H\textsubscript{2}O\textsubscript{2} and Engrailed 2 paracrine activity synergize to shape the zebrafish optic tectum

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Although a physiological role for redox signaling is now clearly established, the processes sensitive to redox signaling remains to be identified. Ratiometric probes selective for H\textsubscript{2}O\textsubscript{2} have revealed its complex spatiotemporal dynamics during neural development and adult regeneration and perturbations of H\textsubscript{2}O\textsubscript{2} levels disturb cell plasticity and morphogenesis. Here we ask whether endogenous H\textsubscript{2}O\textsubscript{2} could participate in the patterning of the embryo. We find that perturbations of endogenous H\textsubscript{2}O\textsubscript{2} levels impact on the distribution of the Engrailed homeoprotein, a strong determinant of midbrain patterning. Engrailed 2 is secreted from cells with high H\textsubscript{2}O\textsubscript{2} levels and taken up by cells with low H\textsubscript{2}O\textsubscript{2} levels where it leads to increased H\textsubscript{2}O\textsubscript{2} production, steering the directional spread of the Engrailed gradient. These results illustrate the interplay between protein signaling pathways and metabolic processes during morphogenetic events.

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Reactive oxygen species (ROS), including hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), once only considered as deleterious compounds, have recently raised novel interest due to their action as bona fide-signaling molecules\textsuperscript{1-3}. Ratiometric probes selective for H\textsubscript{2}O\textsubscript{2} have revealed its complex spatiotemporal dynamics during neural development and adult regeneration\textsuperscript{4,5}. In addition, modifying H\textsubscript{2}O\textsubscript{2} levels disturbs cell plasticity and morphogenesis\textsuperscript{6-7}. Proteins targeted by H\textsubscript{2}O\textsubscript{2} during development, by mechanisms still largely elusive, belong to many categories and include homeoproteins (HPs)\textsuperscript{8-9}. HPs play important roles in the control of cellular and regional identity during development. Although first characterized as purely cell autonomous transcription factors\textsuperscript{9,10}, HPs are also transferred between cells via non-conventional secretion and internalization routes, providing them with direct paracrine activity\textsuperscript{11}. Intercellular transfer is a general property of HPs, and its efficiency is context-dependent\textsuperscript{12,13}. Within recipient cells, transferred HPs may act not only as transcription factors but also on processes as diverse as translation, DNA repair, mitochondrial activity, and epigenetic modification\textsuperscript{14}. We have previously shown that the zebrafish midbrain–hindbrain boundary (MHB) displays high levels of H\textsubscript{2}O\textsubscript{2} that cannot be lowered without affecting tectum topography\textsuperscript{6}, a process that also requires the graded distribution of the Engrailed HPs (EN in amniotes, Eng in fish)\textsuperscript{15-18}. We thus asked whether H\textsubscript{2}O\textsubscript{2} levels and Engrailed distribution interplay with each other by conducting an analysis of both parameters at the time of the tectum antero–posterior polarization. Importantly, the Engrailed extracellular gradient is instrumental in stabilizing tectum polarity in frogs and chicks\textsuperscript{19}, requires Eng2b paracrine activity in the zebrafish\textsuperscript{20}. We combined ex vivo and in vivo approaches to demonstrate an unsuspected role of H\textsubscript{2}O\textsubscript{2} in Engrailed homeoprotein spreading during tectum development. Engrailed is released from cells with high H\textsubscript{2}O\textsubscript{2} levels and transfer to cells with low H\textsubscript{2}O\textsubscript{2} levels in which it stimulates H\textsubscript{2}O\textsubscript{2} production, thereby controlling its own polarized traffic. In addition, we identify cysteine 175 as a key residue in the redox regulation of Engrailed traffic.

