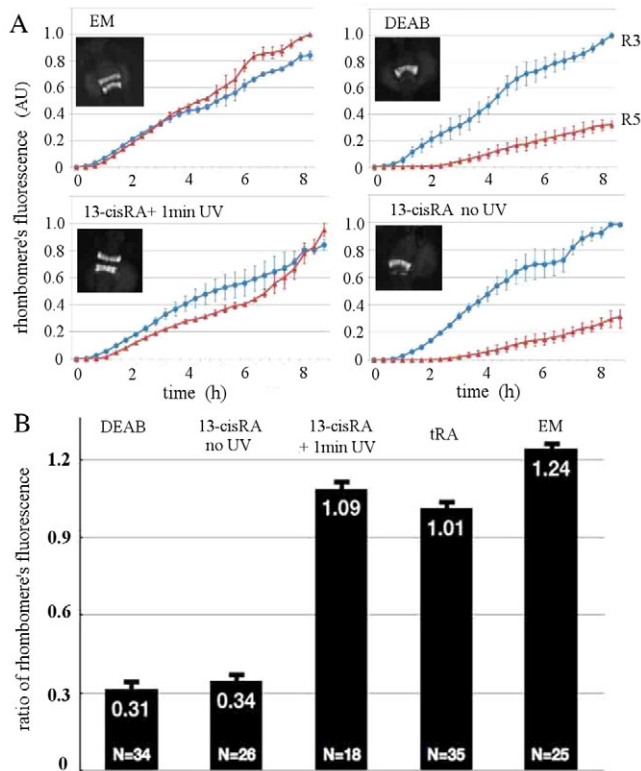


Fig. 4. Quantification of RA response in the GFP transgenic line. (A) Dynamics of the total expression of GFP in r3 and r5 in transgenic zebrafish embryos incubated from sphere stage in 10 μ M DEAB (except for the control incubated in embryo medium, EM). Some of the embryos were exposed at 90% epiboly for 5 minutes to 13-cis RA (cisRA) and illuminated (or not) for 1 minute with a UV lamp. GFP fluorescence can be detected at \sim 6 somites, which defines the time origin in these data. From the almost linear increase with time of the fluorescence in r3 and r5, we deduce that the ratio of fluorescence of r5 to r3 is a good proxy for the response to all-trans RA. Error bars indicate s.d. from the dynamics measured in three different embryos for each condition. Insets show typical fluorescent images of the embryos at 24 hpf. (B) To quantify the response of the embryos to all-trans RA exposure we measured the ratio of total fluorescence in r5 versus r3 in 24-hpf embryos. It is minimal in DEAB and in the presence of 5 nM 13-cis RA, but is almost normal (maximal) in embryos transiently incubated in 1 nM all-trans RA (tRA) or 5 nM 13-cis RA plus 1-minute UV illumination. Error bars indicate s.e.m.

In agreement with our previous observations, transient exposure of the embryos at 90% epiboly to 1 nM all-trans RA (or to 5 nM 13-cis RA followed by 1-minute UV illumination) is enough to partially rescue the expression of genes in r5 ($R_f=1.00\pm 0.05$).

Short exposure to all-trans RA before bud stage rescues hindbrain development

In the experiments described above the embryos were transiently exposed to all-trans RA at 90% epiboly, a stage that has been shown to be critical for proper rescue of the hindbrain (Grandel et al., 2002; Maves and Kimmel, 2005). To test whether the timing of exposure was critical, we exposed embryos incubated in DEAB at various developmental stages for 5 minutes to 1 nM all-trans RA. To quantify the rescue, we measured R_f at 24 hpf. Although rescue is maximal at 90% epiboly, partial rescue is also observed in embryos

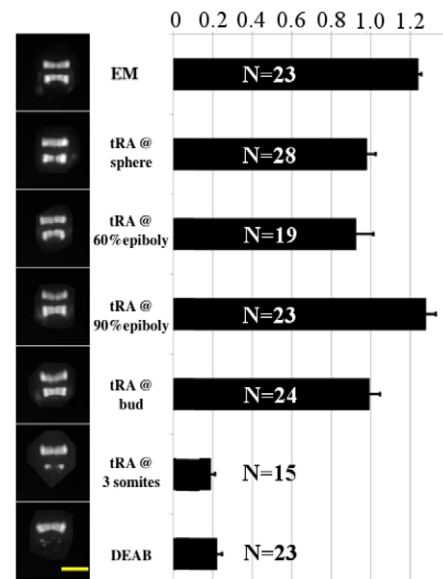


Fig. 5. Rescue of hindbrain upon an all-trans RA pulse at various developmental stages. Rescue of hindbrain development in zebrafish embryos expressing GFP in r3 and r5, continuously incubated in 10 μ M DEAB from sphere stage (4 hpf) and exposed for 5 minutes to 1 nM all-trans RA (tRA) at various developmental stages (except for the control incubated in embryo medium, EM). On the left are shown typical patterns of GFP expression at 24 hpf. On the right is shown the average ratio of total fluorescence in r5 versus r3. Note that exposure to all-trans RA until bud stage yields an almost normal pattern of hindbrain development, whereas exposure to all-trans RA at the 3-somite stage (11 hpf) does not rescue normal development of r5. Error bars indicate s.e.m. Scale bar: 200 μ m.

