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RESEARCH ARTICLE

Xhe2 is a Member of the Astacin Family of Metalloproteases that Promotes X*enopus* Hatching

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Summary: Transcription factors Pax3 and Zic1 are among the earliest genes activated at the neural plate border. In *Xenopus*, they are necessary and sufficient to promote the formation of multiple neural plate border cell types, including the neural crest, cranial placodes, and hatching gland. Pax3 is especially critical for the formation of the hatching gland, a group of cells that produce proteolytic enzymes essential to digest the egg vitelline envelope and jelly coat in order to release the tadpole into the environment. In a screen designed to identify downstream targets of Pax3, we isolated a member of the astacin family of metalloproteases, related to *Xenopus* hatching enzyme (Xhe), that we named *Xhe2*. *Xhe2* is exclusively expressed in hatching gland cells as they first emerge at the lateral edge of the anterior neural plate, and persists in this tissue up to the tadpole stage. Knockdown experiments show that *Xhe2* expression depends entirely on Pax3 function. Gain-of-function studies demonstrate that Pax3 can induce premature hatching through the upregulation of several proteolytic enzymes including Xhe2. Interestingly, Xhe2 overexpression is sufficient to induce early hatching, indicating that Xhe2 is one of the key components of the degradation mechanism responsible for breaking down the vitelline membrane. genesis 52:946–951, 2014. © 2014 Wiley Periodicals, Inc.

Key words: hatching; Pax3; astacin; metalloprotease; *Xenopus*

INTRODUCTION

The ectoderm of the vertebrate embryo can be subdivided into three domains at the end of gastrulation: the non-neural ectoderm, the neural plate, and the neural plate border. The non-neural ectoderm and neural plate will give rise to the epidermis of the skin and the central nervous system, respectively. In amphibians, the neural plate border contains at least three groups of cells: the neural crest, cranial placode, and the hatching gland. The neural crest is a migratory cell population that contributes cartilage, bone, and connective tissue to the face, neurons and glial cells to the peripheral nervous system, and pigment cells to the skin (LeDouarin *et al.*, 2004). The cranial placodes give rise to the paired sensory organs (olfactory, lens, inner ear), and make an important contribution to the cranial peripheral nervous system (Saint-Jeannet and Moody, 2014). The hatching gland is a transient structure that produces proteolytic enzymes implicated in the partial digestion of the egg vitelline membrane that protects the embryo early in life. This proteolysis coupled with early spontaneous muscular contractions of the embryo causes the vitelline envelope to rupture releasing the tadpole into the environment (Carroll and Hedrick, 1974; Drysdale and Elinson, 1991). These three groups of cells can be identified at early neurula stage with the appropriate molecular markers (Hong and Saint-Jeannet, 2007). While neural crest and cranial placode progenitors are derived from the deep layer of the ectoderm, hatching gland cells arise from the outer layer of the ectoderm.

In *Xenopus* embryos, the segregation of these three cell populations depends primarily on the activation of

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two transcription factors at the neural plate border: Pax3 and Zic1 (Garnett *et al*., 2012; Hong and Saint-Jeannet, 2007; Monsoro-Burq *et al*., 2005; Sato *et al*., 2005). Both factors are co-expressed in the neural crest forming region, Pax3 is also expressed in the presumptive hatching gland cells, and Zic1 in the vicinity of the prospective cranial placodes (Hong and Saint-Jeannet, 2007). Several studies have shown that Pax3 and Zic1 are necessary and sufficient to promote hatching gland and cranial placode fates, respectively, while their combined activity is essential to specify the neural crest (Hong and Saint-Jeannet, 2007; Monsoro-Burq *et al*., 2005; Sato *et al*., 2005).

In a screen designed to identify downstream targets of Pax3 at the neural plate border we isolated a member of the astacin family of metalloproteases related to *Xenopus* hatching enzyme (Xhe; Katagiri *et al*., 1997; Sato and Sargent, 1990). Here we describe the expression and function of this protease that we named *Xenopus* hatching enzyme 2 (Xhe2). *Xhe2* is exclusively expressed in hatching gland cells, and in gain-of-function studies we demonstrate that Xhe2 is sufficient, downstream of Pax3, to promote digestion of the vitelline membrane.