**Results and discussion**

**H\textsubscript{2}O\textsubscript{2} levels shape Engrailed 2 distribution in the tectum.** The graded distribution of Eng2 evolves during development, particularly between 24 and 26 h post fertilization (hpf) (Fig. 1a, b and Supplementary Fig. 1). As previously shown in several vertebrates\textsuperscript{15-18}, Eng2 level is maximum at the MHB where it is known to be synthesized and decreases along the posterior–anterior axis until becoming undetectable at the most anterior part of the tectum. The temporal analysis of Eng2 distribution throughout the tectum (24–28 hpf) showed that, at the MHB, Eng2 level is highest at 24 hpf and decreased at 26 and 28 hpf (Fig. 1b). These modifications in Eng2 distribution were concomitant with remarkable changes in H\textsubscript{2}O\textsubscript{2} levels in the same structure (Fig. 1d, e). In the whole tectum, H\textsubscript{2}O\textsubscript{2} levels increased over time (Fig. 1e), but with a marked gradient from the MHB to the most anterior part of the tectum (Fig. 1d). We noticed that in zebrafish embryos, the clear graded distribution of nuclear Eng2 proteins is accompanied by an extranuclear gradient in the tectum along the antero–posterior axis (Fig. 1a–a′′′′), previously observed in chick and Xenopus embryos\textsuperscript{19} and shown to correlate with Engrailed intercellular transfer ex vivo\textsuperscript{21}. Using DAPI staining as a marker of the nuclear compartment, nuclear and extranuclear Eng2 signals were quantified separately. The ratio of nuclear to extranuclear Eng2 staining showed that the cellular distribution of Eng2 varied both spatially and temporally along the antero–posterior axis of the tectum from 24 to 28 hpf (Fig. 1c).

The nuclear/extranuclear ratio was highest at 26 hpf and decreased at 28 hpf while H\textsubscript{2}O\textsubscript{2} increased between 24 and 28 hpf. We thus concluded that EN2 distribution is regulated, at least partially, by H\textsubscript{2}O\textsubscript{2} levels between 24 and 28 hpf, a temporal window during which both are highly dynamic.

We next tested whether altering endogenous H\textsubscript{2}O\textsubscript{2} levels would affect Eng2 distribution. Thanks to the use of the improved ratiometric H\textsubscript{2}O\textsubscript{2} sensor HyPer\textsuperscript{17,22} we were able to detect, in vivo, the modulation of H\textsubscript{2}O\textsubscript{2} levels after treatment with a pan-NADPH oxidase inhibitor (Nox-i) (Fig. 1f). The result of mild decrease in H\textsubscript{2}O\textsubscript{2} levels led to a marked modification of Eng2 distribution, enhancing Eng2 signal at the MHB but raising the gradient slope in the tectum (Fig. 1g). The higher amount of Eng2 at the MHB was not a consequence of a higher transcription rate since a quantitative RT-PCR performed on the two sets of embryos (Nox-i treated and control embryos) revealed no difference in the amount of eng2a and eng2b mRNA (Supplementary Fig. 2). This suggested that lowering endogenous H\textsubscript{2}O\textsubscript{2} levels reduced Eng2 spreading from a neural cell production, most likely by modifying its intercellular trafficking. To test this hypothesis, we analyzed the nuclear/extranuclear distribution of Eng2 over the tectum in embryos treated or not with Nox-i (Fig. 1h). In Nox-i-treated embryos, the cellular distribution of Eng2 was significantly altered (Fig. 1h), characterized by a specific increase of Eng2 in the nuclear fraction, indicating that the correct propagation of Eng2 is dependent on H\textsubscript{2}O\textsubscript{2} having reached a given threshold. In summary, mild reduction in H\textsubscript{2}O\textsubscript{2} levels induced both a strong modification in the allocation of Eng2 between the nuclear and extranuclear compartments, and a distorted Eng2 distribution over the tectum. These results suggested that Eng2 intercellular transfer is regulated by H\textsubscript{2}O\textsubscript{2}. To directly address the involvement of H\textsubscript{2}O\textsubscript{2} in Engrailed intercellular trafficking, we used ex vivo cell culture models which permit an accurate quantification of this process.