incubated for 5 minutes in all-trans RA as early as sphere stage ($R_f\approx 1$), but not in embryos transiently exposed to all-trans RA at 3 somites ($R_f\approx 0.2$) (Fig. 5). Surprisingly, the length of exposure to all-trans RA does not seem to affect the rescue. Embryos incubated at sphere stage in 0.2 nM all-trans RA for 5, 10, 25 and 40 minutes exhibited similar levels of GFP rescue ($R_f\approx 0.55$) (supplementary material Fig. S3). This suggests that rapid equilibrium is achieved with the external concentration of all-trans RA. Notice, however, that after exposure RA is retained for hours, even though the embryos are incubated in a DEAB solution lacking any RA.

Short UV illumination of all-trans RA prevents rescue of hindbrain development

The previous experiments implied that although all-trans RA can rapidly enter the embryo, it does not diffuse out, suggesting that all-trans RA might be sequestered until required for proper development at later stages. Since UV illumination can partially isomerize all-trans RA to its inactive cis-isomers, we tested this hypothesis by attempting to hinder hindbrain rescue by illuminating for 1 minute with UV light a DEAB-treated embryo transiently exposed at sphere stage to 1 nM all-trans RA, thereby photo-isomerizing at different (later) stages \sim 75–80% of the stored all-trans RA. As surmised if photo-isomerization is performed no later than bud stage, rescue of r5 is incomplete ($R_f\approx 0.5$) and corresponds to the level observed when all-trans RA is isomerized immediately after incubation at sphere stage (Fig. 6). However, if photo-isomerization is attempted at the 3-somite stage, r5 is rescued as it would in the absence of UV illumination (i.e. in the

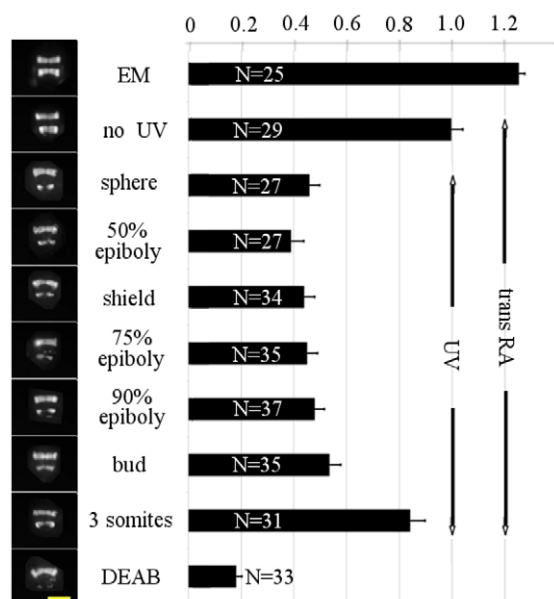


Fig. 6. Partial abortive rescue of hindbrain upon UV illumination of all-trans RA. Hindbrain development in zebrafish embryos expressing GFP in r3 and r5, continuously incubated in 10 μ M DEAB from sphere stage (4 hpf) and exposed for 5 minutes to 1 nM all-trans RA (tRA) at sphere followed at various developmental stages by UV illumination for 1 minute (which isomerizes, i.e. deactivates, \sim 80% of all-trans RA). On the left are shown typical patterns of GFP expression at 24 hpf. On the right is shown the average ratio of total fluorescence in r5 versus r3. Note that UV illumination of the embryos until bud stage prevents full rescue of r5. Fuller rescue of hindbrain development by all-trans RA is however possible if UV illumination is performed at the 3-somite stage. Error bars indicate s.e.m. Scale bar: 200 μ m.

presence of 1 nM all-trans RA; $Rf=0.85\pm 0.05$). These observations were validated (data not shown) by in situ hybridization of genes (*krox20*, *vhnf1*) known to be involved in early hindbrain development (Begemann et al., 2004; Maves and Kimmel, 2005).