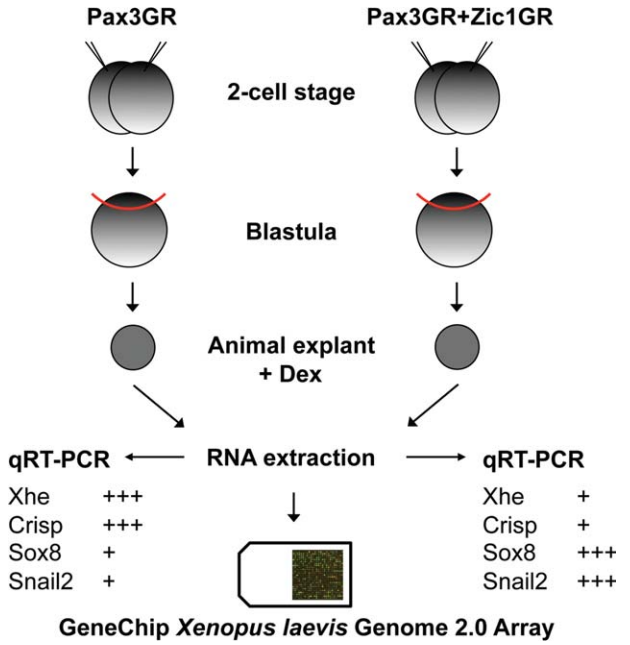

FIG. 1. Strategy to isolate Pax3 targets in the developing hatching gland. *Xenopus* embryos were injected at the 2-cell stage with mRNA encoding GR (not shown), Pax3GR, and Zic1GR (250 pg each), alone or in combination. At the blastula stage (Stage 9), animal cap explants were dissected and cultured for 8 h in the presence of dexamethasone. While Pax3 alone induces hatching gland-specific genes (*Xhe* and *Crisp*), the combination of Pax3 and Zic1 promotes neural crest fate (*Snail2* and *Sox8*) at the expense of hatching gland fate (Hong and Saint-Jeannet, 2007). RNA were extracted from each sample, analyzed by Qpcr, and subsequently used to screen a GeneChip Xenopus laevis Genome 2.0 Array (Affymetrix) as previously described (Bae *et al*., 2014).

MATERIALS AND METHODS

Plasmid Constructs

Xenopus laevis Xhe2 (pSPORT6-Xhe2) was purchased from Open Biosystems (Thermo Scientific, Waltham, MA, USA). An expression construct was generated by sub-cloning the coding region of *Xhe2* into pCS2+ vector (pCS2+Xhe2). pCS2+Pax3GR is a hormone inducible construct in which Pax3 open reading frame is fused to the hormone-binding domain of human glucocorticoid receptor (Hong and Saint-Jeannet, 2007). The activity of such fusion proteins can be regulated by addition of dexamethasone in the culture medium of whole embryos (Kolm and Sive, 1995).

Xenopus Embryo Injections, Explants Culture, and Dexamethasone Treatment

Embryos were staged according to Nieuwkoop and Faber (1967) and raised in 0.1× NAM (Normal Amphibian Medium; Slack and Forman, 1980). *Pax3GR* (0.5 ng; Hong and Saint-Jeannet, 2007), *Xhe2* (1 ng), and *β-galactosidase* (0.5 ng) mRNAs were synthesized in vitro using the Message Machine kit (Ambion, Austin, TX). Pax3 morpholino antisense oligonucleotide (Pax3MO) was purchased from GeneTools (Philomath, OR). Pax3MO has been previously characterized and its specificity is well documented (Monsoro-Burq *et al*., 2005). Moreover, this batch of Pax3MO was validated in a recent study that characterized targets of Pax3 and Zic1 in the developing neural crest (Bae *et al*., 2014). Synthetic mRNAs and antisense oligonucleotides were injected into the animal pole of one blastomere at the 2-cell stage. In all experiments, embryos were also co-injected with *β-galactosidase* mRNA to identify the manipulated side. Embryos injected with *Pax3GR* mRNA were cultured in 0.1× NAM containing 10 μM dexamethasone (Dex; Sigma-Aldrich, St. Louis, MO). Sibling embryos injected with Pax3GR mRNA, cultured in the absence of dexamethasone were used as control.