**Engrailed 2 intercellular transfer is asymmetrically regulated by H\textsubscript{2}O\textsubscript{2}.** To evaluate the sensitivity of Engrailed trafficking to H\textsubscript{2}O\textsubscript{2} levels, we performed assays in HeLa cells, where this process has been best characterized\textsuperscript{13,21,23}. H\textsubscript{2}O\textsubscript{2} levels were fine-tuned with two strategies. To enhance H\textsubscript{2}O\textsubscript{2} levels, we added d-Alanine (d-Ala) to cells expressing a membrane-bound form of d-amino-acid oxidase (Lck-DAO) (Supplementary Fig. S3a, b)\textsuperscript{24}. To reduce H\textsubscript{2}O\textsubscript{2} levels, we expressed Catalase (CAT) deprived of its peroxisome targeting sequence (CAT\textsubscript{ΔC}) targeted to the plasma membrane (Lck-CAT\textsubscript{ΔC}, Supplementary Fig. S3c, d). We also reduced H\textsubscript{2}O\textsubscript{2} levels by adding purified CAT to cell culture or by treating cells with Nox-i (Supplementary Fig. S3e, f). Under all conditions, H\textsubscript{2}O\textsubscript{2} levels were monitored with HyPer expressed in the cytoplasm\textsuperscript{25}. The two steps of intercellular transfer, secretion, and internalization, were analyzed separately using dedicated assays set up with the Engrailed 2 homeoprotein (EN2).

To study EN2 secretion, this process was quantified with a new strategy (transRUSH\textsuperscript{26}) adapted from the RUSH system\textsuperscript{27} (Fig. 2a). Two tags were added to EN2: one (SBP-tag) that hooks the protein at the inner side of the plasma membrane when co-expressed with a membrane-bound Streptavidin hook, and another tag (HiBiT), a small nanoluciferase fragment that allows light production upon interaction with its large counterpart fragment (LgBiT) addressed to the extracellular side of the plasma membrane. In this system, intracellular-trapped EN2 is released upon biotin addition, and secretion is monitored by light production upon interaction of EN2-HiBiT with LgBiT at the cell surface. Increasing H\textsubscript{2}O\textsubscript{2} levels...
Fig. 1 H$_2$O$_2$ levels shape the Engrailed 2 distribution in the tectum. a Immunodetection of Eng2a and Eng2b (green) and DAPI staining (red) in zebrafish embryos (24 hpf) revealed different nuclear/extranuclear distributions along the anteroposterior axis (a’–a”: insets of sections at higher magnification; MHB midbrain hindbrain boundary, HB hindbrain). Eng2 staining alone is shown in Supplementary Fig. 1. b Quantification of total Eng2 levels (inferred from Eng immunostaining) along the anteroposterior axis of the tectum at 24, 26, and 28 hpf. All values were normalized to the maximum value for 26 hpf. c Ratio of Eng2 nuclear over extranuclear signals at 24, 26, and 28 hpf. d H$_2$O$_2$ levels in the tecta of zebrafish embryos from 24 to 28 hpf. H$_2$O$_2$ levels were inferred from the YFP$_{500}$/YFP$_{420}$ excitation ratio of HyPer7 in time-lapse recordings. Arrowhead: MHB position. The quantification is presented in e. f H$_2$O$_2$ levels in the tecta of control (Ctrl) and two hours Nox-i (100 nM) treated zebrafish 26 hpf embryos. g Quantification of immunodetected Eng2 along the anteroposterior axis of the tectum in control (Ctrl) and Nox-i-treated embryos (26 hpf). h Ratio of Eng2 nuclear/extranuclear signals in control (Ctrl) and Nox-i-treated embryos (26 hpf). Double arrows in g and h indicate the domain where nuclear to extranuclear ratios are statistically different (confidence interval varying from 0.0001 to 0.05 from the MHB to more anterior positions). Scale bars, 50 μm. *p-value ≤ 0.05.
from its interaction with a cytosolic LgBiT (Fig. 2f). Increasing H$_2$O$_2$ levels (via D-Ala addition) reduced EN2 internalization (Fig. 2g), while decreasing them (by treatment with either purified CAT or Nox-i) enhanced EN2 internalization (Fig. 2h, i). These results were confirmed by direct visualization of FITC-tagged EN2 uptake (Supplementary Fig. S4). In summary, redox levels modulate the two steps of EN2 trafficking in an uneven manner: low levels of H$_2$O$_2$ stimulate internalization and reduce secretion, while high levels of H$_2$O$_2$ have the opposite effects. The dual role of H$_2$O$_2$ levels on EN2 trafficking observed in cell culture nicely fits with the in vivo effects of Nox-i treatment presented above (Fig. 1h): the nuclear accumulation in producing cells close to the MHB and the reduction of Eng spreading through the tectum are best explained by an inhibition of Eng2 secretion supporting the view that H$_2$O$_2$ levels directly regulate Eng2 distribution in the zebrafish embryonic tectum.