We have furthermore used our approach to estimate the concentration of all-trans RA at a given developmental stage: 75% epiboly. We compared the rescue of r5 when an embryo is transferred into a DEAB solution at 75% epiboly (with and without subsequent UV illumination) with that of an embryo incubated from an early stage (blastula) in DEAB and exposed at 75% epiboly to a pulse of all-trans RA of various concentrations. From this comparison (supplementary material Fig. S4) we deduced that the concentration of all-trans RA in the embryo at 75% epiboly is \sim 0.05 nM.

These results demonstrate that quantitative control of all-trans RA concentration and activity can be achieved by UV-induced isomerization: all-trans RA can be activated by photo-isomerization of 13-cis RA as well as it can be deactivated by its own photo-isomerization. We note, however, that because UV-induced isomerization yields a steady state of RA isomers (Neveu et al., 2008) (supplementary material Fig. S1), one cannot deactivate the all-trans RA generated from photo-isomerization of cis-isomers.

Rescue of hindbrain development by local activation of RA

Local release has been achieved by implanting beads soaked with all-trans RA in various tissues, in particular in the posterior part of

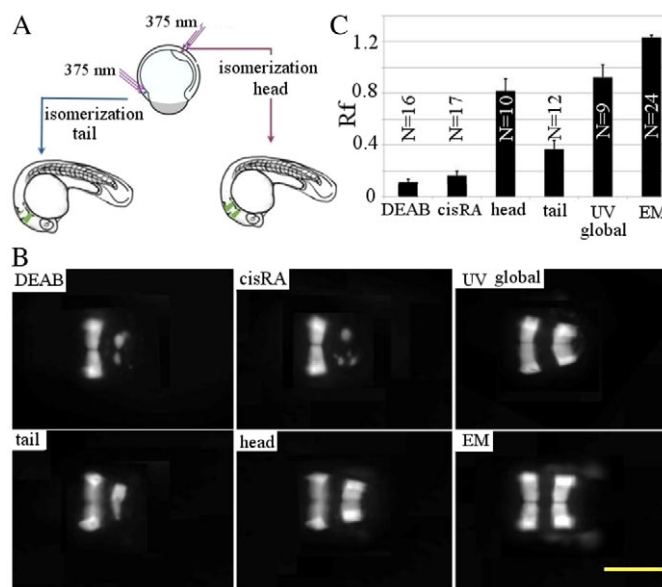


Fig. 7. Local activation of RA via isomerization. Local activation of all-trans RA in zebrafish embryos at 80-90% epiboly (incubated from 4 hpf in 10 μ M DEAB) by UV laser illumination at 375 nm following a 5-minute 13-cis RA (cisRA) pulse (5 nM) at 75% epiboly. (A) Schematics describing how embryos were illuminated in localized areas during embryogenesis (see Materials and methods for details). (B) Representative images of embryos incubated in EM (control), in EM plus DEAB, subjected to a 13-cis RA pulse and global UV illumination or to local UV laser-induced isomerization in the tail or head regions. (C) Quantification of the average ratio (Rf) of total fluorescence in r5 versus r3 for the different conditions in B. Error bars indicate s.e.m. Scale bar: 200 μ m.

the brain to investigate the rescue of development in DEAB-treated embryos (White et al., 2007). These experiments led White et al. to suggest that all-trans RA is a bona fide morphogen, an anteroposterior gradient (in conjunction with similar gradients of Fgf8 and Cyp26) of which determines the proper location and development of the rhombomeres. Even though much can be, and indeed has been, learned from such experiments, the high concentration of all-trans RA in the beads (between 10 μ M and 1 mM) and the lack of control of the all-trans RA gradient hinder the test of the elaborate models proposed for the action of all-trans RA during the development of this (and other) organs [e.g. during somitogenesis (Dequéant and Pourquié, 2008)]. Although our purpose here is not to test these models, we show below that our approach for all-trans RA control via UV photo-isomerization offers a means to locally activate (or deactivate) all-trans RA at specific times and locations during embryogenesis.

Zebrafish embryos were incubated from sphere stage in 10 μ M DEAB and exposed at 75% epiboly for 5 minutes to 13-cis RA (5 nM). They were then returned to a DEAB solution and individually placed in separate wells of an array in agarose (Herrgen et al., 2009). They were then illuminated at 80-90% epiboly for 20 seconds with a focused UV laser beam (375 nm) in selected regions of the gastrula where precursors of the head or tail are located. The UV illumination locally isomerizes 13-cis RA into all-trans RA. To test for all-trans RA activity we then looked for the rescue of GFP expression in r5 at 24 hpf in the transgenic line

described above. The rescue of r5 ($R_f \approx 0.9$) upon global UV illumination is similar to the rescue observed upon local UV laser illumination in the precursor of the head region (Fig. 7). A much less substantial rescue ($R_f \approx 0.35$) is observed upon laser illumination in the precursor of the tail region, possibly owing to light scattering that might isomerize some 13-cis RA in the head region. These results imply that all-trans RA is sequestered locally before being used as a possible morphogen as implicated in the development of the hindbrain. As a control, other embryos were injected at the one-cell stage with mRNA encoding Kaede (Chudakov et al., 2010) and similarly illuminated with the UV laser beam (the fluorescence of Kaede then switches from green to red). Although the green fluorescence of Kaede interferes with the GFP fluorescence of r3 and r5 and thus cannot be used in conjunction with GFP, the fluorescence of the photoactivated Kaede allows us to verify at later stages that the head or tail regions have indeed been UV illuminated (supplementary material Fig. S5).