Lineage Tracing and Whole-Mount In Situ Hybridization

Embryos at the appropriate stage were fixed in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM MgSO4, 3.7‰ formaldehyde) and successively processed for Red-Gal (Research Organics, Cleveland, OH) staining and in situ hybridization. Antisense DIG-labeled probes (Genius kit; Roche, Indianapolis, IN) were synthesized using template cDNA encoding *Xhe* (Katagiri *et al*., 1997), *Crisp* (Schambony *et al*., 2003), and *Xhe2* (pSPORT6-Xhe2). Whole-mount in situ hybridization was performed as previously described (Harland, 1991). Images were captured using an Olympus SZX9

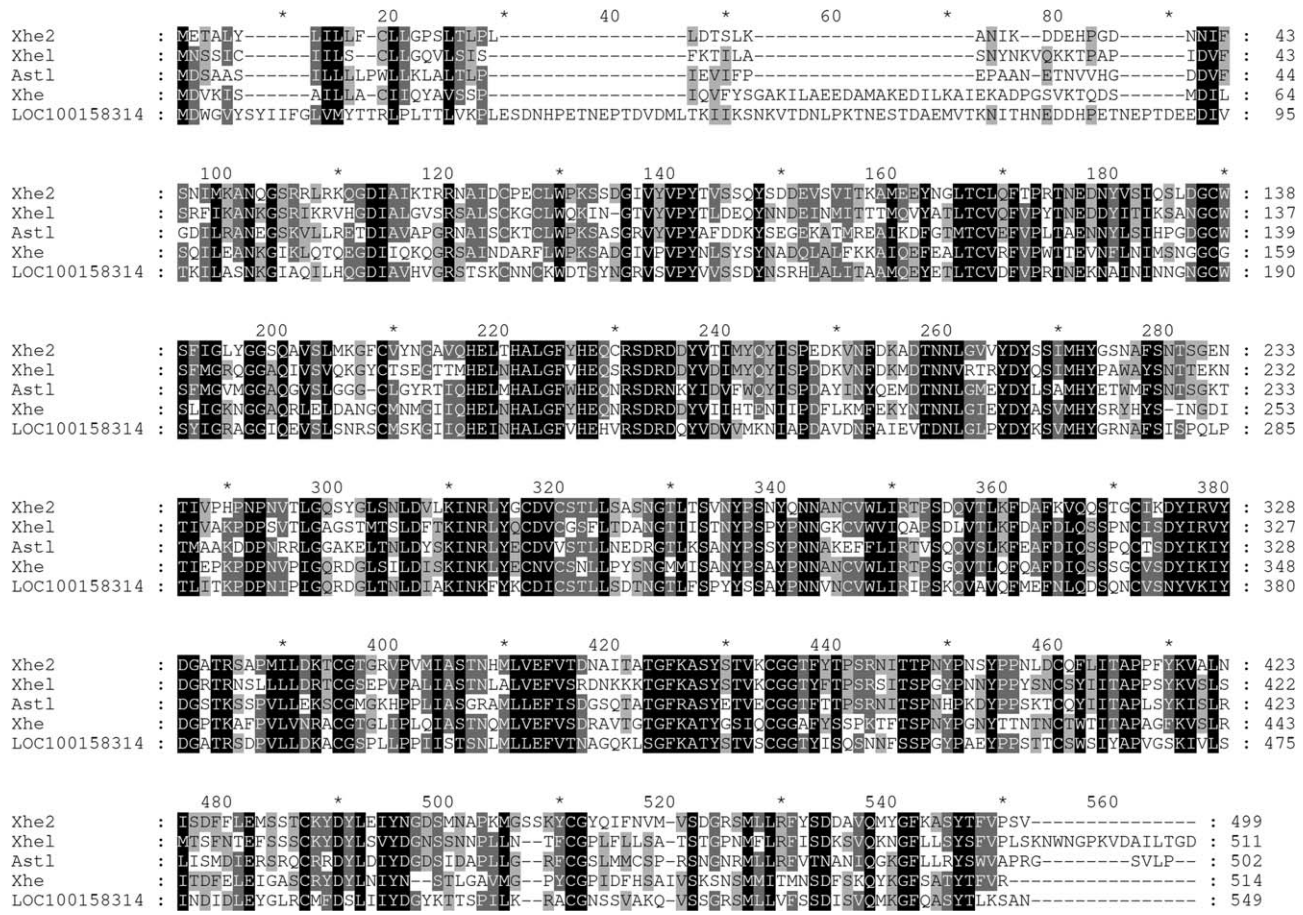

FIG. 2. Sequence comparison of proteins related to Xhe2. The predicted amino acid sequences from *Xenopus laevis Xhe2, Xhel, Ast-1, Xhe*, and *LOC100158314* genes were aligned using ClustalX2. Amino acid conservation among these proteins is indicated.

microscope and a QImaging Micro Publisher 3.3 RTV camera.