Cysteine 175 is involved in the redox regulation of Engrailed spreading. Reversible oxidation of cysteine residues is the main target of ROS action within proteins, affecting their conformation and activity. Only one cysteine is conserved through evolution in Engrailed proteins. It is located at position 175 in chicken EN2, next to the hexapeptide motif, that is essential both for EN2 transcriptional activity and intercellular transfer (Fig. 3a). To test whether this conserved cysteine is required for EN2 functions, we first evaluated the DNA-binding and transcriptional activities of a C175S EN2 mutant (EN2C>S) using a negative control EN2W>K, a W169>K,W>172>K double mutant deficient for transcriptional activity and intercellular transfer. In the electrophoretic mobility shift assay (EMSA), EN2C>S bound its target nucleic acid sequence in the presence of its PBX co-factor with the same efficiency as EN2 (Fig. 3b). In co-transfection experiments, EN2 C>S stimulated transcription from the MAP1b promoter (a known target of Engrailed) to the same extent as EN2 (Fig. 3c). The transfer ability of EN2C>S was then analyzed in secretion and internalization assays. As shown in Fig. 3d–g, EN2C>S secretion became insensitive to H$_2$O$_2$ levels. Reducing EN2 cysteine by DTT decreased its internalization to EN2C>S levels, which was insensitive to DTT treatment (Fig.3h). Part of this effect might involve the ability of EN2 to dimerize as a covalent EN2 dimer (Supplementary Fig. S5a). Indeed, promoting covalent redox-insensitive EN2 homodimerization using a chemical cross-linker (Supplementary Fig. S5b) stimulated internalization compared to native EN2 (Fig. 3i). In summary, the EN2C>S mutation had no broad effect on the transcriptional activity of EN2, but it...
Fig. 3 A conserved cysteine is involved in the redox regulation of Engrailed transfer. 

a. Conserved cysteine residue in Engrailed 2 proteins across species (the hexapeptide is underlined).

b. Gel-shift assay comparing the DNA-binding properties of EN2, EN2_C>S and DNA-binding deficient EN2_W>K.

c. Quantification of EN2_C>S and EN2 transcriptional activity on the MAP1b promoter.

d–f. Quantification of EN2 or EN2_C>S secretion via the transRUSH method from control cells d or cells expressing Lck-DAO with or without D-Ala e or inactive or active Catalase (CATmut or CAT, respectively)

f. Quantification of EN2 or EN2_C>S internalization g after DTT pretreatment h or EN2 dimerization i. *p-value ≤ 0.05; **p-value ≤ 0.01; ***p-value ≤ 0.001; and ****p-value ≤ 0.0001.
lessened EN2 intercellular transfer and abolished its sensitivity to H$_2$O$_2$.

**Engrailed 2 internalization increases H$_2$O$_2$ levels.** Signaling pathways are not just linear chains of events, but use crosstalk, feedback, and reciprocal interactions to control biological processes$^{33}$. In this context, because Engrailed is known to influence the oxidative metabolism of recipient cells$^{11}$, we wondered whether its trafficking$^{34}$ could have an impact on intracellular H$_2$O$_2$ levels. To address this question, EN2 was added to the medium of cells expressing HyPer throughout their cytoplasm. EN2 addition, but not of either mutant proteins deficient for internalization (EN2$_{W>K}$ and EN2$_{C>S}$), induced a quick increase in H$_2$O$_2$ levels (Fig. 4a, b). To verify that EN2 internalization was responsible for this increase, this step was specifically blocked by preincubation with the 4G11 monoclonal antibody$^{19}$. Under these conditions, EN2 addition no longer enhanced intracellular H$_2$O$_2$ levels (Fig. 4c, d). EN2 import is thus necessary and sufficient to modify cytoplasmic H$_2$O$_2$ levels in recipient cells ex vivo. We then asked whether Eng intercellular transfer also modulates H$_2$O$_2$ levels in vivo. Extracellular anti-En single-chain antibody 4F11scFv, which blocks Engrailed transfer$^{35}$, was expressed in the Eng domains using eng2a mini-enhancer$^{36}$ in zebrafish embryos expressing HyPer7. This led to
a strong reduction in H₂O₂ levels in the tectum of embryos expressing the blocking antibody, but not of embryos expressing a fluorescent protein as a control (Fig. 4e, f).