DISCUSSION

We have described a method for the quantitative, temporal and local control of RA activity in a developing embryo. This method relies on photo-isomerization of an inactive isomer of RA (13-cis RA) to yield ~20% and ~30% of the active all-trans and 9-cis RA isomers. By transient incubation of the embryos in all-trans RA versus 9-cis RA, we observed that the all-trans RA isomer could rescue the developmental defects observed in a zebrafish embryo incubated in DEAB (an inhibitor of RA synthesis) at concentrations 10-fold lower than 9-cis RA. We deduce that, in the experiments reported here, the main effect of 13-cis RA isomerization is to yield ~20% of the active all-trans RA isomer. This conclusion is supported by RT-qPCR results showing that the expression levels of RA-responsive genes (*vhnf1*, *hoxb1a*) were equal when equal concentrations of all-trans RA and 13-cis RA were UV illuminated (they yield similar distributions of isomers). When only 13-cis RA was illuminated we had to use a 5-fold higher concentration to observe equal expression of the studied genes as when using all-trans RA.

Our results (RT-qPCR on all-trans RA-responsive genes and the GFP intensity ratio in r5 versus r3) show that UV isomerization of 13-cis RA is similar to a transient (5-minute) incubation in all-trans RA at one-fifth of the concentration. This is expected because ~20% of 13-cis RA is isomerized into all-trans RA upon UV illumination. Surprisingly, however, the embryo can be partially rescued by such a pulse of all-trans RA if it is given as early as sphere stage (but no later than bud stage). Because after 5 minutes of incubation in all-trans RA (or 13-cis RA) the embryo is put back into a DEAB solution, one might have expected the internalized RA to diffuse out on a similar time-scale. This, however, is not the case as implied by two lines of evidence. First, the embryo is rescued by such pulses of all-trans RA, which is believed to be required at the end of gastrulation (Grandel et al., 2002). Second, if, between the exposure to all-trans RA (at sphere) and the end of gastrulation, the embryo is UV illuminated then only very partial rescue is observed (as would be expected from a decrease in the internal concentration of all-trans RA due to isomerization). We deduce that RA is sequestered in the embryo in the time interval (a few hours) between exposure and the end of gastrulation. Sequestration per se is not surprising, as many proteins are known to bind intracellular all-trans RA with sub-nanomolar affinities [e.g. CRABP (Dong et al., 1999)]. Extracellular all-trans RA-binding proteins have also been described (Bernstein et al., 1995; Ong et al., 2000). However, these proteins have much lower affinities for

cis-isomers. Their presence would therefore not explain why 13-cis RA could be activated hours after a 5-minute incubation. Binding to these proteins would equally fail to explain why equilibrium with an external concentration of all-trans RA is reached within 5 minutes, whereas the RA can be retained for hours after the embryos are transferred into a solution lacking any RA.

Beyond the role possibly played by all-trans RA sequestration in normal development, our results suggest that spatiotemporal control of all-trans RA concentration is possible via its photo-isomerization. This was demonstrated by the rescue of hindbrain patterning upon photo-isomerization of 13-cis RA in the head and to a lesser extent in the tail region. This should open the way for a detailed investigation of various developmental models in which all-trans RA is known to play an important role, such as hindbrain patterning and somitogenesis. The local release of a known concentration of all-trans RA at a specific location in the embryo and at a specific time during the developmental program will allow more precise characterization of the response to test the relevant models of development (Allenby et al., 1993; Goldbeter et al., 2007). There are a few caveats to these investigations though: because all-trans RA is sequestered and regulated in the embryo, it is better to conduct these types of studies in embryos devoid of endogenous all-trans RA (e.g. those treated with DEAB) so that the local perturbations achieved by photo-isomerization of 13-cis RA (or all-trans RA) are not modulated by an ill-defined, local, endogenous all-trans RA response (such as the compensatory release or sequestration of endogenous all-trans RA); moreover, because photo-isomerization of 13-cis or all-trans RA also generates 9-cis RA, one should keep in mind and investigate separately the possibly synergistic role of 9-cis RA, a natural ligand of RXR.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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