Histology

Embryos were embedded in Paraplast+, sectioned (12 μm) on an Olympus rotary microtome, counter stained with Eosin and mounted in Permount. Images were captured on a Nikon SMZ1500 microscope equipped with a Nikon Ds-U3 camera.

RESULTS AND DISCUSSION

Identification and Characterization of Xenopus laevis Xhe2

We recently performed a microarray screen designed to identify targets of Pax3 and/or Zic1 (Bae *et al*., 2014), two transcription factors that are necessary and sufficient to specify neural plate border cell types in *Xenopus* (Hong and Saint-Jeannet, 2007; Monsoro-Burq *et al*., 2005; Sato *et al*., 2005). One aspect of the screen was based on the observation that while expression of Pax3 favored hatching gland fate in *Xenopus* animal cap explants, the simultaneous expression of Pax3 and Zic1 repressed hatching gland-specific genes to promote neural crest fate (Fig. 1; Hong and Saint-Jeannet, 2007). Genes that follow this pattern of induction were expected to represent genes required for hatching gland formation downstream of Pax3. Among these genes we found an uncharacterized protein LOC494813 (NP_001088112), related to *Xenopus* hatching enzyme (Xhe, also known as, USV.2; Katagiri *et al*., 1997; Sato and Sargent, 1990), here referred as *Xhe2*. Xhe2 possesses an open reading frame encoding 499 amino acids (Fig. 2a). When compared to *Xenopus laevis* proteins, Xhe2 shares 53% identity with Xhe-like (Xhel; NP_001082458), 48% with Ast-like (Astl; NP_001093347), 46% with Xhe (UVS.2; NP_001081221), and 42% with LOC100158314 (NP_001121238) an uncharacterized protein. All these proteins belong to the astacin family of metalloproteases. Astacins represent a large family of proteolytic enzymes described in a variety of organisms and include several subfamilies of closely related members, such as the bone morphogenetic protein/tolloid-like enzymes, the

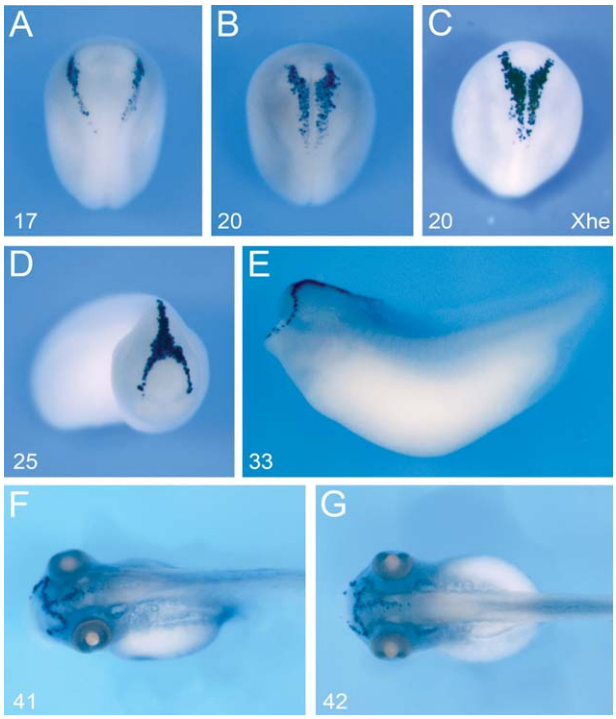

FIG. 3. Developmental expression of *Xhe2*. (**a**) *Xhe2* is first detected at the neurula stage into two regions lateral to the anterior neural plate. (**b**) As the neural tube closes, the two lateral domains converge at the dorsal midline in a pattern identical to that of *Xhe* (**c**). Embryos are viewed from the dorsal side, anterior to top. (**d**) By stage 25, the two posterior domains have completely fused dorsally to form the characteristic inverted Y shape of the hatching gland. Frontal view, dorsal to top. (**e**) Lateral view of a stage 33 embryo illustrating the dorsal anterior expression domain of *Xhe2*. This expression pattern persists up to the tadpole stage (**f**), though around Stage 42 the number of Xhe2 expressing cells starts to decrease (**g**). Dorsal views, anterior to left. The developmental stages (Nieuwkoop and Faber, 1967) are indicated in the lower left corner of each panel (a–g).