In summary, Engrailed internalization enhanced intracellular levels of H₂O₂, modifying the properties of the recipient cells.

Conclusion

Together, these results demonstrate that Engrailed is transferred from cell to cell in the zebrafish tectum in an H₂O₂-dependent manner, and itself acts rapidly to establish an H₂O₂ gradient in vivo. We propose that the global wave of H₂O₂ that takes place during development (Fig. 1d, e) sets the spatio-temporal window for heightened Engrailed transfer. Engrailed internalization increases H₂O₂ levels in recipient cells, making them more competent to secrete Engrailed and reducing Engrailed uptake once they have reached an H₂O₂ threshold. Both events propagate the Engrailed signal forward (posterior to anterior), which leads to a directional spread of the Engrailed gradient.

As a consequence, the extent and intensity of the morphogenetic action of Engrailed mediated via its non-cell autonomous diffusion is shaped by its reciprocal interactions with the main redox signaling molecule (Fig. 4g). We still do not know if this finding can be generalized to other HPs endowed with paracrine activity but, if so, may give new insights into how tissue morphogenesis and cell metabolism influence each other. Given the role of HPs expression in evolution, it is tempting to consider how such a mechanism may have contributed to link the second rise in atmospheric oxygen to the concomitant increase in metazoan complexity.

Methods

DNA constructs, recombinant proteins, and biochemical assays. Details of the DNA constructs are given in Supplementary Table S1. Hsp70-tagged recombinant proteins were produced in BL21 (DE3) grown in MagicMedia (Invitrogen) 24 h, 28 °C and purified on HisTrap columns (GE Healthcare), eluted by NiCl₂ gradient and dialyzed for 2 days (20 mM phosphate buffer, 100 mM NaCl pH 7.5). For protein FITC labeling, 100 μM of purified protein was incubated with a two-fold molar excess of fluorescein isothiocyanate in carbonate buffer (50 mM pH 9.5, 100 mM NaCl) overnight at 4 °C and free FITC was removed by dialysis (48 h, 4 °C). The activity of FITC incorporation was determined by SDS-PAGE and spectrophotometric analysis. The molecular weight of purified protein was confirmed by a ratio between 1.5 and 2 for all proteins. Gel-shift assays were performed as described in ref. 39. Redox-sensitive En2c covalent dimerization was obtained with the homobifunctional crosslinker 1,8-bis[maleimidodithylylglycol (Thermo #22336), according to manufacturer instructions.

Fish care and manipulation. Fish husbandry: Zebrafish were maintained and staged as previously described. Experiments were performed using the standard AB wild-type strain. The embryos were incubated at 28 °C. Developmental stages were determined and indicated as hours postfertilization (hpf). The animal facility obtained permission from the French Ministry of Agriculture for all the experiments described in this manuscript (agreement no. C7 5-05-12). Nucleic acid injection (2 ng/μl) were injected into one-cell stage embryos to induce ubiquitous expression. EN2-EKT2 fusions were activated at 90% epiboly by adding cyclofen in as described in ref. 20.