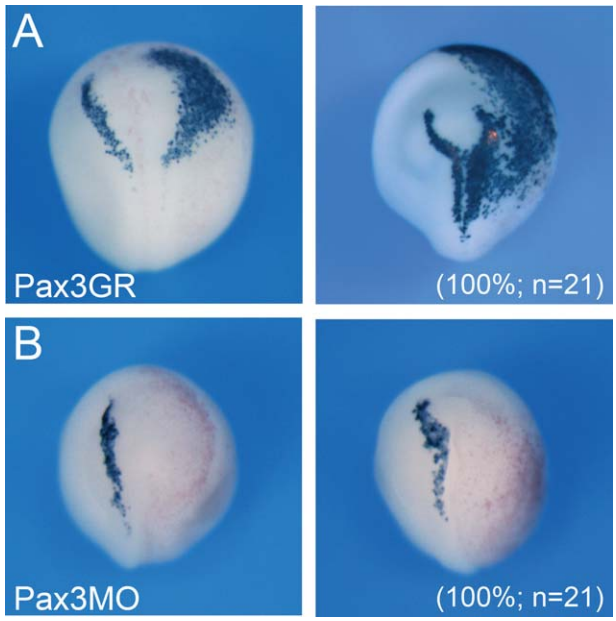

FIG. 4. *Xhe2* expression is regulated by Pax3. (**a**) Unilateral injection of *Pax3GR* mRNA resulted in a dramatic expansion of *Xhe2* expression domain (100% of the embryos; $n = 21$). (**b**) Conversely, morpholino-mediated knockdown of Pax3 caused a loss of *Xhe2* expression on the injected side (100% of the embryos; $n = 21$). The injected side is on the right, as indicated by the presence of the lineage tracer (Red-gal). Embryos are viewed from the dorsal side, anterior to top.

meprins, and the hatching proteases. Proteolytic enzymes play essential roles in living organisms, where they have been implicated in the control of cell cycle, morphogenesis, cell migration, ovulation, fertilization, and apoptosis (Gomis-Rüth *et al.*, 2012).

Developmental Expression Xhe2 and its Regulation by Pax3

To evaluate the expression of *Xhe2*, we performed whole-mount in situ hybridization on embryos at various stages of development. *Xhe2* was first detected at the neurula stage into two narrow stripes on each side of the anterior neural plate (Fig. 3a). As the neural tube starts to close the two lateral domains converge at the dorsal midline and eventually fused dorsally to form the characteristic inverted Y shape of the hatching gland (Fig. 3b,d,e). This expression pattern is identical to that of *Xhe* (Fig. 3c) and *Crisp*, two hatching gland specific genes (Katagiri *et al.*, 1997; Schambony *et al.*, 2003). The expression and restriction to the hatching gland persist up to the tadpole stage, when the number of *Xhe2*-positive cells starts to progressively decrease (Fig. 3f,g).

To confirm that *Xhe2* is a genuine target of Pax3, we performed perturbation experiments in the embryo by injection of *Pax3GR* mRNA (the hormone-inducible versions of Pax3) or Pax3-specific morpholino antisense oligonucleotides. While Pax3 overexpression caused a dramatic up-regulation of Xhe2 expression, Pax3 knockdown resulted in a loss of *Xhe2* expression on the injected side (Fig. 4). These results confirm the position of *Xhe2* downstream of Pax3 in the regulatory cascade controlling hatching gland formation. To determine whether *Xhe2* is a direct target of Pax3, we analyzed the genomic sequence around *Xhe2* gene. We identified three putative Pax3 binding sites in a region spanning 3.5 kb of sequence upstream of *Xhe2* transcription start site (not shown), suggesting that Pax3 may directly regulate *Xhe2* expression.

Xhe2 Overexpression Promotes Early Hatching

One of the most remarkable phenotypes of Pax3 gain-of-function is a premature hatching of the embryo from its vitelline membrane (Hong and Saint-Jeannet, 2007). We have previously shown that this phenotype is a direct consequence of Pax3's ability to activate

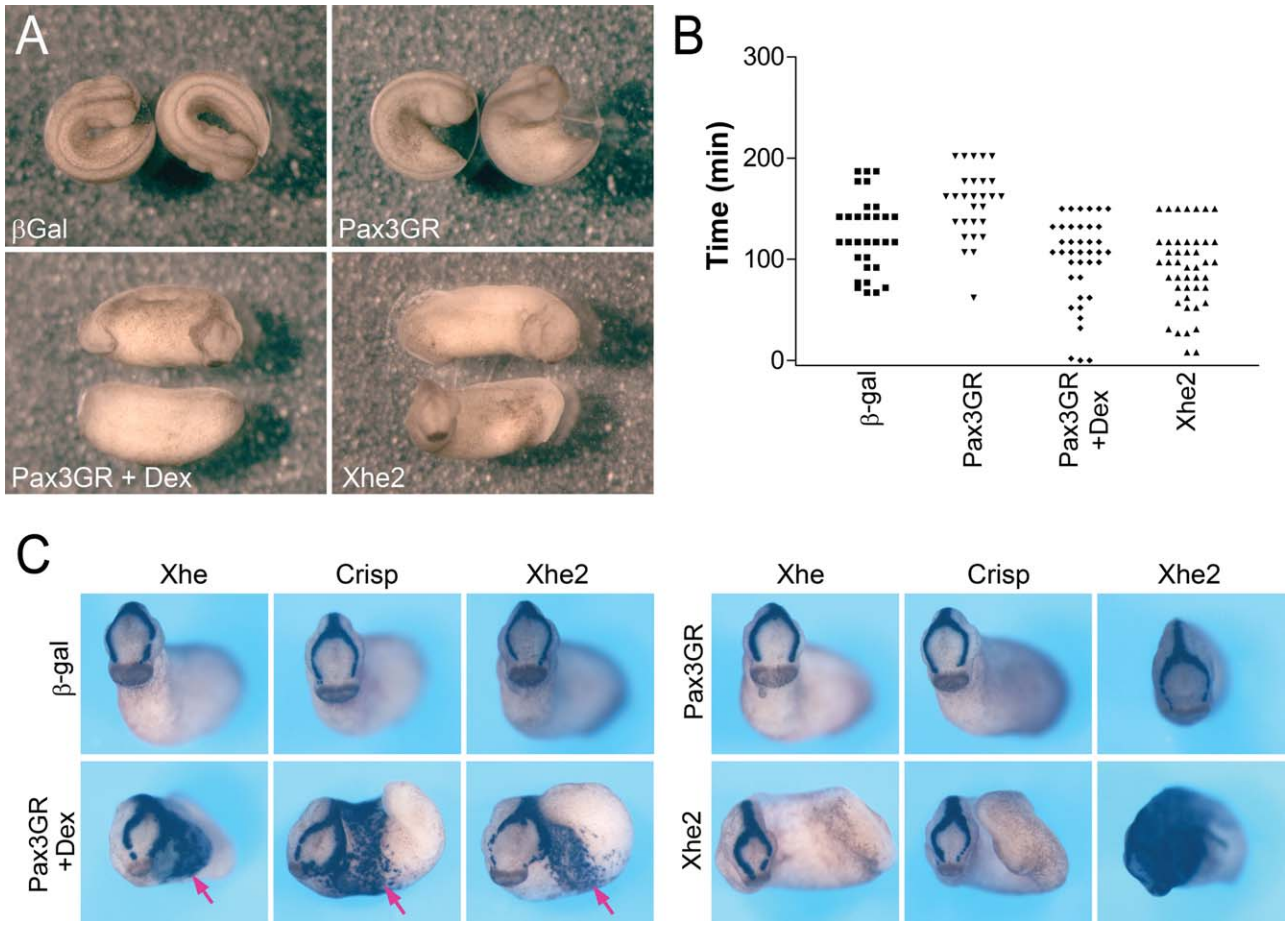

FIG. 5. *Xhe2* overexpression promotes early hatching. (**a**) Embryos injected with *Xhe2* mRNA or injected with *Pax3GR* mRNA and treated with dexamethasone (Pax3GR +Dex) at the gastrula stage hatch earlier than their siblings cultured in the absence of dexamethasone (*Pax3GR*), or injected with *β-galactosidase* mRNA (β-gal). Note that while Xhe2 and Pax3GR+Dex embryos are fully extended, Pax3GR and β-gal embryos are still bent due to the constraint of the vitelline membrane. (**b**) Quantification of the results. Among the four experimental conditions the time at which the first embryo hatched was recorded at time zero. Each symbol represents one embryo. (**c**) Stage 25 embryos that received unilateral injection of *Pax3GR* at the 2-cell stage and treated with dexamethasone (Pax3GR +Dex) exhibited a strong ectopic expression of *Xhe*, *Crisp*, and *Xhe2* on the injected side (arrows), while in sibling embryos cultured in the absence of dexamethasone (Pax3GR), or injected with *β-galactosidase* mRNA (β-gal), the expression of these genes was unperturbed. Early hatching in *Xhe2* mRNA injected-embryos is a direct consequence of Xhe2 overexpression. Here, the *Xhe2* in situ hybridization probe recognizes the injected *Xhe2* mRNA. Frontal views, dorsal to top.