Pharmacological treatments: To decrease H₂O₂ levels, embryos were incubated in VAS-2870 (Nax-i) (100 nM) from Enzo Life Sciences (#BML-E3995-0010, Enzo Life Sciences, Inc.; Farmingdale, NY, USA) or an equivalent amount of DMSO as a control. To increase H₂O₂ levels, cells expressing D-amino acid oxidase (DAO) were treated with 10 μM of D-aspartic acid (Sigma-Aldrich #A7377) before the internalization or blocking anti-Engrailed antibody. 4G11 was deposited to the DSHB by Jessel TM and Brenner-Morton S. Quantitative internalization assay: Cells (13,000 per well) stably expressing doubly tagged EN2 (SBP-EN2-HiBiT) were plated on 96-well plates (Greiner Bio-one) coated with polyornithine (10 μg/ml) and induced for constitutive protein expression with doxycycline. After 10 h, the cells were transfected with bidirectional expression plasmids of the transRUSH series making use of the scMV enhancer, all expressing transmembrane fusions of LgBiT (outside) and core streptavidin (inside) downstream the CMV minimal promoter, and expressing in the opposite direction—downstream the scMV promoter—either one of Lck-tagged DAO, active CAT devoid of a peroxisomal signal, or its inactive counterpart. After 24 h, media were removed, and cells were incubated with fresh medium at 37 °C. Luciferase activity was measured 1 h later with a 96-well plate luminometer (Tristar, Berthold) as described in the HiBiT assay kit (Promega). The cells were then lysed to measure intracellular protein expression. Normalization with biotin-untreated wells enabled us to calculate the secretion index and report the secretion efficiency.

Quantitative internalization assay: Cells (30,000 per well) stably expressing LgBiT were plated in 96-well plates in six-well plates (Ibidi). After 24 h, the medium was removed, and cells were incubated with a fluorescent protein (1 μM) diluted in DMEM without serum for 30 min at 37 °C before visualization on a CSU-W1 Yokogawa spinning disc coupled to a Zeiss Axio Observer Z1 inverted microscope equipped with a sCMOS Hamamatsu camera with a ×25 (Zeiss 0.8 Imm DIC WD: 0.57 mm) objective. DOPS 100 mM 405 nm and 150 mM 491 nm lasers and a 525/50 bandpass emission filter were used. Quantification and statistical analyses: Total Engrailed was quantified by measuring the mean fluorescence of control and experimental samples. Ordinary one-way ANOVA followed by Tukey’s multiple comparison test was performed to evaluate the significant differences between the conditions along the tectum. H₂O₂ levels were quantified by measuring the mean ratio value of HyPer7 fluorescence at each time point and normalized to the mean ratio value determined before treatment. A t-test was then performed to statistically determine the differences between the conditions over time.
with a 96-well plate luminometer (Tristar, Berthold) with a HiBiT assay kit (Promega).

H2O2 imaging with the HyPer probe. HyPer fluorescence was excited with 501/16 and 420/40 bandpass excitation filters, and the corresponding YFP emission was measured using a 530/35 bandpass emission filter. Spinning-disk images were acquired using a ×63 objective (×63/1.4 oil WD: 0.17 mm) on a Spinning-Disk CSU-W1 (Yokogawa) equipped sCMOS Hamamatsu 2048×2048 camera. To calculate the HyPer ratio, images were treated as previously described42. Transcriptional activity: MAP1B promoter activation by EN2 or EN2C>S was quantitated in co-transfection experiments as previously described32 except that the reporter construct expressed Nanoluciferase instead of Luciferase.

Statistics and reproducibility: Data were analyzed using GraphPad Prism 6 and expressed as the mean ± standard error of the mean (SEM). Statistical significance was calculated using a two-sided paired Student t-test. For multiple conditions, ordinary one-way ANOVA followed by Tukey comparison test was performed to evaluate the significant differences. Sample sizes and number of replicates are given in Supplementary Table 4. For each experiment, at least two independent experiment with similar results were performed.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Source data are provided with this paper. The DNA constructs and cell lines and transgenic fish are available upon request. All other data underlying the findings of the study are available from the corresponding author upon reasonable request.

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**Author contributions**

S.V. and A.J. conceived the project and designed the experiments. I.Q., M.T., I.A., A.J. and M.V. prepared the DNA constructs used in this study. I.A., M.T. and C.R. performed the experiments. V.B. and V.Y.V.P. provided the HyPer sensor and useful advice. L.A., M.T., C.R., M.V., A.J. and S.V. analyzed the experimental data. A.P., M.V., A.J. and S.V. wrote the paper.
Competing interests
The authors declare no competing interests.

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