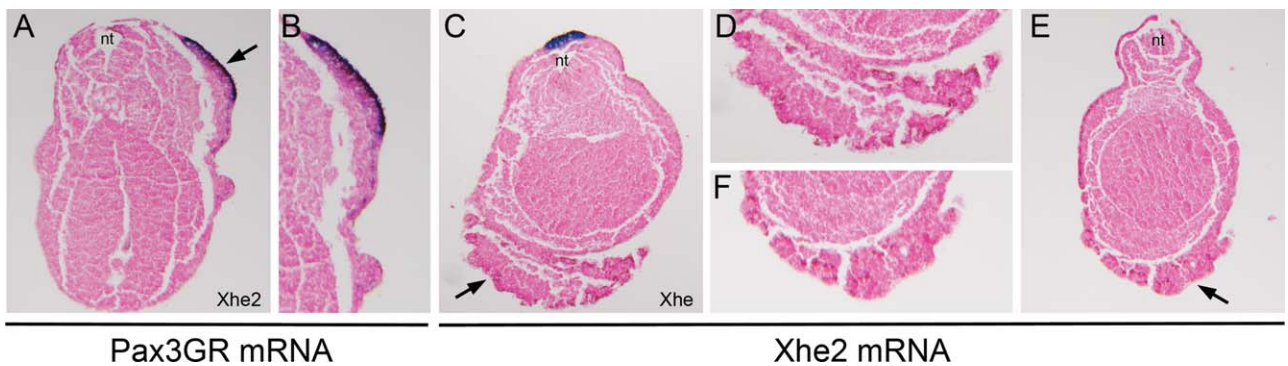

FIG. 6. Histology of tailbud stage embryos overexpressing Pax3GR or Xhe2. (**a**,**b**) Transverse section of an embryo injected with Pax3GR mRNA and stained with Xhe2. Xhe2 ectopic expression (arrow) is associated with a structure that resembles hatching gland cells. (**b**) Higher magnification view of the Xhe2 ectopic cells in panel (**a**). (**c–f**) Transverse section of two embryos injected with Xhe2 mRNA and stained with Xhe. Xhe2 overexpression results in hyperplasia of the ectoderm (arrows). (**d**) and (f) are higher magnification views of panel (c) and (**e**), respectively. All panels are transverse sections, dorsal to top. nt; neural tube.

transcription of *Xhe* (Katagiri *et al*., 1997) and *Crisp* (a cystein-rich secretory protein; Schambony *et al*., 2003). Here we confirm these findings and show that in addition Pax3 also induces the expression of *Xhe2* (Fig. 5a–c).

To further investigate the function of Xhe2 we performed overexpression experiments. Unilateral injection of full-length *Xhe2* mRNA in one blastomere at the two-cell stage resulted in premature hatching of embryos (Fig. 5a,b). Xhe2-induced hatching occurred several stages earlier than in sibling control embryos injected with β-gal mRNA, in which hatching normally happens around Stage 30–33 (Nieuwkoop and Faber, 1967). Interestingly, Pax3GR- and Xhe2-injected embryos exhibited the same pattern of premature hatching (Fig. 5b) suggesting that these factors act in the same pathway.

Since Pax3GR and Xhe2 overexpression have very similar outcome in the embryos, we wished to determine whether they mediate their activity by similar mechanisms. We performed histology on *Pax3GR*- and *Xhe2*-injected embryos at the tailbud stage, and in both cases we observed hyperplasia of the ectoderm, which was however more pronounced in the context of embryos overexpressing Xhe2 (Fig. 6). The ectopic *Xhe2*-positive cells in *Pax3GR*-injected embryos appeared to be organized in structures that resemble hatching glands (Fig. 6a,b), while the ectopic structures observed in Xhe2-injected embryos had a much looser organization (Fig. 6c–f). This suggests that Pax3GR may induce true hatching gland cells secreting all the appropriate hatching enzymes (Fig. 5c). In contrast, Xhe2 overexpressing cells are merely synthesizing Xhe2 (Fig. 5c), which is sufficient to promote early hatching. Moreover, the overproduction of this metalloprotease appeared to have deleterious effects on adjacent tissues since the overall architecture of these embryos was severely disrupted (Fig. 6c–f).

Altogether these findings indicate that Xhe2 is a novel and essential component of the proteolytic degradation mechanism to digest the vitelline membrane. It is likely to act in concert with other proteolytic enzymes such as Xhe and related family members, to mediate its activity and release the tadpole into the environment.

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LITERATURE CITED

Bae CJ, Park BY, Lee YH, Tobias JW, Hong CS, Saint-Jeannet JP. 2014. Identification of targets of Pax3 and Zic1 in the developing neural crest. Dev Biol 386:473–483.

Carroll EJ, Hedrick JL. 1974. Hatching in the toad *Xenopus laevis*: Morphological events and evidence for a hatching enzyme. Dev Biol 38:1–13.

Drysdale TA, Elinson RP. 1991. Development of the *Xenopus laevis* hatching gland and its relationship to surface ectoderm patterning. Development 111: 469–478.

Garnett AT, Square TA, Medeiros DM. 2012. BMP, Wnt and FGF signals are integrated through evolutionarily conserved enhancers to achieve robust expression of Pax3 and Zic genes at the zebrafish neural plate border. Development 139:4220–4231.

Gomis-Rüth FX, Trillo-Muyo S, Stöcker W. 2012. Functional and structural insights into astacin metallopeptidases. Biol Chem 393:1027–1041.

Harland RM. 1991. In situ hybridization: An improved whole-mount method for *Xenopus* embryos. Methods Cell Biol 36:685–695.

Hong CS, Saint-Jeannet JP. 2007. The activity of Pax3 and Zic1 regulates three distinct cell fates at the neural plate border. Mol Biol Cell 18:2192–2202.

Katagiri C, Maeda R, Yamashika C, Mita K, Sargent TD, Yasumasu S. 1997. Molecular cloning of *Xenopus* hatching enzyme and its specific expression in hatching gland cells. Int J Dev Biol 41:19–25.

Kolm PJ, Sive HL. 1995. Efficient hormone-inducible protein function in *Xenopus laevis*. Dev Biol 171: 267–272.

LeDouarin NM, Creuzet S, Couly, G, Dupin E. 2004. Neural crest cell plasticity and its limits. Development 131:4637–4650.

Monsoro-Burq AH, Wang E, Harland R. 2005. Msx1 and Pax3 cooperate to mediate FGF8 and Wnt signals during *Xenopus* neural crest induction. Dev Cell 8: 167–178.

Nieuwkoop PD, Faber J. 1967. Normal table of *Xenopus laevis* (Daudin). Amsterdam: North Holland Publishing Company.

Saint-Jeannet JP, Moody SA. 2014. Establishing the preplacodal region and breaking it into placodes with distinct identities. Dev Biol 389:13–27.

Sato S, Sargent TD. 1990. Molecular approach to dorsoanterior development in *Xenopus laevis*. Dev Biol 137:747–753.

Sato T, Sasai N, Sasai Y. 2005. Neural crest determination by co-activation of Pax3 and Zic1 genes in *Xenopus* ectoderm. Development 132:2355–2363.

Schambony A, Hefele JA, Gentzel M, Wilm M, Wedlich D. 2003. A homologue of cysteine-rich secretory proteins induces premature degradation of vitelline envelopes and hatching of *Xenopus laevis* embryos. Mech Dev 120:937–948.

Slack JM, Forman D. 1980. An interaction between dorsal and ventral regions of the marginal zone in early amphibian embryos. J Embryol Exp Morphol 56: 283–299.